

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:

Scott Michael Krummey

Date

The Confluence of Protective Immunity and Alloreactivity: Analysis of Memory CD4⁺ Th17 and
CD8⁺ T Cells During Heterologous Immunity

By

Scott Michael Krummey

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Immunology and Molecular Pathogenesis

Mandy L. Ford, Ph.D.
Advisor

Lawrence H. Boise, Ph.D.
Committee Member

Brian D. Evavold, M.D., Ph.D.
Committee Member

Jacob E. Kohlmeier, Ph.D.
Committee Member

Christian P. Larsen, M.D., D.Phil.
Committee Member

Accepted:

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

Date

The Confluence of Protective Immunity and Alloreactivity: Analysis of Memory CD4⁺ Th17 and
CD8⁺ T cells During Heterologous Immunity

By

Scott Michael Krummey

B.A. (*magna cum laude*), Colgate University, 2007

Advisor: Mandy L. Ford, 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

Graduate Division of Biological and Biomedical Science

Immunology and Molecular Pathogenesis

2014

Abstract

The Confluence of Protective Immunity and Alloreactivity: Analysis of Memory CD4⁺ Th17 and CD8⁺ T Cells During Heterologous Immunity

By Scott M. Krummey

Effective immunomodulation following solid organ transplantation presents a tremendous clinical challenge. Microbe-elicited T cells that cross-react with allogeneic antigen and mediate graft rejection, a process termed allogeneic heterologous immunity, are a strong barrier to transplantation acceptance and tolerance. Although this phenomenon has been described in both murine models and human studies, the specific parameters that control allogeneic heterologous immunity are poorly understood. While the susceptibility of CD8⁺ memory T cells to CD28/CTLA-4 blockade with belatacept (a CTLA-4 Ig derivative) are well defined, the role of Th17 memory cells in costimulation blockade resistant rejection is not known. We found that the frequency of Th17 memory cells was specifically associated with acute cellular rejection in patients treated with belatacept. Murine and human Th17 cells were resistant to costimulation blockade with CTLA-4 Ig both *in vitro* and *in vivo*, due at least in part to higher expression of the coinhibitory receptor CTLA-4. An additional undefined facet of allogeneic heterologous T cell responses is the role of T cell priming affinity. As heterologous immunity requires a T cell to recognize two distinct p:MHC complexes, T cell priming affinity is hypothesized to be a critical feature of heterologous immunity. Here we report that compared to high affinity memory CD8⁺ T cells, memory T cells primed with low affinity antigen are capable of potent heterologous rechallenge responses. We found that large CD45 isoforms, represented as CD45RB^{hi}, predominated on low affinity primed memory cells, and that CD45RB^{hi} expression conferred a proliferative advantage during secondary rechallenge characterized by distinct IL-2/IL-2R α production. Low affinity primed secondary effectors also upregulated the TNF receptor 2 (TNFR2) and were more dependent on TNF:TNFR2 signals during graft rejection than high affinity primed secondary effectors. Together, these data provide important phenotypic and functional description of pathogen elicited T cell subsets in the context of transplantation. As the successful modulation of pathogenic T cell responses depends on a more granular understanding of T cell functionality, this work provides important description of the role of T cell subsets in the context of heterologous immunity that may generate novel therapeutic strategies.

The Confluence of Protective Immunity and Alloreactivity: Analysis of Memory CD4⁺ Th17 and
CD8⁺ T Cells During Heterologous Immunity

By

Scott Michael Krummey

B.A. (*magna cum laude*), Colgate University, 2007

Advisor: Mandy L. Ford, 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
Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis

2014

Acknowledgments

The scientific results of this thesis are certainly dependent on the contributions of a great number of lab members and collaborators. First and foremost is my advisor and mentor, Dr. Mandy L. Ford, who I owe tremendous thanks for the opportunity to work on exciting projects from my first weeks in the lab and for providing the resources with which to pursue interesting findings for over three years. Through our countless discussions, I have learned a tremendous amount not only about immunology but also about being a professional scientist. I greatly look forward to continuing to work together in the future.

In the Emory Transplant Center, many current lab members have provided valuable helping hands and technical assistance with experiments current members including Maylene Wagener, Danya Liu, Ching-Wen Chen, Linda Stempora, and Cindy Breeden. Many former lab members were also very helpful during early stages of these projects, including Dr. William Kitchens, Dr. Ivana Ferrer, and Tamara Floyd. Many current and former investigators within the ETC provided very helpful discussion about projects and presentations at various stages of this work, including Dr. Andrew B. Adams, Dr. Allan D. Kirk, Dr. Raul Badell, Dr. Thomas C. Pearson, Dr. Aneesh K. Mehta, Dr. Stuart J. Knechtle, Dr. Neal Iwakoshi, and Dr. Leslie Kean. On our human project, Shannon Bond and Dr. John Roback were essential for continuing these studies. Members of the Emory Transplant Center Biorepository were also crucial for studying transplant patients including Jennifer Cheeseman, Peter Jang, Jason Conger, and Jacob Danoff. I am also tremendously appreciative of our collaborators at Bristol-Meyers Squibb, including Drs. Steven G. Nadler and Suzanne J. Suchard, whose contribution of pre-clinical reagents has enabled many experiments that would not otherwise be feasible. Drs. Brian Evavold, Jacob Kohlmeier, and Timothy Denning provided very helpful suggestions at critical stages. I also thank collaborators and friends Ryan Martinez, Rakieb Andargachew, and Catherine Gaville for helpful discussions, protocols, and reagents.

I am deeply grateful for the Emory MD/PhD Program leadership for giving me the opportunity to pursue this exciting career path six years ago and providing an environment that truly encourages success, including Dr. Charles Parkos, Dr. Kerry Ressler, and Mary Horton. I also owe thanks to my medical school small group advisor, Dr. Joyce Doyle for much guidance and many wonderful home cooked meals along the way.

Personally, I am most thankful for my wife, Jennifer, who has wholeheartedly supported this lengthy training process. I would be remiss not to mention our canine companion, Stella, who has also provided support and much needed comic relief. Last but certainly not least, I thank my parents, sister, and in-law family, all of whom have been nothing but supportive, and without whom I would not have had the opportunity to pursue this path.

Table of Contents

Chapter 1. Introduction.....	1
Chapter 2. High CTLA-4 Expression on Th17 Cells Results in Increased Sensitivity to CTLA-4 Coinhibition and Resistance to Belatacept.....	31
Introduction.....	31
Materials & Methods	33
Results.....	36
<i>Alloreactive Th17 memory cells are resistant to belatacept</i>	
<i>Th17 memory cells significantly upregulate the coinhibitor CTLA-4</i>	
<i>CD45RA⁺ Th17 cells are uniquely sensitive to CTLA-4 coinhibition</i>	
<i>Renal transplant recipient-derived lymph node CD45RA⁺ Th17 cells are resistant to belatacept</i>	
<i>An elevated Th17 memory cell frequency correlates to rejection during belatacept therapy in renal transplant recipients</i>	
Discussion	41
Figures.....	44
Chapter 3. Candida-Elicited Murine Th17 Cells Express High CTLA-4 Compared to Th1 Cells and Are Resistant to Costimulation Blockade	50
Introduction.....	50
Materials & Methods	53
Results.....	57
<i>Candida antigen immunization yields costimulation blockade resistant graft rejection</i>	
<i>Candida immunization elicits a Th17 skewed phenotype compared to M.Tb.</i>	
<i>M.Tb and Candida elicit similar frequencies of effector memory cells</i>	
<i>Candida elicited Th17 cells have a more pathogenic phenotype compared to M.Tb elicited Th17 cells.</i>	
<i>Th17 cells express greater amounts of CTLA-4 than Th1 cells.</i>	
<i>Th17 cells are less susceptible to CTLA-4 Ig and more sensitive to CTLA-4 coinhibition than Th1 cells.</i>	
<i>Pathogen elicited Th1 and Th17 cells similarly regulate CD154 expression</i>	
<i>Neutrophils are recruited to skin grafts in a costimulation blockade independent manner by Candida-elicited T cell responses.</i>	
Discussion	65
Figures.....	68
Chapter 4. High CTLA-4 Expression Corresponds with Diminished FOXO3 Expression on Human Th17 Memory Cells.....	81
Introduction	81
Results & Discussion	82
<i>Human Th17 cells are resistant to selective CD28 blockade</i>	
<i>Th1 and Th17 cells are similarly activated by CD28 signals</i>	
<i>FOXO3 expression correlates inversely with CTLA-4 expression</i>	
<i>FOXO3 expression is diminished in Th17 cells</i>	
Materials & Methods	85
Figures	88

Chapter 5. Low Affinity Memory CD8⁺ T Cells Mediate Robust Heterologous Immunity	92
Introduction.....	92
Results.....	94
<i>Low affinity CD8⁺ T cell priming efficiently generates memory cells</i>	
<i>Low affinity memory CD8⁺ T cells have a distinct central memory phenotype</i>	
<i>Low affinity memory CD8⁺ T cells are tuned to generate robust secondary recall responses</i>	
<i>CD45RB is a stable marker of the affinity experience of memory CD8⁺ T cells</i>	
<i>High CD45RB expression tunes CD8⁺ T cells to respond to heterologous rechallenger antigen</i>	
<i>Low affinity primed secondary effector cells have a distinct phenotype</i>	
<i>Low affinity primed secondary effector CD8⁺ T cells produce high levels of IL-2 and upregulate IL-2Rα</i>	
<i>Low affinity polyclonal CD8⁺ T cells express high levels of CD45RB</i>	
Discussion.....	102
Materials & Methods	105
Figures.....	109
Chapter 6. Enhanced Requirement for TNF Signaling on Low Affinity Memory CD8⁺ T Cells During Heterologous Immunity	120
Introduction.....	120
Materials & Methods	122
Results.....	124
<i>Low affinity primed memory CD8⁺ T cells produce high levels of TNF</i>	
<i>Low affinity primed memory CD8⁺ T cells produce high levels of TNF</i>	
<i>Low affinity primed secondary effectors upregulate TNFR2 expression</i>	
<i>Low affinity primed memory CD8⁺ T cells are dependent on TNFR2 signaling in order to optimally mediate graft rejection during heterologous rechallenger</i>	
Discussion.....	128
Figures.....	130
Chapter 7. Discussion	134
References.....	149

Figure Index

Chapter 2.

- Figure 2.1. CD45RA⁻ Th17 Cells are Alloreactive and Resistant to Belatacept.*
Figure 2.2. Th17 Memory Cells Express High Levels of CTLA-4.
Figure 2.3. CD45RA⁻ Th17 Cells are Resistant to Inhibition by Belatacept and More Dependent on Coinhibition by CTLA-4.
Figure 2.4. Renal Transplant Recipient Lymph Node CD45RA⁻ Th17 Cells are Resistant to Belatacept.
Figure 2.5. Renal Transplant Patients Experiencing Acute Rejection on Belatacept-based Regimens Have Elevated Frequencies of Th17 Memory Cells.
Supplemental Figure 2.1. Th1 cells are inhibited and CD45RA⁻ Th17 cells are resistant to a range of belatacept concentrations

Chapter 3.

- Figure 3.1. Candida elicited CD4⁺ T cells can mediate costimulation blockade resistant graft rejection.*
Figure 3.2. Candida elicits a higher frequency of Th17 cells than M.Tb.
Figure 3.3. M.Tb and Candida elicit a similar CD4⁺ T_{EM} phenotype.
Figure 3.4. Candida elicits more pathogenic Th17 cells than M.Tb.
Figure 3.5. Th17 cells express higher levels of CTLA-4 and are less susceptible to CTLA-4 Ig than Th1 cells.
Figure 3.6. Th17 Cells are more susceptible to CTLA-4 coinhibition than Th1 cells.
Figure 3.7. Th1 and Th17 cells express similar levels of CD154 and are inhibited by CD154 blockade.
Figure 3.8. Candida polarized mice have greater neutrophil recruitment to skin grafts in the presence of CTLA-4 Ig.
Supplemental Figure 3.1. M.Tb and Candida immunization and costimulation blockade treatment yields similar levels of CD8⁺ OT-I cells and anti-OVA antibodies.
Supplemental Figure 3.2. M.Tb and Candida elicited Th1 and Th17 cells express similar levels of T-bet and contain similar frequencies of IL-2 and TNF co-producers.
Supplemental Figure 3.3. M.Tb and Candida elicited Th17 cells express more CCR6 and CTLA-4.
Supplemental Figure 3.4. Th1 and Th17 cells from M.Tb and Candida are similarly inhibited by CTLA-4 Ig and augmented by anti-CTLA-4.

Chapter 4.

- Figure 4.1. CD28 mediates divergent signaling outcomes on Th1 and Th17 memory cells.*
Figure 4.2. Th1 and Th17 cells are similarly activated by TCR and CD28 signals.
Figure 4.3. CTLA-4 expression correlates inversely with FOXO3 expression.
Figure 4.4. Th17 memory cells express less of the transcription factor FOXO3.

Chapter 5.

- Figure 5.1. Low affinity priming efficiently generates central memory CD8⁺ T cells.*
Figure 5.2. Low affinity primed CD8⁺ T cells mount potent secondary responses against high affinity antigen.
Figure 5.3. High CD45RB expression tunes low affinity primed memory CD8⁺ T cells for heterologous rechallenge responses.

Figure 5.4. Low affinity primed secondary effectors downregulate CD45RB and have a distinct effector phenotype.

Figure 5.5. Low affinity primed secondary effectors have a distinct IL-2 dominant phenotype.

Figure 5.6. Low affinity polyclonal CD8⁺ T cells express high levels of CD45RB.

Supplemental Figure 5.1. Low affinity priming generates quality memory CD8⁺ T cells.

Supplemental Figure 5.2. High and low affinity primed memory CD8⁺ T cells express similar levels of TCR tuning molecules.

Supplemental Figure 5.3. CD45RB expression correlates with T cell priming affinity in vitro.

Supplemental Figure 5.4. High and low affinity primed secondary effectors produce similar levels of IFN- γ and TNF.

Chapter 6.

Figure 6.1. Low affinity primed memory CD8⁺ T cells mediate graft rejection.

Figure 6.2. Low affinity primed memory CD8⁺ T cells produce high levels of TNF in response to heterologous rechallenge.

Figure 6.3. Low affinity primed CD8⁺ secondary effectors upregulate surface TNFR2 expression.

Figure 6.4. Low affinity primed memory CD8⁺ T cells rely on TNFR2 signaling

Chapter 1. Introduction

In only the past half century, solid organ transplantation has transformed from an experimental medical field into curative treatment for dozens of end-stage diseases. Central to this advance is the discovery that the adaptive immune system, T and B cells, play a critical role in mediating graft rejection. Much of the progress made towards maintaining functioning allografts has been made through the development of pharmacologic therapy to suppress the alloreactive T and B cell responses. Despite this progress there is a recent dearth of new immunomodulatory therapies in the last three decades. While the one-year rate of graft survival has steadily risen, the long-term survival of transplanted organs has simultaneously stagnated. This is due largely to the toxicities associated with mainstay immunomodulatory therapy, calcineurin inhibitors. As well, understanding of molecular and cellular factors that contribute to graft rejection has plateaued. There is clearly a great need for more a more detailed understanding of the factors governing alloreactive immune responses, which will lead to higher resolution analyses of novel targets for immunomodulation.

Great progress has also been made in our understanding of the nature of the adaptive response that provides immunity to pathogens. Molecular and phenotypic analysis, enabled largely by the advent of techniques such as multi-color flow cytometry, has deepened understanding of T cells beyond simply CD4⁺ and CD8⁺ populations to encompass a wide and growing array of subsets with specific functions and mechanisms of interacting with other populations. In particular, there has been a great focus on understanding CD4⁺ and CD8⁺ T cell memory cells, as these are critical for advances in enhancing immunity to pathogens. While there are certainly caveats, a tremendous amount of knowledge has been gained from TCR transgenic mouse models of acute and chronic infections, which allows the detailed analysis of pathogen specific T cells over the course of infection. These models have generated a fundamental

understanding of T cell biology that applies to immune responses in other contexts, such as autoimmune disease and transplantation. Compared to the fields of protective immunity and autoimmunity, models of transplantation have lagged in the high-resolution techniques that enable visualization of the whole course of an alloreactive response.

Protective immunity and transplantation, however, have become conceptually linked through a body of work that demonstrates that pathogen-elicited memory T cell responses play a large role in alloreactive T cell responses, a phenomenon termed allogeneic heterologous immunity. Strikingly, pre-existing T cell memory has been shown in several models to be a barrier to transplantation tolerance with immunomodulatory therapies. It seems that while adaptive immune responses have evolved to provide robust memory responses to rechallenge with the same or cross-reactive pathogens, these same properties might also be detrimental to long-term graft survival. Unfortunately, this appears to be a problem that requires highly granular understanding of the immune system, as specific pathogens and subsets T cell memory may be problematic, while others may not. This finding necessitates framing the study of alloreactive memory responses around what is known of protective immunity responses, with the goal of deepening our understanding of the molecular and cellular parameters that would make specific targets for alloreactive immunomodulation.

Pathogen-Elicited Memory and Allogeneic T Cell Responses are Closely Intertwined

Characteristics of Memory T Cells

A hallmark characteristic of adaptive immunity and T cell responses is the ability to form long-lived memory responses. During an infection, CD4⁺ and CD8⁺ T cells that recognize their cognate antigen clonally expand and differentiate into effector cells (1-3). Following clearance of antigen during the effector phase, these populations undergo significant contraction during which the majority die by apoptosis, while a small fraction survive as memory T cells. The advantage of this process is an enhanced ability to mount responses against repeated infection with the same

pathogen or cross-reactive antigen. This enhanced response by memory T cells has been well characterized as more potent due to the enhanced cytolytic ability of memory T cells and an elevated precursor frequency for a given antigen (4-7). Currently, two major subsets of CD4⁺ and CD8⁺ T cell memory are thought to exist: central memory (T_{CM}), which reside primarily in secondary lymphoid tissues and have high proliferative capacity, and effector memory (T_{EM}), which reside in peripheral tissues and are more potently cytotoxic (1, 3, 8). A more detailed discussion of these phenotypes is included below.

In the case of interaction with cross-reactive antigen, microbe-elicited T cells have been shown to cross-react with different pathogens (9-12) or also with allogeneic antigen (13-16). Thus, these very properties that make T cell memory particularly poised to provide enhanced immunity against cross-reactive challenge make it less susceptible to immunomodulatory strategies.

Evidence for Alloreactivity Among T Cell Memory

While it has long been appreciated that T cells are potent mediators of alloreactivity and graft rejection, much work has been performed to understand the relative contribution of primary effectors and memory cells to alloreactive responses (17). Seminal studies using cord blood, which is absent of memory T cells, and alloantigen restimulation suggested that alloreactivity was principally found within the memory T cell compartment (17, 18). Recent work by Metes and colleagues examined the frequencies of alloreactivity among purified naïve, central memory, effector memory, and terminal effector memory populations, revealing that approximately equal frequencies of alloreactive T cells exist among these subsets (19). Nevertheless, this work establishes that a significant component of alloreactive T cell responses arise from memory T cells.

Alloreactive memory T cells can be generated through a variety of mechanisms that can be divided into three major categories. The first most traditional means is through sensitization with alloantigen. Reports from both experimental models (20, 21) and clinical patients (22)

revealed worse outcomes in recipients of a prior graft, consistent with “second set” rejection. Alloreactive memory T cells can also arise during pregnancy, during which time the female can be primed against paternal antigens carried by the fetus (23). Furthermore, a recent study also found that immunity generated following platelet transfusion was sufficient to induce rejection following subsequent bone marrow transplantation in murine recipients, even across minor histocompatibility antigens (24). Since platelet transfusion is a common occurrence prior to liver transplantation in particular, these data indicate that the subpopulation of frequently transfused transplant recipients may be at an increased risk for memory T cell-mediated graft rejection.

Second, alloreactive memory T cells may be generated in an antigen-independent manner when in a lymphopenic environment exists in the host. In these instances, naïve $CD4^+$ and $CD8^+$ alloreactive precursors are induced to undergo IL-7-dependent homeostatic proliferation and differentiation into memory T cells (25, 26). Seminal studies from Turka and colleagues showed that both adoptive transfer of lymphocytes into T and B-cell devoid SCID recipients and experimental depletion of lymphocytes in murine transplant recipients resulted in rapid reconstitution of peripheral T cell compartments with memory T cells (27). These findings may be clinically relevant in that lymphopenia can be induced in patients following infection with a viral pathogen such as HIV, or following therapeutic depletion of T cells for the treatment of autoimmunity or transplantation, and residual naïve T cells might be induced to undergo rapid division and acquisition of a memory-like phenotype (28). Studies from non-transplant models have revealed that these “pseudomemory” T cells have functional characteristics similar to those of memory T cells as well (25, 26). Thus, lymphopenia-induced immunologic memory seems to be both as phenotypically and functionally competent as true antigen-dependent memory.

Finally, microbe-elicited T cells might cross-react with allogeneic antigen, a phenomenon termed allogeneic heterologous immunity. There is growing evidence that microbial stimulation history might significantly influence the character of alloreactive memory. It has long been recognized that a significant portion of the T cell compartment can respond to microbial antigen

(29) and that memory cells raised against microbial antigens can be cross-reactive with allogeneic antigen (13, 30-32). Specific examples of T cells that were generated against EBV, CMV, or tetanus peptide:MHC and are cross-reactive with allogeneic peptide:MHC have been described functionally (33-35) and structurally (36-38). The significance of this phenomenon was significantly elevated by the observation that nearly half of viral-specific CD4⁺ and CD8⁺ memory cells are cross-reactive with allogeneic antigen (16, 39, 40). Murine models of heterologous immunity have demonstrated that virus-primed T cells can mediate graft rejection (15, 41). In addition, recent studies demonstrated that alloreactive T cells are inherently polyspecific for peptide-MHC complexes (42-44), a finding that makes them permissive to scenarios of heterologous immunity.

Alloreactive Memory is Highly Donor Specific

Despite intrinsic cross-reactive potential of TCRs, studies of virus-specific human memory T cell clones revealed that while allo-cross reactivity was indeed very common, this cross-reactivity was usually confined to a single HLA molecule (45). Thus, while several studies have now shown that that alloreactivity exists among memory, the extent to which donor-reactive memory T cells are present appears to be highly dependent on the donor tissue tested. This is an important finding because it had previously been hypothesized that due to the lower activation threshold of memory T cells, many different alloantigens might be capable of stimulating memory T cells. In a 2007 study, Benichou and coworkers stimulated memory T cells from 11 different non human primates with a panel of 14 different stimulator cells, and found that the donor-reactive memory T cell precursor frequencies within a given individual spanned an over 40-fold range depending on the allostimulator used (46). Interestingly, the authors also reported that naïve alloreactive T cell precursor frequencies did not range as widely across the different responder: stimulator pairs tested, suggesting that the observed difference in donor-reactive memory T cell precursor frequencies was not due to intrinsic differences in the alloreactive T cell

repertoires of these animals, but instead was likely due to differences in their immunologic histories (46).

Alloreactive T Cells May Be Uniquely Polyspecific

In addition, recent studies out of the Allen lab have provided insight into the properties of alloreactive T cells that makes them permissive to scenarios of allogeneic heterologous immunity (47). Felix et al screened H-2^b selected CD4⁺ T cells for clones that reacted with I-E^k restricted antigen (42). Surprisingly, they found that allogeneic reactive T cells were able to recognize multiple, distinct peptides with unrelated sequence homology. Another systematic investigation of alloreactive H-2b restricted CD4⁺ T cells against H-2d antigen demonstrated that alloreactive recognition is an inherent property of certain T cells, as demonstrated by distinct TCR CDR3 domain sequences, and is peptide dependent (43). This demonstration that T cells can have an intrinsic ability to recognize p:MHC in a polyspecific manner suggests that alloreactive T cell responses might be preferentially mounted by T cells that are permissive to scenarios of heterologous immunity.

The Role of CD4⁺ T Cell Subsets in Heterologous Immunity

The CD4⁺ T Cell Compartment Contains Distinct Th Subsets

T cells differentiate into distinct phenotypes based on the surrounding environment that is present during their initial interaction with cognate antigen, and largely maintain this phenotype during challenge with recall or cross-reactive antigen (3, 48-50). In particular, functional and phenotypic diversity within the CD4⁺ compartment has been well appreciated. Seminal work by Mossman and Coffman established that CD4⁺ Th1 and Th2 subsets produced discrete cytokine profile that defined their function (51, 52). This work established the concept not only of functional heterogeneity among CD4⁺ T cell but also that these subsets regulate the development

and function of other subsets. Since this discovery, additional CD4⁺ subsets have been identified, including CD4⁺CD25⁺FoxP3⁺ Tregs and Th17 cells (1).

CD4⁺ T cell memory has received relatively less attention than CD8⁺ T cell memory (discussed below). Several groups have demonstrated that Th1 effector memory cells arise from Th1 effectors during clonal expansion (53, 54). While the IL-7R α (CD127) is also permissive for CD4⁺ memory cell formation, the transcription factors T-bet, Blimp-1 and eomesodermin do not delineate CD4⁺ memory cell precursors in the same manner as CD8⁺ memory (55, 56). Two recent demonstrated that CD4 central memory cells have a T follicular helper-like CCR7⁺CXCR5⁺ phenotype (57, 58). These molecular changes are partially responsible for memory T cell superior functional capabilities, long life span, and reduced activation requirements compared to naïve counterparts (2).

The Th17 lineage was identified out of inconsistencies in the study of Th1 cells. Pathogenesis of EAE was regarded as a Th1 mediated disease despite the paradoxical observations that exogenous IL-12 or IFN- γ inhibited disease (59, 60), while genetic deletion or neutralization of IFN- γ enhanced disease (61, 62). Work from two groups established that IL-23 and IL-17 were produced by a distinct subset of inflammatory CD4⁺ cells (63, 64) and that passive transfer of IL-17 producing CD4⁺ T cells could drive EAE (63). Studies by Harrington and Park established that Th17 lineage development was reciprocal from Th1/Th2 transcription factors (65, 66). Thus, the CD4⁺ compartment is understood to have proinflammatory Th1, Th2, and Th17 lineages as well as regulatory/suppressive CD25⁺FoxP3⁺ Tregs.

While Th17 cells were initially shown to drive autoimmune inflammation, a great deal of work has identified phenotypic heterogeneity within the Th17 lineage. Several groups have established that Th17 cells can function as protective or pathogenic cells, termed “classic” and “alternative” by Kuchroo and colleagues, depending on the particular cytokine composition of

their environment and their expression of effector molecules besides IL-17 (67). The role of two key cytokines, IFN- γ and IL-10, will be discussed herein.

Several studies have identified Th17 cells that co-express the Th1 transcription factor T-bet and produce IFN- γ . These IL-17⁺IFN- γ ⁺ have been associated with sites of inflammation in multiple disease models of EAE (68-70) and colitis (71, 72). IL-17/IFN- γ double producers have also been identified in IBD patients (73) and MS patients (74). Co-expression of IFN- γ by Th17 cells has been shown by several groups to be dependent on IL-23 exposure during differentiation (75-77). A recent study by Hirota et al recently demonstrated that these double producers are generated from Th17 cells that begin expressing IFN- γ . Using a fate-mapping IL-17 mouse, they showed that during EAE IL-17⁺IFN- γ ⁺ cells were derived from cells that first expressed IL-17 only and that a significant portion of Th17 cells shut down IL-17 production (78). On a transcriptional level, it was recently shown that IL-17⁺IFN- γ ⁺ cells express lower levels of ROR- γ t, which was shown to be due to T-bet inhibition of ROR- γ t via Runx1 sequestration (78). While IL-17⁺IFN- γ ⁺ cells have been associated with sites of inflammation and are postulated to be more pathogenic, it has not been shown conclusively whether the production of IFN- γ by Th17 cells is protective or pathogenic.

The anti-inflammatory cytokine IL-10 has also been shown to play an important role in a subset of protective Th17 cells. Seminal work from McGeachy and colleagues demonstrated that exposure to IL-6 and TGF- β 1 in the absence of IL-23 drives myelin-reactive CD4⁺ cells to produce IL-10, and that they fail to induce CNS inflammation in a model of EAE (69, 77). A recent study demonstrated that IL-17⁺ and IL-17⁺IFN- γ ⁺ cells are sensitive to IL-10 suppression owing to higher IL-10R expression (79). A recent study added another layer to the role of TGF- β in Th17 pathogenicity with the finding that TGF- β 3 production by Th17 cells was an IL-23-dependent pathological determinant distinct from TGF- β 1 (80). An important study in human cells demonstrated that different Th17 polarizing pathogens can affect the Th17 phenotype.

Zielinski et al showed that *Candida albicans*-specific Th17 cells co-produced IFN- γ while *Staphylococcus aureus*-specific Th17 cells produced IL-10 (81). In summary, a growing number of studies have demonstrated heterogeneity among Th17 cells. The most recent model is that in the absence of IL-23 signaling, Th17-mediated inflammation is self-limited by IL-10 production; conversely, IL-23 exposure during or after differentiation promotes a pro-inflammatory “alternate” Th17 phenotype that is capable of driving severe inflammation (67).

Despite this detailed work identifying Th17 subsets, major outstanding questions remain in this area, mostly centered on the behavior of these subsets in vivo. For one, despite categorization as protective “classical” and pathogenic “alternate” phenotype, major functional differences between these subsets have not been demonstrated in disease settings. For example, what are the functions of IL-17 in each setting, and how do co-expressed IFN- γ and IL-10 shape an immune response? Additionally, a greater understanding is needed of the signals and transcriptional programs that favor one subset over another. The elegant work of Lee and Hirota and colleagues demonstrate that the answer is likely to require a very fine resolution, as they found that many genes were differentially expressed in pathogenic and classical Th17 cells. Finally, the plasticity between Th17 phenotypes has not been thoroughly addressed in vivo. Are these subsets capable of shifting between functional subsets in vivo as the environment changes, e.g. subsequent infections?

CD4⁺ T Cell Subsets Have Distinct Costimulation Requirements

Our understanding of CD4⁺ T cell costimulation comes largely from the study of Th1 cells, as these are the most abundant CD4⁺ subset in naïve mice and the majority of this work was conducted before the discovery of Th17 cells. This work established the CD28/CTLA-4 pathway as the prototypic T cell costimulation pathway (82). CD28 is constitutively expressed on T cells and transmits costimulatory signals that enhance IL-2 production and T cell proliferative

responses. In contrast, CTLA-4 is maintained in intracellular vesicles that rapidly flux to the surface upon TCR stimulation, and mediate coinhibitory signals that antagonize T cell responses. While much work has identified mechanisms by which CTLA-4 acts in a cell intrinsic manner, very recent studies have identified cell extrinsic mechanisms of CTLA-4 action (discussed below).

There is growing evidence, however, that Th17 cells have costimulation requirements that differ from those of traditionally studied Th1/Th2 cells. An early study of murine Th17 cells found that IL-17 and IFN- γ production were similarly dependent on CD28 (66). More recent studies of naïve murine CD4⁺ T cells under Th17 polarizing conditions have found that both CD28 (83, 84) and CTLA-4 can inhibit differentiation (85). Paulos and colleagues demonstrated that in the presence of ICOS ligation, CD28 signaling inhibited Th17 differentiation (86). A recent study by Santarlasci and colleagues revealed that human Th17 clones are uniquely sensitive to CD28 signaling compared to Th1 clones (87). The conflicting results in these studies might be explained by the differences in experimental conditions, such as naïve murine CD4⁺ T cell cultured in the presence of exogenous cytokines versus human CD4⁺ clones maintained in long term culture with repeated anti-CD3/CD28 stimulation. However, these results raise the possibility that the CD28/CTLA-4 pathway plays a different role on Th17 cells compared to Th1 cells.

Another intriguing body of work has demonstrated that Th17 cells might rely on additional costimulatory pathways for optimal differentiation. Work from Paulos et al demonstrated that ICOS was expressed on resting Th17 cells and required for optimal Th17 differentiation from naïve CD4⁺ cells under polarizing conditions (86). In murine models, Th17 cells were found to be dependent on ICOS signaling in a model of collagen induced arthritis (88). Interestingly, several studies have implicated CD5 as a critical costimulatory pathway for Th17 development. De Wit et al showed that CD5 ligation was capable of augmenting Th17 differentiation under polarizing conditions (84). Two studies by Chatterjee identified

costimulation through two SLAM family receptors, SLAMF3 and SLAMF6, as capable of inducing Th17 polarization via enhanced ROR γ t recruitment of the IL-17 locus (89, 90).

The overall thrust of this work is that CD4⁺ subsets might have different functional requirements for costimulation. Several groups have demonstrated divergent roles for the CD28/CTLA-4 pathway on Th17 cells compared to Th1 cells. However, major discrepancies regarding the nature of these differences remain to be resolved. The concept of subset-specific cosignaling requirements has been most well studied in FoxP3⁺ Treg. Classically, Treg have been shown to rely on CD28 for development [citation]. However recent work found that CD28 has a post-maturational role on Treg function. Tregs are also unique for their constitutive expression of the coinhibitor CTLA-4 and depend on it for cell-extrinsic suppressive function (discussed in depth below). Certainly, more exciting work remains to be done to further understand the programmatic cosignaling requirements of Th lineages.

The Emerging Identification of Th17 Cells in Alloreactive Responses

The observation that alloreactive T cell memory is comprised of cross-reactive pathogen-elicited T cells raises the possibility that circulating pathogen elicited Th17 cells can cross-react with allogeneic antigen in the same manner as Th1 cells. The degree of contribution of Th1/Th17 cells to alloreactivity is likely dependent on both the individual and the organ transplanted. Certain disease indications for solid organ transplantation likely skew the CD4⁺ compartment in a particular direction. For example, autoimmune Type 1 diabetes or SLE have both been shown to have Th17 components, and asthma is known to elicit Th17 responses in the lungs. Additionally, the microbial stimulation history of an individual plays a role in the character of the Th17 memory compartment that can cross-react with allogeneic antigen. Th17 responses are elicited by several ubiquitous pathogens, including *Candida albicans* and *Staphylococcus aureus* (81). Finally, the character of alloreactivity might depend on the particular solid organ that is

transplanted. For example, Th17 immunity to commensal gastrointestinal or respiratory microbes might be particularly relevant in the liver or lung, respectively.

The likely dependence on multiple contextual factors makes the study of human alloreactive Th17 responses difficult on a population level, and likely requires more detailed analysis of patients stratified by the parameters described above. There is a growing list of reports implicating Th17 cells in allogeneic responses. Elevated IL-17 has been described in rejecting human lung and liver allografts (91-93) and correlated with kidney allograft dysfunction (94). In murine studies, IL-17 producing cells have been identified after allogeneic stimulation (95). Murine IL-17 producing cells were shown to mediate rejection in both Tbet^{-/-} (95-98) and wild-type animals (99).

The Role of CD28 in CD4⁺ T cell Responses

The idea that T cells require a second signal for full activation was proposed decades ago, and led to the discovery that T cell antigen receptor stimulation in conjunction with costimulation was required for productive activation of T cells. CD28 was the first costimulatory molecule described for T cells (100, 101). Although five additional members of the CD28 family have been identified (CTLA-4, ICOS, PD-1, BTLA, and JAM-L) in addition to a variety of other costimulatory molecules in the TNFR and CD2 family, among others. This discussion will focus on the role of CD28, as it has been the most well studied costimulatory molecule in protective immunity and a target for immunomodulation in autoimmunity and transplantation.

The CD28/CTLA-4 pathway is the prototypic cosignaling pathway in T cells. T cells that receive inadequate CD28 costimulation via suboptimal ligation of CD80 and CD86 (B7.1 and B7.2, respectively) become anergized or apoptotic (102). CTLA-4 has been characterized as a counter-signal to CD28 costimulation, as CTLA-4 also binds CD80/CD86 (103). In contrast to CD28, which is constitutively expressed in most populations of T cells at rest and following activation, CTLA-4 is not expressed on the surface of resting T cells, but is maintained in

intracellular Golgi-associated vesicles beneath the plasma membrane. Upon antigen stimulation, CTLA-4 surface expression is induced through both *de novo* gene expression and cycling of existing CTLA-4 protein to the cell surface.

CD28 and CTLA-4 form homodimers and bind the ligands CD80 and CD86. In contrast to CTLA-4 (discussed below), CD28 is constitutively expressed on T cells and signals through two Y-based motifs in its cytoplasmic tails. Despite its widespread study as a costimulatory molecule on a cellular level, CD28 signal transduction remains enigmatic. Studies have implicated that signaling through the YMN motif depends on PI3K activates NF κ B to turn on transcription of IL-2 (104-107) and Bcl-XL (108-110). Other studies have revealed that the PYAP motif signals through PKC θ to recruit Lck to the immunological synapse and enhances TCR signaling (111). A recent study by Pagan et al demonstrated that CD4⁺ T cells proliferate and mediate NF κ B translocation independent of either the YMN and PYAP motifs (112), suggesting that CD28 signaling occurs through yet undiscovered sites in the cytoplasmic tail.

The requirement of CD28 signaling to initiate primary T cell responses has been appreciated in many model systems. TCR engagement without CD28 ligation leads to a state of anergy or apoptosis (113). Early systems utilized influenza (114, 115), VSV (116), and gHV68 (117) infections to show that the absence of CD28 signaling, either through CTLA-4 Ig administration or CD28^{-/-} mice, leads to clonal expansion of CD4⁺ and CD8⁺ T cells as well as their function and antibody responses (118). One exception to this work is LCMV, with which was shown to not require CD28 for CD8⁺ responses and viral clearance. However, it is likely that this is due to the unusually high levels of LCMV induced TCR stimulation in its natural host, leading to greater antigen load and persistence (118). Seminal work by Lanzavecchia demonstrated that TCR stimulation must achieve a threshold for T cell activation to occur, while the presence of CD28 lowers that threshold significantly (119). Together, this work established

the importance of costimulation and established CD28 as the prototypic costimulatory molecule for primary CD4⁺ and CD8⁺ T cell responses.

Several early studies investigating the role of CD28 for memory responses concluded that T cell memory was largely independent of CD28 signaling, a finding that has formed a paradigm in immunology for decades (120-123). One such *in vivo* study challenged OT-I containing VSV-OVA immune mice with oral OVA in the presence or absence of CTLA-4 Ig. Based on blast size and *ex vivo* cytolytic function, the authors concluded that CD8⁺ memory cells were independent of CD28 (122). Similar studies examining the *ex vivo* killing capacity of T cells revealed that on a per cell basis, cytolytic function is not affected by the absence of CD28 costimulation (120, 121, 123). A study in LCMV demonstrated that WT and CD28^{-/-} mice formed similar populations of long-lived CD8⁺ memory T cells and were able to survive a lethal challenge dose of LCMV (124).

Indeed, recent studies have modified this understanding and provided considerable evidence that memory T cell responses are quite dependent on CD28 signaling (125-129). Borowski et al carefully examined the requirements of CD28 during memory recall response to HSV and influenza infection using CD28 blockade or adoptive transfer into CD80/CD86 deficient hosts. CD8⁺ T cell responses were significantly reduced (3-fold and 9-fold, respectively) following rechallenge (125). Using a model of vaccinia infection, Fuse et al showed that CD28^{-/-} mice produced similar frequencies of memory cells, however CD28^{-/-} memory cells expressed less CD122 and CD27 and produced less IL-2 after restimulation (128). A recent study in CD4⁺ memory showed that the amount of IL-2 (but not IFN- γ) was diminished during *ex vivo* restimulation of influenza specific CD4⁺ T cells in the presence of CTLA-4 Ig (129).

While the early experiments described above formed the basis of the popular notion that T cell memory is independent of CD28, there are several caveats of these studies raised by Boesteanu and colleagues that soften the interpretation of these experiments. First, *ex vivo* cytolytic assays utilized adjusted CD8⁺ T cell numbers, failing to take into account differences in secondary challenge expansion with and without CD28 signaling. Second, primary responses to

LCMV were shown to be independent to CD28, unique among most model infections, which calls into question the interpretation of CD28 independent memory responses in this system. Third, studies in CD28^{-/-} T cells carry the caveat of reduced fitness of these cells due to the requirement of CD28 during thymic selection (118).

Taken together, the evidence is growing that early interpretations of CD28 independence of memory were overstated. While further work must be done to further clarify this question (such as the generation of a inducible CD8⁺ specific CD28 knockout mouse), two alternate interpretations exist. First, it is possible that the desire to extrapolate findings from model systems is overstated and that the costimulation requirements are truly different between memory generated against different pathogens. Second, the description of CD28 costimulation requirements might not be binary but rather a continuum, by which memory cells are less dependent on CD28 (or more poised to rely on other costimulatory pathways), but still affected by CD28 signals for optimal function.

CTLA-4, which shares binding partners with CD28, has been less extensively studied than CD28. This is largely due to its expression only on activated CD4⁺ and CD8⁺ T cells. Indeed, CTLA-4 was initially described as an activation marker because of the induction of its message upon antigen engagement (102, 130, 131). Early studies with blocking monoclonal antibodies also confounded the function of CTLA-4, with several investigators proposing that CTLA-4 was an induced costimulatory molecule because of augmented responses following antibody engagement (131-133). Subsequent studies revealed that cross-linking of CTLA-4 inhibited T cell proliferation and IL-2 production (134, 135). However, the description of lymphoproliferation of both CD4⁺ and CD8⁺ subsets in the CTLA-4^{-/-} mouse established the coinhibitory role of CTLA-4 (136). Subsequent decades have revealed many functions for CTLA-4 with little progress in the way of consensus or unification.

The Role of CTLA-4 in CD4⁺ T Cell Responses

CTLA-4 expression is induced not only by antigen stimulation, but also by CD28 signaling (137). Indeed, multiple studies have shown that CD28 triggering is required for maximal CTLA-4 expression and coinhibition (137, 138). The pathways that control *de novo* CTLA-4 expression are not completely understood (139). Seminal work demonstrated that the mTOR inhibitor rapamycin or the NFAT pathway inhibitor cyclosporine function to reduce CTLA-4 expression (140). Consistent with a role for the Akt/mTOR signaling pathway to control CTLA-4 expression, the FOXO family of transcription factors was recently shown to bind to the upstream regulatory region of CTLA-4 and induce expression (141, 142).

CTLA-4 is a Critical Inhibitor of Alloreactive T Cell Responses

CTLA-4 has been established as a critical molecule for controlling antigen specific T cell responses (143, 144) and in models of autoimmune disease, pathogen-responses, and cancer (102, 103, 145). In transplantation, the function of CD28 as a critical regulator of T cell activation led to many studies investigating its potential as a target to induce long term graft survival (102). Indeed, many early studies investigating mechanisms of tolerance found enhanced graft survival using the CD80/CD86-binding molecule CTLA-4 Ig. While it was largely presumed that this was due to the inhibition of CD28 signals, work from several groups demonstrates that the inhibitory effects of blocking CD28 require CTLA-4 signals. In a cardiac allograft model of tolerance induction with CTLA-4 Ig and DST, Judge et al. showed that CTLA-4 signals early following transplantation were required for long term graft survival. Interestingly, donor CD80, but not CD86, signals were critical for the effect of CTLA-4 in this model (146). Similarly, cardiac allografts transplanted into CD28^{-/-} recipients displayed accelerated rejection kinetics when CTLA-4 signals were blocked (147).

More recently, CTLA-4 has been demonstrated to be a critical regulator of alloreactive T cell responses (Table 1). In a model of islet allograft tolerance with anti-CD45RB, CTLA-4 was selectively upregulated and CTLA-4 signals were required for allograft survival (148, 149). Two

recent studies utilizing selective CD28 blocking reagents have demonstrated long-term survival of skin and cardiac allografts (150, 151). In both models, the efficacy of CD28 blockade was dependent on CTLA-4 signals, as concurrent CD28 and CTLA-4 blockade abrogated the enhanced graft survival. Together, these studies establish the importance of CTLA-4 as a regulator of alloreactive T cell responses, and demonstrate that CTLA-4 coinhibitory signals are critical for multiple strategies that enhance allograft survival.

The Traditional Model of CTLA-4 Coinhibition

CTLA-4 has long been understood to function as a coinhibitor that restrains T cell responses, owing to early studies using monoclonal antibodies to block CTLA-4 that augmented murine and human T cell proliferation (139), and the profound auto-proliferative phenotype of CTLA-4 knockout mice, which develop severe polyclonal T cell infiltration in multiple tissues (136). However, a precise understanding of the mechanism of CTLA-4 coinhibition has been elusive, as a number of proposed mechanisms of coinhibition have been proposed.

Here we define the traditional model of CTLA-4 cell-intrinsic coinhibition that is based on two phenomena (Figure 1): 1) the higher binding affinity of CTLA-4 vs. CD28 for the shared ligands CD80/CD86, and 2) the transmission of negative intracellular signals through the CTLA-4 cytoplasmic tail (for a detailed review, see Reference 4). While CD28 and CTLA-4 share the same ligand binding motifs, CD28 binds its ligands monovalently, while CTLA-4 binds bivalently. Multiple groups have demonstrated that CTLA-4 expression can prevent CD28 ligand binding, possibly through its recruitment to the immunological synapse (139). Once CTLA-4 engages its ligands, inhibitory intracellular signaling cascades are initiated that involve the ITIM motif of CTLA-4 and SHP-2, PP2A, and/or cbl-b (139). This model has established that the localization, expression kinetics, and biochemistry of CTLA-4 inhibit T cell responses. However, recent studies have uncovered additional roles for CTLA-4 that significantly broaden our understanding of CTLA-4 as a global regulator of T cell responses.

Mechanisms of Cell Extrinsic CTLA-4 Function

While early studies employing selective CTLA-4 blockade or genetic ablation demonstrated that CTLA-4 functions to dampen T cell proliferative responses, considerable evidence demonstrates that CTLA-4 also functions in a non-cell autonomous, or cell extrinsic, mechanism. Although CTLA-4 KO T cells have an autoreactive phenotype, two groups reported that in bone marrow chimeras of CTLA-4 KO and sufficient cells, CTLA-4 KO cells do not hyper-proliferate (152, 153). This finding demonstrates that the expression of CTLA-4 on T cells is sufficient to provide coinhibition to CTLA-4 deficient T cells. A cell extrinsic function for CTLA-4 found on activated T cells has also been demonstrated in vitro (154).

Recently the importance of the cell extrinsic function of CTLA-4 in vivo has been further investigated. Using a co-adoptive transfer approach of antigen-specific CTLA-4 deficient and WT CD4⁺ T cells, Corse et al. showed that the presence of CTLA-4 on WT CD4⁺ T cells limited the hyper-proliferation of CTLA-4 KO cells to WT levels (155). Interestingly, gene expression profiling on CD4⁺ CTLA-4 KO and WT cells demonstrates that the absence of CTLA-4 induces a signature of cell cycle progression during in vivo immune responses. Similarly, Wang et al. showed in dual adoptive transfer experiments that the presence of CTLA-4 on CD4⁺ Tregs limited the proliferative response of CTLA-4 KO Tregs (156). However, only CTLA-4^{+/+} Tregs, but not CTLA-4^{+/+} Tregs, were sufficient to suppress disease in a RIP-OVA model of diabetes.

In a seminal study, Qureshi et al. provided a compelling mechanistic explanation for cell extrinsic CTLA-4 coinhibition by demonstrating that CTLA-4 facilitates the trans-endocytosis of CD80/CD86 from antigen presenting cells (157). The authors demonstrated that in both human and murine T cells, CTLA-4 selectively removes its ligands from the surface of neighboring cells and targets it for degradation in the endocytic vesicles of the recipient cell. This mechanism is sufficient for suppressor function of CD4⁺ Tregs and Tregs, as blockade of CTLA-4 with

monoclonal antibodies preserved the expression of CD80/CD86 on APCs and enhanced costimulatory function of T cells.

Together, these studies demonstrated that CTLA-4 expressed by Teff acts during primary CD4⁺ clonal expansion in a cell extrinsic suppressive manner. One potential cell extrinsic CTLA-4 mechanism of coinhibition is the induction of IDO expression in APCs mediated through B7 signals, which inhibits T cell responses by limiting tryptophan availability (158). However, outcomes beyond inhibiting proliferation – including limiting effector functions – remain to be shown. As well, CD8⁺ effectors and memory cells upregulate CTLA-4 following TCR engagement, and it remains to be seen whether CTLA-4 on CD4⁺ or CD8⁺ cells limits expansion of CD8⁺ T cells. While CTLA-4 is recognized as a critical attenuator of alloreactive T cell responses, strategies to maximize cell extrinsic CTLA-4 function in settings of transplantation have not been fully explored. For example, strategies aimed at transiently inducing cellular expression of CTLA-4 at the time of transplantation could effectively inhibit alloreactive T cell responses.

CTLA-4 as T Cell:APC Conjugation Inhibitor

Recently an entirely novel mechanism of CTLA-4 inhibition of T cell activation has been elucidated for CD4⁺ T effector (Teff) cells involving the control T cell:APC conjugation. T cell activation requires a sustained stable conjugation between the T cell and cell presenting its cognate antigen. In the absence of cognate antigen, T cells rapidly scan antigen-presenting cells in search of cognate antigen. Recognition of a TCR and its cognate pMHC induces expression of molecules that enable the T cell to arrest its motion and facilitate sustained conjugation. Seminal work from Schneider and colleagues demonstrated that CTLA-4 plays an important role in reversing the TCR-mediated stop signals that occurs when a T cell encounters peptide:MHC complex (159). Using two-photon microscopy, this groups showed that CTLA-4 negative cells increased their contact times upon contacting antigen while CTLA-4 positive cells were unable to

increase APC dwell times. These observations corroborate earlier observations that CTLA-4 is able to associate with the immunological synapse, and provide another distinct cell intrinsic mechanism of action for CTLA-4.

The Role of CTLA-4 on Tregs

The CD4⁺ T cell compartment is comprised of pro-inflammatory effector as well as anti-inflammatory suppressor cells, the best characterized of which are CD4⁺CD25⁺FoxP3⁺ regulatory T cells. FoxP3⁺ Tregs play a critical role in maintaining self-tolerance in models of autoimmunity and transplantation (160). Prior to the discovery of FoxP3 as the master regulator of CD4⁺CD25⁺ suppressor function, CTLA-4 was shown to be constitutively expressed on Tregs (161-163). The identification of FoxP3 as a master factor of Treg function revealed that CTLA-4 is in fact a transcriptional target, leading to the investigation of CTLA-4 in FoxP3⁺ Tregs (164).

While experiments using CTLA-4 blockade in vivo and in vitro produced conflicting results, more recent work has established that CTLA-4 is required for Treg-mediated suppression of immune responses (164). In a FoxP3⁺ conditional knockout mouse model of CTLA-4 deletion, Wing et al. definitively demonstrated that CTLA-4 deficiency in FoxP3⁺ Treg cells is sufficient to allow the development of spontaneous and fatal autoimmunity, reminiscent of CTLA-4 KO mice (165). Conditional deletion of CTLA-4 on Tregs also leads to enhanced tumor immunity and an enhanced allogeneic proliferative response (165). Recently, several groups have shown that reduced expression of CTLA-4 on Tregs leads to dysfunctional Treg function in vitro and in vivo (141, 142, 166). Similarly, suppression by human T cells requires CTLA-4 but not FoxP3 (167). Recently, Zhang et al showed that CD28-deficient Tregs were unable to maintain self-tolerance (166). CD28-deficient Tregs had diminished CTLA-4 expression and fail to survive following a skin graft challenge in the presence of CD28 sufficient Tregs.

While these studies have established that CTLA-4 is required for Treg mediated immunosuppression, it remains to be seen whether the suppressive effect of Tregs is mediated by

solely by high CTLA-4 expression or whether CTLA-4 has a unique biochemical properties when expressed on Tregs. Many studies have provided evidence of the former scenario, that CTLA-4 expression on non-FoxP3⁺ Tregs is sufficient to induce a suppressor function. For example, Tai et al. demonstrated that activated Teffs had in vitro suppressive capacity similar to Tregs, and both cell types required the extracellular, but not the intracellular, CTLA-4 domains (154). Studies examining cell extrinsic suppressive function of CTLA-4 have thus far not identified a unique role for CTLA-4 on Tregs (155, 156). This is further supported by the finding that Teffs and Tregs demonstrate a similar ability to trans-endocytose CD80/CD86 molecules via CTLA-4 (157). Together, these findings suggest that suppression is a function of high CTLA-4 expression, not a unique property of CTLA-4 when expressed on Tregs, as evident by the fact that high CTLA-4 expression can induce suppressive function on otherwise effector T cells.

Interestingly, recent work from Rudd and colleagues has demonstrated a divergent function of CTLA-4 on Tregs and Teffs that is independent of expression level (168). Using two-photon microscopy in an antigen-specific system to monitor T cell migration, the investigators were able to sensitively detect the conjugation patterns and kinetics of T cell:APC conjugates. While the presence of CTLA-4 limited the conjugation time with APCs for CD4⁺ Teffs, Tregs were resistant to CTLA-4-mediated reverse stop signals. This study supports a model in which APC conjugation of Teffs is limited by CTLA-4, while longer conjugation times of Tregs are better able to occupy APC binding sites. While this striking finding is consistent with the function of Tregs as occupying antigen binding sites on APCs and out-competing Teffs for costimulatory receptors, it indicates that the reverse stop potential of a cell does not rely solely on the CTLA-4 expression level. This work is the most thorough demonstration that CTLA-4 might have a different functional role on Tregs compared to non-Tregs, and future studies will examine the mechanistic basis for this result, whether due to a biochemical property of CTLA-4 expressed by Tregs and Teffs, or the presence of additional molecules that can override CTLA-4 function.

The Expression of CTLA-4 on Th17 cells

Th17 cells are a pro-inflammatory CD4⁺ lineage that provides immunity to fungi, including *Candida albicans*, and extracellular bacteria. Th17 cells can be potent mediators of pathogenic T cell responses in autoimmune disease, and have been shown to participate in graft rejection in several animal models and in human studies (169, 170).

Recently it has been demonstrated that Th17 cells are resistant to CD28/CTLA-4 blockade with CTLA-4 Ig in vitro and in vivo (83, 138, 171). Human and murine Th17 cells express high levels of CTLA-4 compared to primary and memory Th1 cells (138, 171). In an antigen-specific pathogen immunization model, murine Th17 cells also expressed more CTLA-4 than Th1 cells and correlated with CTLA-4 Ig resistant skin graft rejection (171). Interestingly, the expression level of CTLA-4 correlated to the level of augmentation of proliferation in the presence of CTLA-4 blockade with antibodies, suggesting that CTLA-4 acts in a cell intrinsic manner on Th1 and Th17 cells. Consistent with the finding that Th17 cells are reliant on CTLA-4 signals, Ying et al found that CD28^{-/-} Th17 cells were augmented by CTLA-4 blockade with CTLA-4 Ig (85).

Interestingly, costimulation blockade with the CTLA-4 Ig derivative belatacept has demonstrated mixed efficacy in Th17 mediated disease (102). Renal transplant recipients who experience rejection while being treated with belatacept, but not calcineurin inhibitors, had an elevated frequency of Th17 memory cells in their blood, suggesting that Th17 memory cells participate in graft rejection in the setting of CD28/CTLA-4 blockade therapy (138). Together, these studies suggest that CTLA-4 Ig, which was designed to induce deletion and anergy through blockade of CD28 signals, might have unintended consequences on specific T cell populations through blockade of endogenous coinhibitory signals.

Unanswered Questions About CTLA-4 Function

CTLA-4 has been established as a critical regulator of peripheral tolerance, and offers tremendous therapeutic potential as a target that can be harnessed to limit alloreactive T cell responses. In addition to the traditional model of CTLA-4 function, recent studies outlined here have broadened our understanding of the mechanisms of action of CTLA-4 coinhibition. An updated “new” model of CTLA-4 function can be segregated into three components: 1.) CTLA-4 can inhibit cell autonomous T cell activation, 2.) CTLA-4 expressed on FoxP3⁺ Tregs and Teffs can act in a cell extrinsic manner to suppress T cell responses 3.) CTLA-4 has distinct expression patterns on T cell subsets with functional consequences. Perhaps the greatest challenge facing our complete understanding of CTLA-4 biology is that none of these modes of action are mutually exclusive. As such, more work is needed to elucidate the contexts under which each of these mechanisms do or do not occur.

For investigators, the most salient conceptual dichotomy is how to reconcile cell-intrinsic and cell-extrinsic CTLA-4 functions. As these studies were largely temporally separated – with earlier studies focused on traditional biochemical cell autonomous functions of CTLA-4 and more recent studies elucidating cell extrinsic functions -- more careful investigation of the potential interplay of these two phenomena is needed. For example, do cell-intrinsic and -extrinsic functions occur simultaneously? Do they occur preferentially in particular T cell subsets? Are there synergistic or antagonistic effects of these mechanisms on T cell activation?

Additionally, regarding the cell extrinsic role of CTLA-4, further work needs to elucidate the differences between Teffs and FoxP3⁺ Tregs. While it is well established that Tregs require CTLA-4 for suppression, it remains unclear whether the function of CTLA-4 is critically different on Tregs than Teffs. Several groups have now shown that the expression of CTLA-4 is sufficient for any CD4⁺ T cell to have a suppressive function, while work from Lu et al. provides the most compelling subset-specific functional difference to date. The goal of costimulation-blockade based biologic therapies includes limiting effector T cell populations while retaining functional Tregs, greater understanding of distinctions of CTLA-4 function on these populations is critical.

In conclusion, our understanding of “the original” T cell coinhibitory receptor has deepened substantially in recent years. The updated model of CTLA-4 demonstrates the importance of continued mechanistic investigations of established therapeutic pathways, as new understanding can lead to further advances in clinical applications. In the context of the higher-than-anticipated acute rejection rates observed in belatacept-based regimens for renal transplant patients, these new insights suggest that perhaps perturbations in the cell-extrinsic functions of CTLA-4 or Th17 cells might be playing a role. While many challenges remain to unify the model of CTLA-4 coinhibition, it remains a therapeutic target with tremendous promise to effectively limit alloreactive T cells.

Blockade of CD28/CTLA-4 Signals in Autoimmunity and Transplantation

CD28 blockade with CTLA-4 Ig has been extensively studied in transplantation (102). Over twenty years ago it was first demonstrated that CTLA-4 Ig can prevent allograft rejection in murine models (172, 173) and non-human primates (174). Since then, CTLA-4 Ig derivatives have been effective in the clinical treatment of several autoimmune diseases. CTLA-4 Ig derivative abatacept is an approved therapy for rheumatoid arthritis, and has been studied in pre-clinical models of lupus, EAE, and Type I diabetes. Interestingly, in murine studies CTLA-4 Ig was ineffective at preventing EAE disease relapse (175) and was ineffective in a clinical trial of relapsing-remitting MS (102). In the NOD model of type I diabetes, CTLA-4 Ig resulted in disease exacerbation despite inhibition of Th1 and Th2 cytokines, a finding that is attributed to the inhibition of Tregs (161, 176). These results suggest that the complexities of autoimmune T cell responses might render some conditions more amenable to CD28/CTLA-4 blockade, and underscores the importance of gaining greater understanding mechanisms of pathogenesis as more specific immunomodulatory agents are developed.

The CTLA-4 Ig derivative belatacept (LEA29Y) was designed to maximize CD80 and CD86 binding for solid organ graft rejection prophylaxis (177). In a non-human primate model

(NHP) of renal transplantation, belatacept was superior to CTLA-4 Ig in preventing graft rejection (174). Recent work in NHP models have demonstrated that belatacept couples with the mTor inhibitor sirolimus to prolong graft survival without the need for induction therapy (178, 179). Murine studies have demonstrated the potential for integrin blockade as an adjunct therapy for costimulation blockade with CTLA-4 Ig. Kitchens and colleagues showed that anti-LFA-1 or anti-VLA-4 enabled indefinite graft survival in minor antigen mismatch and full allogeneic models (180, 181). Reisman et al demonstrated that anti-LFA-1 treatment resulted in the egress of naïve T cells from the peripheral LNs to the circulation, as well as an increase in Tregs in graft-draining nodes (182). Anti-LFA-1 therapy has also been demonstrated efficacy in NHP renal transplant models (183).

During clinical development, belatacept was shown to have comparable graft survival as well as a diminished cardiovascular toxicity profile compared to CNIs, and was FDA approved for renal transplantation in 2011 (184-186). Some caveats have been observed in the course of belatacept therapy, however. First, belatacept is associated with increased PTLD in patients who are EBV naïve at the time of transplantation. As well, belatacept is associated with an increased incidence of acute cellular rejection early following transplantation. Investigation into the mechanisms of this rejection might reveal certain T cell subsets that are pathogenic and resistant to CD28/CTLA-4 blockade.

The Role of CD8⁺ T Cells in Allogeneic Heterologous Immunity

The CD8⁺ T Cell Pool is Comprised of Heterogeneous Subsets

CD8⁺ T cell responses are critical for immunity to viruses and intracellular bacteria, so-called Type 1 responses (3, 17, 18). There is growing evidence that microbial stimulation history might significantly influence the character of alloreactive memory. It has long been recognized that a significant portion of the human T cell compartment can respond to microbial antigen (29). For example, frequencies of EBV and CMV reactive CD8⁺ T cell memory ranges from 10-40%

(29). This finding has particular significance to transplantation, as work from several groups has demonstrated that memory cells raised against microbial antigens can be cross-reactive with allogeneic antigen, a process termed heterologous immunity (13, 30-32). Several studies have observed that EBV and CMV-elicited CD8⁺ T cells can cross-react with MHC Class I allogeneic antigen complexes (30-32, 187). EBV and CMV specific CD4⁺ memory T cells can also recognize allogeneic MHC Class II antigen (39, 40). Surprisingly, in a systematic evaluation of cross-reactivity to allogeneic MHC molecules, Amir et al found that 45% of CD4⁺ and CD8⁺ T cell clones were reactive to allogeneic antigen (16).

While this work has established that microbe elicited T cells that survive to memory can cross-react with allogeneic p:MHC complexes, work in murine systems has demonstrated that allogeneic heterologous immunity can have important consequences for graft rejection (4, 13). Seminal work from Adams and colleagues demonstrated that LCMV T cell memory was capable of mediating graft rejection in a fully allogeneic skin graft model (41). Importantly, this study showed that CD8⁺ memory is a barrier to tolerance induction with costimulation blockade and mixed chimerism. Resistance to tolerization regiment was greater in mice serially infected with other viruses (VSV or VV), demonstrating that heterologous immunity can be a potent mediator of graft rejection. Brehm et al extended these studies through demonstration of cross-reactivity between LCMV epitopes and allogeneic antigen. The specific LCMV-specific clones that cross-react varies with both the allogeneic target and the private TCR specificities of the host (15), demonstrating that heterologous immunity is a highly specific phenomenon. Although providing limited mechanistic insight and involving a murine pathogen with limited clinical relevance, these observation provides striking evidence that microbial stimulation history has profound implications for allogeneic T cell responses and immunomodulatory strategies to prevent graft rejection.

The Role of T cell Affinity in Forming T Cell Memory and Allogeneic Heterologous Immunity

Allogeneic heterologous immunity requires the TCR recognition of two distinct peptide:MHC complexes, that of pathogenic peptide:MHC and allogeneic peptide:MHC. While very high and low affinity T cells are eliminated during thymic selection against self peptide:MHC, the affinity of T cells for allogeneic peptide:MHC is not physiologically restrained. Indeed studies have shown that allogeneic recognition occurs at a broad range of TCR affinities from 1-49 μM (36-38). By comparison, viral epitope recognition occurs at 1-20 μM and cancer epitope recognition has been established at 20-200 μM , while self-epitopes occur at lower affinities of greater than 250 μM (12). The independent recognition of multiple peptide:MHC complexes as occurs during allogeneic heterologous immunity suggests that each of these interactions has a unique affinity. For example, low affinity microbial memory may recognize allogeneic antigen at higher affinity, or vice versa.

There are few examples of the structure and affinity of allogeneic heterologous immunity pairs. Perhaps the most well studied example is of human CD8^+ T cell clone elicited against EBV peptide that recognizes two allogeneic peptide:HLA combinations. Structural studies reveal that the LC13 T cell clone recognizes all three peptide:MHC combinations in an identical manner via an induced-fit mechanism of molecular mimicry (37). This investigation revealed that an allogeneic peptide:MHC recognition can occur in a similar manner as recognition of pathogen peptide:MHC. Interestingly, recognition of an allogeneic peptide:MHC was higher affinity (1 μM) than for its priming EBV peptide:MHC (70 μM) (37, 188). The affinity of the murine 2C TCR for its allogeneic H-2L^d was measured at 2 μM , compared to 83 μM for sygeneic H-2K^b (36). While few in number, these studies have provided important insight into the nature of allogeneic peptide:MHC recognition by the TCR and modified the hypothesis that allogeneic recognition is intrinsically different from conventional peptide:MHC interactions by virtue of being peptide-independent binding. These structural studies reveal that allogeneic T cell

recognition is very similar, if not superimposable on conventional peptide:MHC recognition (12, 47).

The functional outcome of allogeneic peptide:MHC recognition by a memory T cell likely depends on a variety of factors regarding the environment. Considering the requirement of the TCR to recognize a cross-reactive peptide:MHC at a different affinity than its priming antigen, this raises the importance of understanding the qualities of CD8⁺ T cell memory primed at a variety of TCR affinities. While a great body of work has been amassed regarding CD4⁺ and CD8⁺ T cell memory using monoclonal TCR systems, these systems do not take into account phenotypic and functional differences that are a consequence of altered affinity (189, 190).

Several very recent studies have carefully investigated the functional characteristics of low affinity effector and memory cell generation. Using an infection model of OVA altered peptide ligands (APLs) for the OT-I system, Zehn and colleagues showed that low affinity primed CD8⁺ T cells (ranging from 2.8-650 fold lower EC₅₀) were capable of forming long-term memory (191). Low affinity cells had similar upregulation of activation markers and costimulation molecules during the primary effector phase. Low affinity primed cells, however, exited the periarteriolar lymphoid sheath (PALS) and entered the bloodstream before day 4 post-infection, while high affinity cells are not detected in the blood until later. Importantly, low affinity primed OT-I cells were capable of robust recall responses. When rechallenged with heterologous OVA APL-expressing pathogen, equal precursor frequencies of both low and high affinity primed CD8⁺ cells responded with a magnitude equivalent to the affinity of the rechallenge antigen. These observations establish that low affinity CD8⁺ memory cells are maintained and are capable of functional qualities similar to those of high affinity cells. While this work established the role of TCR affinity in dictating the kinetics of a primary response, the phenotypic and functional characteristics of low affinity primed memory CD8⁺ T cells are poorly understood.

A thorough investigation of CD8⁺ memory against influenza revealed that CD27 costimulation is a necessary for low affinity cells to be maintained into memory (192). CD8⁺ T

cells responses primed in the absence of CD27:CD70 interactions were devoid of low affinity cells and less clonally diverse. Interestingly, CD27 was required for the formation of effector memory cells specifically, raising the possibility that low affinity cells are biased towards an effector memory phenotype. An elegant study by Gottschalk et al investigated the functional consequences of antigen density and TCR affinity (193). Using the 5C.C7 TCR that recognizes its natural ligand MCC as well as the APL 102S, they compared two doses of peptide that induced the same amount of proliferation. Compared to the APL at higher density, the high affinity ligand MCC induced greater sensitivity to IL-2, as visualized by greater CD122/CD25 expression levels, and elicited more stable T:DC interactions in vivo. Gene array analysis of these two conditions revealed differential induction of a network of genes to the high affinity ligand. This work carefully demonstrates that within the scope of TCR functional avidity, TCR affinity and antigen density have much different functional consequences. More importantly, TCR affinity may be more sensitively sensed by the T cell than antigen density or duration.

Recent work in APL systems has provided further characterization of the functional consequences of TCR affinity in the context of autoimmunity. Gronski et al studied several APLs of the LCMV epitope GP33 (194). Using a RIP-gp model of diabetes, P14 cells primed with an intermediate but not low affinity APL were capable of inducing disease, although at a lower frequency than WT mice. Interestingly, the intermediate APL primed T cells were similarly cytotoxic to the high affinity primed T cells but proliferated less in vivo. These results demonstrate that effector function and proliferative capacity can be decoupled on T cells.

In the OVA APLs in RIP-OVA mice, King et al showed that a certain threshold of TCR affinity induced asymmetric cell division during activation. This asymmetric division was marked by greater CD8 and VLA-4 expression and a higher frequency of SLEC formation, which resulted in better infiltration of peripheral tissues and induction of diabetes (195). This study is the first study implicating asymmetric cell division as a consequence of TCR affinity and provides another mechanism of the development of quality CD8⁺ effector responses.

Enouz et al extended the investigation of OVA APL by generating a TCR transgenic mouse that is low affinity for the OT-I OVA epitope, termed OT-3 (196). The affinity of OT-3 cells for OVA is at the threshold for negative selection, however not all cells were negatively selected. OT-3 cells are capable of forming memory and responding to challenge antigen. Surprisingly, OT-3 cells in the periphery were capable of responding to lower affinity APLs to produce cytokine, lyse target cells, and induce diabetes. These results demonstrate that the threshold of TCR affinity to activate a response in the periphery is well below that of negative selection in the thymus, a finding which highlights the importance of understanding low affinity memory cells.

In scenarios relevant to allogeneic heterologous immunity, the role of TCR affinity has not been carefully assessed. For example, are pathogen elicited CD8⁺ T cells more functionally poised to respond to cross-reactive allogeneic antigen of lower or higher affinity? Are low affinity memory cells relevant in this scenario? What immunomodulatory strategies are effective against alloresponses of varying affinities? These outstanding questions are critical to advancing both understanding of allogeneic T cell responses and new strategies to inhibit graft rejection.

Chapter 2. High CTLA-4 Expression on Th17 Cells Results in Increased Sensitivity to CTLA-4 Coinhibition and Resistance to Belatacept Introduction

During a secondary T cell response, memory T cells maintain the functional and phenotypic properties that reflect their priming conditions (48). Recent studies have shown that pathogen-primed memory T cells can cross-react with alloantigen (16, 41) and that alloreactive T cells are inherently more polyspecific for peptide:MHC than conventional T cells (42, 44), suggesting that the alloreactive memory T cell pool reflects the pathogen-specific stimulation history of an individual. The heterogeneity of T cell memory recall responses is critically important for transplant recipients who receive lifelong immunosuppression to prevent T cell mediated graft rejection.

The recently approved CTLA-4 Ig derivative belatacept inhibits graft-specific immune responses by blocking CD28/CTLA-4 signals on T cells, and offers significantly improved long-term graft function and fewer toxicities compared to calcineurin inhibitors. However, belatacept is associated with a high incidence of pathologically severe acute rejection within one year of transplantation (186). While the mechanism of this rejection is currently unknown, the kinetics and severity of this phenomenon suggests that a CD28/CTLA-4 blockade resistant population of T cells mediates this rejection.

Although classically studied CD4⁺ Th1 responses are known to depend on CD28 signals for optimal secondary recall responses (41, 129), the costimulation requirements of Th17 cells are less understood. Intriguingly, recent studies have suggested differences in the costimulation signals that mediate differentiation of naïve Th0 cells into Th1 or Th17 cells (83-87, 89). While this work has focused on cosignalling during primary differentiation into Th17 cells, little is known about the costimulation requirements of memory Th17 cells during subsequent recall responses.

In this study we investigated the relative contribution of Th17 cells to alloreactivity and their susceptibility to costimulation blockade with belatacept. We demonstrate that Th17 memory cells express high levels of the coinhibitory receptor CTLA-4, which results in resistance to belatacept and is associated with rejection in renal transplant recipients. This study demonstrates that the costimulatory requirements of CD4⁺ Th1 and Th17 subsets are distinct, and highlights the differential susceptibilities of heterogeneous microbe-elicited memory populations to immunomodulation with costimulation blockade.

Materials & Methods

Human Study Approval. Healthy donor peripheral blood mononuclear cells (PBMC) and patient PBMC and lymph node samples were isolated following protocols approved by the Emory University Institutional Review Board (IRB #00006248).

Human Alloreactive Proliferation Assay. Monocyte-derived dendritic cells (MDDCs) were derived from 3×10^6 fresh PBMC in a 6 well plate in RPMI supplemented with 10% human AB serum (Mediatech, VA), 2.4 mM L-glutamine. Non-adherent lymphocytes were washed off 4 hours later, and adherent cells were cultured with 50 ng/mL of IL-4 and 100 μ g/mL of GM-CSF (R&D Systems) for 5-7 days at 37 °C. Responders were derived from healthy donor fresh PBMC CFSE labeled with 5 μ M CFSE (Invitrogen) for 3 min and co-cultured with allogeneic MDDC at a 3:1 ratio in 96 well flat-bottomed plates for 4 d at 37 °C. Some cultures were restimulated with 30 ng/mL PMA and 400 ng/mL Ionomycin (Sigma) for 4 h, and 10 μ g/mL GolgiStop (BD Biosciences) was added for the final 3 h. To determine frequency of divided CD4⁺ fractions in response to allogeneic stimulation, cells were gated on CD4⁺CD45RA⁺CFSE^{low} or CD4⁺CD45RA⁻CFSE^{low}, followed by either IFN- γ ⁺ or CCR6⁺IL-17⁺ as described. To determine the effect of belatacept following allogeneic stimulation, cells were first gated on CD4⁺CD45RA⁺IFN- γ ⁺ (CD45RA⁺ Th1), CD4⁺CD45RA⁻IFN- γ ⁺ (CD45RA⁻ Th1), or CD4⁺CD45RA⁻CCR6⁺ Th17 (CD45RA⁻CCR6⁺ Th17) followed by CFSE^{low} divided cells. The effect of belatacept on CD4⁺ subsets following allogeneic stimulation was calculated as $(1 - (\% \text{ CFSE}^{\text{low}} \text{ with belatacept} / \% \text{ CFSE}^{\text{low}} \text{ with no treatment})) \times 100$.

Human Polyclonal Stimulation and Costimulation Blockade. Fresh or frozen PBMC from healthy donors cells were cultured in 96 well flat-bottomed plates in RPMI supplemented with 10% human AB serum (Mediatech, VA) and 2.4 mM L-glutamine. Frozen PBMC were rested

overnight before stimulation. Cells were stimulated with either 1 $\mu\text{g}/\text{mL}$ (PBMC) or 2 $\mu\text{g}/\text{mL}$ (lymph node T cells) functional grade anti-CD3 (OKT3; eBiosciences) in the presence of belatacept (100 $\mu\text{g}/\text{mL}$; Bristol-Myers Squibb, NY) or human IgG1-Fc control (BioXCell, Lebanon, NH), or with anti-CD3/CD28 Dynabeads (Invitrogen) in the presence of 10 $\mu\text{g}/\text{mL}$ anti-CTLA-4 (BN13; BioXCell, Lebanon, NH) or mouse IgG1 (BioXCell, Lebanon, NH), as indicated. Cells were washed twice with media and restimulated with PMA/Iono for 4 h as described above. CD4⁺ T cell subsets were defined by the following gating strategy: CD45RA⁺ Th1, CD4⁺CD45RA⁺IFN- γ ⁺; CD45RA⁻ Th1, CD4⁺CD45RA⁻ IFN- γ ⁺; CD45RA⁻CCR6⁺ Th17, CD4⁺CD45RA⁻CCR6⁺IL-17⁺. The change in frequency of CD4⁺ populations was calculated as (% Cytokine⁺ Blockade/% Cytokine⁺ IgG)x100 of the indicated population.

Surface and Intracellular Staining of Healthy Donor and Patient Cells. Fresh PBMC were isolated from normal healthy donors under IRB approval using CPT tubes and plated at 1×10^5 /well in round-bottom 96 well plates. PMA/Iono and GolgiStop were added for 4 h as described above. Surface staining was performed for 20 min at 23°C using the following antibodies: CD4 (L200 or RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD19 (HIB19), CD28 (CD28.2) or IgG1 κ , CD45RA (MEM-56 or HI100), and CCR6 (TG7/CCR6) or IgG2b κ . Cells were prepared for intracellular staining following manufacturer's protocol (BD Biosciences Fix/Perm Kit) and stained with the following antibodies: CTLA-4 (CD152, BN13) or IgG2a κ , IFN- γ (45.B3), and IL-17A (eBio64DEC17). All panels depict CD4⁺CD45RA⁺ or CD4⁺CD45RA⁻ populations. For CTLA-4 surface capture assay, anti-CTLA-4 PE was added for the final 3 h of PMA/Iono stimulation, and cells were prepared for intracellular flow cytometry. Samples were analyzed using an LSR II flow cytometer (BD Biosciences), and data was analyzed using FlowJo software (Treestar, San Carlos, CA).

Transplant Recipient Cohort Selection. Patients were selected as rejectors if an episode of acute cellular rejection was diagnosed by renal biopsy following transplantation (1-6 months post-transplant for belatacept-treated patients, mean 2.7 months; or 1-14 months post-transplant for tacrolimus-treated patients, mean 5.8 months). Stable patients were free from rejection episodes through the time of assessment (mean time of assessment 9.5 months). Belatacept stable, belatacept rejectors, and tacrolimus rejectors were not significantly different for gender or age (59 ± 3.0 , 49 ± 5.4 , 54 ± 4.9 y.o., respectively), were all first time transplant recipients, were similarly HLA mismatched, and had comparable common indications for transplantation.

Renal Transplant Recipient PBMC and LN cells. PBMC and LN cells from patients were cryopreserved at -80°C in RPMI with 40% FBS and 10% DMSO. 1×10^6 thawed PBMC were cultured in 96 well round-bottom plates and rested overnight in RPMI with 10% human AB serum (for PBMC; Mediatech, VA) or 10% FBS and 20 U/mL IL-2 (for LN cells; eBiosciences) and 2.4 mM L-glutamine. Cells were washed twice with media before stimulation with either anti-CD3 for 3 d (LN cells, as described above) or PMA/Iono for 4 h (PBMC). Patient PBMC were defined by the following gating strategy: Th1 Memory, $\text{CD4}^+\text{CD45RA}^-\text{CCR6}^-\text{IFN-}\gamma^+$, Th17 Memory, $\text{CD4}^+\text{CD45RA}^-\text{CCR6}^+\text{IL-17}^+$ LN CD4^+ T cell subsets were defined by the following gating strategy: CD45RA^+ Th1, $\text{CD3}^+\text{CD4}^+\text{CD45RA}^+\text{IFN-}\gamma^+$; CD45RA^+ Th1, $\text{CD3}^+\text{CD4}^+\text{CD45RA}^-\text{IFN-}\gamma^+$; CD45RA^+ Th17, $\text{CD3}^+\text{CD4}^+\text{CD45RA}^-\text{IL-17}^+$ The effect of belatacept was calculated as described for healthy donors.

Statistics. Proliferation, cytokine production, and population frequencies between groups were compared using Mann-Whitney non-parametric (two-tailed) analysis. All analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Significance was determined as $*p < 0.05$, $**p < 0.005$, $***p < 0.001$. Data shown and described depict average \pm SEM.

Results

Alloreactive Th17 memory cells are resistant to belatacept

Th17 memory cells are important for fungal and extracellular bacterial immunity (81, 197), and can be potent drivers of autoimmune disease (67, 198). However, their involvement in alloreactivity is not well defined (199). We stimulated CFSE-labeled responders with allogeneic monocyte-derived dendritic cells (MDDC) followed by brief PMA/Iono restimulation to identify alloreactive Th1 and Th17 cells. Alloreactive CD45RA⁻ Th1 cells were found in the CCR6⁻ and CCR6⁺ compartments ($13.8 \pm 2.85\%$ and $15.7 \pm 2.08\%$ of CFSE^{lo}, respectively), while CD45RA⁻ Th17 cells were exclusively found within the CCR6⁺ fraction (Figure 2.1 A-B)(200). We found that $2.78 \pm 0.478\%$ of total alloreactive CCR6⁺ memory cells were Th17 cells (Figure 1B). After 4 days of allogeneic stimulation, CD45RA⁺ Th1, CD45RA⁻ Th1 and CD45RA⁻ CCR6⁺ Th17 cells contained similar frequencies of divided cells ($7.95 \pm 2.05\%$ CD45RA⁺ Th1, $7.66 \pm 1.66\%$ CD45RA⁻ Th1, $11.8 \pm 3.2\%$ CD45RA⁻CCR6⁺ Th17, Figure 2.1 C-D). This demonstrates that like Th1 cells, Th17 cells can participate in alloreactive responses.

The addition of belatacept to allogeneic co-cultures resulted in the inhibition of CD45RA⁺ Th1 and CD45RA⁻ Th1 cells ($72.4 \pm 5.46\%$ and $82.0 \pm 10.5\%$ of no treatment, respectively, Figure 2.1C and 2.1E). In contrast, we found that CD45RA⁻CCR6⁺ Th17 cells were augmented by belatacept in each pair evaluated ($127 \pm 11.8\%$ of no treatment, Th17 vs. CD45RA⁺ Th1 $p = 0.0079$, Th17 vs. CD45RA⁻ Th1, $p = 0.0079$, Figure 2.1C and 2.1E), suggesting that Th17 cells might differentially utilize on the CD28/CTLA-4 pathway compared to other CD4⁺ memory subsets.

Th17 memory cells significantly upregulate the coinhibitor CTLA-4

Multiple studies have indicated that CD4⁺ Th1 memory recall responses require CD28 (41, 129), while less is known about the costimulation requirements during recall of Th17

memory cells. Because belatacept inhibits both CD28 and CTLA-4 signals by binding to their common ligands CD80/CD86 on APCs, we next investigated the CD28/CTLA-4 costimulatory pathways on Th1 and Th17 cells.

Nearly all resting naïve CD45RA⁺ and memory CD45RA⁻ CD4⁺ cells were CD28⁺ and expressed high levels of CD28 (Figure 2.2A). CTLA-4, which is also blocked by belatacept, is maintained in intracellular vesicles that rapidly flux to and from the cell surface during activation. Staining for total intracellular CTLA-4 revealed that nearly half of resting memory CD4⁺ T cells expressed low levels of CTLA-4, and that the level of expression rose after restimulation (Figure 2.2B). Th17 memory cells expressed significantly more CTLA-4 than Th1 memory, as the majority of IL-17⁺ cells ($64.4 \pm 2.55\%$) were CTLA-4^{high} compared to a small fraction of IFN- γ ⁺ cells ($24.1 \pm 3.33\%$)(Figure 2.2B-C)

We next sought to determine if the differences in the expression of CTLA-4 between Th1 and Th17 memory cells were reflected in CTLA-4 levels on the cell surface. Using a modified staining protocol to capture rapidly endocytosed surface CTLA-4, we found that very few resting naïve and memory cells expressed surface CTLA-4, while restimulated memory cells upregulated CTLA-4 (Figure 2.2D). Strikingly, significantly more Th17 memory cells ($84.8 \pm 2.25\%$) were CTLA-4⁺ compared to Th1 memory cells ($37.0 \pm 2.77\%$) (Figure 2.2D). Furthermore, Th17 memory cells expressed significantly more CTLA-4 than Th1 (Figure 2.2E). These data are the first demonstration that Th1 and Th17 memory cells differ significantly in their expression of CTLA-4 following restimulation.

CD45RA⁻ Th17 cells are uniquely sensitive to CTLA-4 coinhibition

We hypothesized that both alloreactive and non-alloreactive Th17 memory cells would be resistant to belatacept due to relatively greater sensitivity to signaling through coinhibitory CTLA-4. Following polyclonal stimulation with soluble anti-CD3, CD45RA⁺ and CD45RA⁻ Th1 cells were inhibited by belatacept ($53.2 \pm 7.53\%$ and $87.11 \pm 4.66\%$ of IgG-Fc, respectively,

Figure 2.3A-B). In contrast, CD45RA⁻ Th17 cell frequencies were significantly augmented ($130 \pm 9.5\%$ of IgG-Fc, $p = 0.0001$ vs CD45RA⁺ Th1 and CD45RA⁻ Th1, Figure 2.2A-B). Both CD45RA⁻ Th1 and CD45RA⁺ Th1 cells were inhibited over a range of belatacept concentrations in a dose-dependent manner, while CD45RA⁻CCR6⁺ Th17 cells were augmented over a range of belatacept doses (Supplemental Figure 2.1). Together, these results demonstrate that the relative contribution of CD28/CTLA-4 signaling is distinct between Th1 and Th17 cells.

To directly test whether CD45RA⁻ Th17 cells are more sensitive to coinhibitory signals through CTLA-4 we next interrogated the function of the CTLA-4 pathway on Th17 cells. While both CD45RA⁺ and CD45RA⁻ Th1 responses were augmented in the presence of anti-CTLA-4, the CD45RA⁻ Th17 cell population was increased to a significantly greater degree than the CD45RA⁻ Th1 population ($168 \pm 15.0\%$ vs $118.5 \pm 8.10\%$ of IgG, $p = 0.0071$, Figure 2.3C-D). Taken together, these results demonstrate that CD45RA⁻ Th17 cells are more sensitive to coinhibition than CD45RA⁻ Th1 cells.

Renal transplant recipient-derived lymph node CD45RA⁻ Th17 cells are resistant to belatacept

Belatacept has been recently approved for post transplant immunosuppression in renal transplant recipients, but it has been associated with more frequent and severe early acute rejection episodes compared to calcineurin inhibitor based regimens (186). The critical site of action of belatacept on primary and memory T cell activation is likely to be the site of T cell:APC interaction in secondary lymphoid tissue (82), leading us to investigate the effect of belatacept on Th1 and Th17 cells derived from lymph nodes in a cohort of patients who received renal allografts.

Lymph node cells harvested from renal transplant recipients prior to the induction of immunosuppressive therapy were stimulated in the presence of CD28/CTLA-4 blockade with belatacept. Similar to T cells in the peripheral blood of healthy donors (Figure 2.3A-B),

belatacept inhibited the proliferation of CD45RA⁺ Th1 cells ($69.1 \pm 3.43\%$ of IgG-Fc) and modestly reduced the frequency of CD45RA⁻ Th1 cells ($94.2 \pm 2.08\%$ of IgG-Fc, Figure 2.4A-B). Lymph node-derived CD45RA⁻ Th17 cells, in contrast, were augmented in the presence of belatacept compared to control Ig molecule ($115.5 \pm 2.43\%$ of IgG-Fc, $p = 0.0049$ vs CD45RA⁻ Th1, Figure 2.4A-B). Thus, in contrast to CD45RA⁺ and CD45RA⁻ Th1 cells, CD45RA⁻ Th17 cells from the secondary lymphoid organs of transplant recipients are also augmented in the presence of belatacept.

An elevated Th17 memory cell frequency correlates to rejection during belatacept therapy in renal transplant recipients

We hypothesized that acute cellular rejection that occurs in patients treated with belatacept might be associated with elevated Th17 memory cell frequencies. To investigate this possibility, we studied a cohort of renal transplant recipients treated with standard immunosuppressive regimens containing either belatacept or the calcineurin inhibitor tacrolimus. Belatacept treated patients were classified based on whether they experienced biopsy-proven acute cellular rejection early following transplantation or were stable (described in Methods). Tacrolimus-treated patients were similarly classified as rejectors by the occurrence of biopsy-proven acute cellular rejection.

At baseline, the frequencies of CCR6⁻ Th1 and CCR6⁺ Th17 memory cells were not significantly different between each of these three groups (Figure 2.5A-B). We next investigated the Th1 and Th17 memory cell frequencies at 1 month post-transplant for belatacept stable patients and the time of rejection in belatacept or tacrolimus rejectors. The frequency of Th1 memory cells was not significantly different between belatacept-treated patients who were stable or rejecting, nor were they different between patients rejecting while being treated with belatacept vs those rejecting on tacrolimus (Figure 2.5C-D). In contrast, the frequency of Th17 memory cells was significantly elevated in belatacept rejectors ($2.80 \pm 0.593\%$) compared to stable

belatacept-treated patients ($0.801 \pm 0.203\%$, $p = 0.0286$) and tacrolimus rejectors ($1.26 \pm 0.261\%$, $p = 0.0286$, Figure 2.5C-D). These data demonstrate that acute rejection episodes in belatacept treated patients are associated with an increased Th17 memory population relative to rejection in tacrolimus-treated patients.

Discussion

In this study, we show that Th17 memory cells, which are elicited by microbes such as *Candida* and *Staphylococcus* (81, 197, 199), are a component of the alloreactive memory T cell compartment. As a population, a similar fraction of Th1 and Th17 cells divide in response to allogeneic stimulation. This observation provides a new dimension to previous descriptions of alloreactive heterologous immunity (41), including a recent study that found that a significant portion of viral specific memory CD4⁺ and CD8⁺ T cells are alloreactive (16). This finding is also congruous with recent work indicating that alloreactive T cells inherently recognize multiple distinct peptide:MHC complexes, a property termed polyspecificity (42, 44). Our study supports the idea that the alloreactive memory T cell pool is comprised of microbe-elicited cells possessing both the Th1 and Th17 phenotype.

The observation that Th17 cells from renal transplant recipients are resistant to belatacept coupled with the association of elevated Th17 memory cell frequencies with acute cellular rejection strongly suggests that Th17 memory cells play a role in clinical belatacept-resistant graft rejection. Indeed, Th17 cells are known to be potent mediators of pathologic immune responses in a number of autoimmune diseases, and the CTLA-4 Ig derivative abatacept has been reported to be ineffective or even exacerbate the Th17-mediated autoimmune diseases MS, IBD, and SLE (102, 201, 202). Thus, this finding provides a potential explanation for immunomodulation of pathologic immune responses in both transplantation and autoimmunity.

While offering insight into Th17 cell biology in both healthy donors and renal transplant recipients, this study does not exclude the possibility that cellular subsets other than Th17 memory cells also play a role in belatacept resistant graft rejection. Similarly, the activation and effector function of belatacept-resistant Th17 cells may secondarily drive the recruitment and activation of additional cell subsets that may also participate in allograft rejection. A recent study by Vondran *et al.* found that patients with stable renal allograft function who were treated with

belatacept for nearly eight years had diminished Th17 cell frequencies compared to patients treated with CNIs (203). In conjunction with our study, these data suggest that elevated Th17 frequencies are not associated with long-term treatment with belatacept in the absence of acute rejection but are increased in the setting of acute rejection. Future studies that provide careful monitoring of peripheral blood and graft-infiltrating cells prior to episodes of rejection in belatacept-treated individuals would help determine if Th17 memory cell frequencies can be used in as a predictive tool in the clinic.

Our data demonstrate that high expression of coinhibitory CTLA-4 on Th17 cells results in an unexpected augmentation of CD45RA⁻Th17 cells in the presence of belatacept, in contrast to inhibition of CD45RA⁺ and CD45RA⁻ Th1 cells. Th17 cells are known to be maintained at relatively low frequencies in vivo, even at sites of inflammation (87, 200), and high expression of the coinhibitor CTLA-4 is likely a mechanism that contributes to this phenomenon. The correlation of a higher expression level with greater augmentation in the context of selective CTLA-4 blockade strongly suggests that CTLA-4 is acting in a cell intrinsic mechanism on Th1 and Th17 populations. However, recently several groups have implicated cell extrinsic mechanisms of action for CTLA-4 (155, 156, 168), and we cannot definitively exclude additional cell extrinsic action in our experiments. Additional studies will be needed to determine mechanistic basis for the increased CTLA-4 expression in Th17 cells.

These data highlight an important caveat for the clinical use of belatacept for the prevention of organ transplant rejection. Most of the acute rejection has occurred early following transplantation, and thus the use of Th17-inhibiting adjunct therapies during this window offers one potential solution to prevent rejection and maintain the long-term advantages of belatacept. As well, therapies that specifically target alloreactive Th17 responses might be particularly valuable in conjunction with belatacept. For example, the IL-12/23 blocker ustekinumab, currently approved to treat psoriasis, might offer such a solution due to the importance of IL-23

for Th17 maintenance. Recently, the use of inhibitors of the bromodomain and extra-terminal domain (BET) family of chromatin remodelers have been demonstrated to suppress human Th17 cells and autoimmune pathology in a murine model (204).

In conclusion, the CD28/CTLA-4 costimulation blocker belatacept is the first advance in post-transplant immunosuppression in many years. The paradoxical increase in pathologically severe acute rejection episodes is instructive of the need to develop higher resolution understanding of the functional characteristics of pathologic T cell subsets in order to more effectively modulate pathological T cell responses.

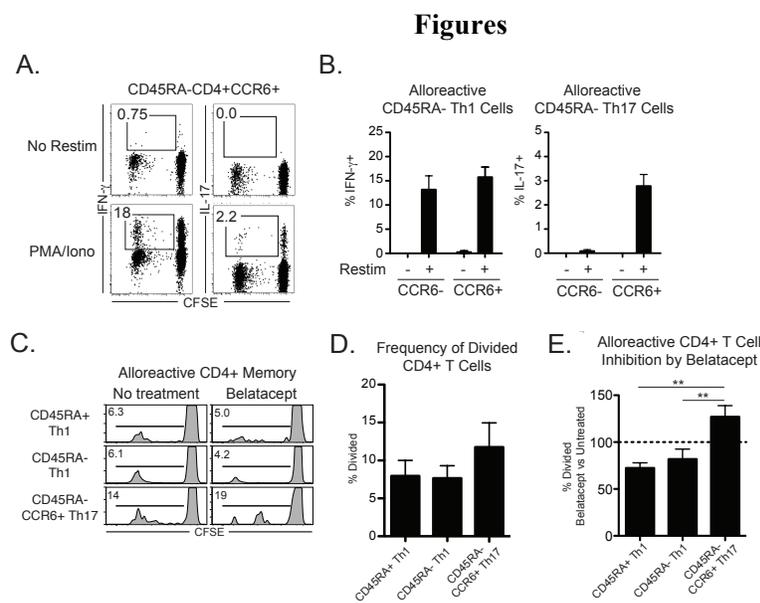


Figure 2.1. CD45RA⁻ Th17 Cells are Alloreactive and Resistant to Belatacept. CFSE labeled T cell responders were stimulated with allogeneic MDDCs for 4 d followed by brief restimulation with PMA/Iono or not. Data depicted are from five independent stimulator/responder pairs. (A-B) Cells were gated on CD4⁺CD45RA⁺CFSE^{low} or CD4⁺CD45RA⁻CFSE^{low}, followed by either IFN- γ ⁺ or IL-17⁺ as described. (A) Representative frequencies of Th1 and Th17 cells among CD45RA⁺CCR6⁺CFSE^{low} cells with and without PMA/Iono restimulation. (B) Summary frequencies of Th1 and Th17 cells within the CD45RA⁻CCR6⁻ and CD45RA⁻CCR6⁺ subsets. (C-E) Cells were first gated on CD4⁺CD45RA⁺IFN- γ ⁺ (CD45RA⁺ Th1), CD4⁺CD45RA⁻IFN- γ ⁺ (CD45RA⁻ Th1), or CD4⁺CD45RA⁻CCR6⁺IL-17⁺ (CD45RA⁻CCR6⁺ Th17), followed by CFSE^{low} divided cells. (C) Concatenated histograms depicting the frequency of CFSE^{low} alloreactive CD45RA⁺ Th1, CD45RA⁻ Th1, and CD45RA⁻ CCR6⁺ Th17 cells in the absence (left column) or presence (right column) of belatacept following allogeneic co-culture. (D) Frequency of divided CD45RA⁺ Th1, CD45RA⁻ Th1 and CD45RA⁻CCR6⁺ Th17 cells (CD45RA⁺ Th1/Th17 p = 0.310, CD45RA⁻ Th1/Th17 p = 0.421). (E) Relative frequency of divided CD45RA⁺ Th1, CD45RA⁻ Th1, and CD45RA⁻CCR6⁺ Th17 following allogeneic stimulation in the presence of belatacept compared to no treatment (CD45RA⁺ Th1/Th17 p = 0.0079, CD45RA⁻ Th1/Th17 p = 0.0079).

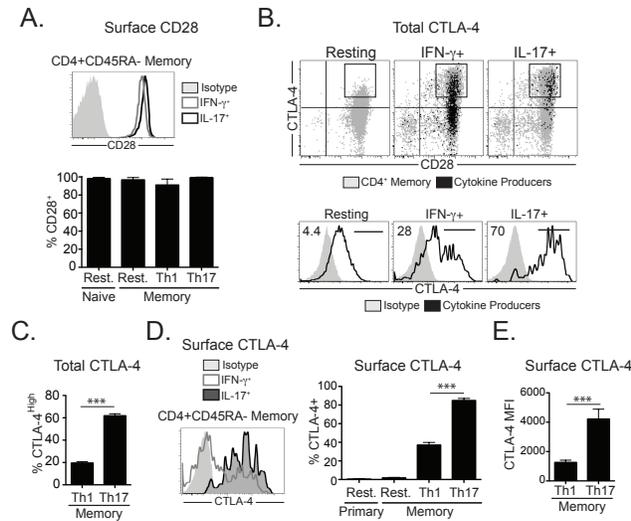


Figure 2.2. Th17 Memory Cells Express High Levels of CTLA-4. Peripheral blood T cells were stimulated briefly with PMA/Iono and analyzed by flow cytometry. CD4⁺ T cell subsets were defined by the following gating strategy: Primary Th1, CD4⁺CD45RA⁺IFN- γ ⁺; Memory Th1, CD4⁺CD45RA⁻IFN- γ ⁺; Memory Th17, CD4⁺CD45RA⁻CCR6⁺IL-17⁺. (A) Representative histogram (top) and summary data (bottom) of surface CD28⁺ CD4⁺ populations resting or restimulated with PMA/Iono ($p = 0.092$, $n = 10$). (B) Top, CD28 and total CTLA-4 expression on resting and restimulated Th1 and Th17 memory cells. Quadrant gate depicts CD28⁺ and CTLA-4⁺ population, square gate defines CTLA-4^{high} population. Bottom, total CTLA-4 expression on resting and restimulated Th1 and Th17 memory cells. Gate depicts CTLA-4^{high} expression ($n = 8$). (C) Frequency of CTLA-4^{high} cells among Th1 and Th17 memory cells ($p = 0.0009$, $n = 8$). (D) Representative frequency (left) and summary frequencies of surface CTLA-4⁺ CD4⁺ populations ($p < 0.0001$, $n = 9$). (E) Mean fluorescence intensity of CTLA-4 on restimulated CD4⁺ IFN- γ ⁺ and IL-17⁺ memory cells ($p < 0.0001$, $n = 9$).

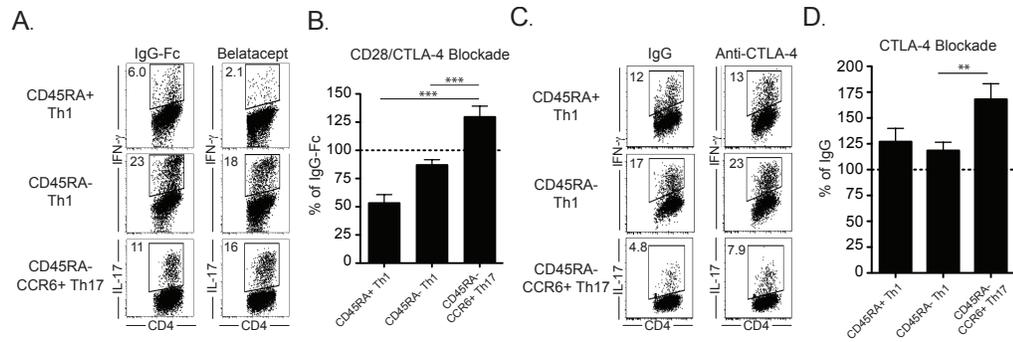


Figure 2.3. CD45RA⁻ Th17 Cells are Resistant to Inhibition by Belatacept and More

Dependent on Coinhibition by CTLA-4. Peripheral blood T cells from healthy donors were

stimulated with anti-CD3 in the presence of blocking molecules or relevant control for 3 d

followed by brief restimulation with PMA/Iono. CD4⁺ T cell subsets were defined by the

following gating strategy: CD45RA⁺ Th1, CD4⁺CD45RA⁺IFN- γ ⁺; CD45RA⁻ Th1,

CD4⁺CD45RA⁻ IFN- γ ⁺; CD45RA⁻CCR6⁺ Th17, CD4⁺CD45RA⁻CCR6⁺IL-17⁺. (A) Representative

frequency of CD45RA⁺ Th1, CD45RA⁻ Th1, and CD45RA⁻CCR6⁺ Th17 after stimulation with

anti-CD3 and belatacept or IgG-Fc control. (B) Relative change in frequencies of CD45RA⁺ Th1,

CD45RA⁻ Th1, and CD45RA⁻CCR6⁺ Th17 cells after anti-CD3 stimulation and treatment with

belatacept compared to IgG-Fc among multiple individuals (CD45RA⁺ Th1/Th17 p = 0.0006,

CD45RA⁻ Th1/Th17 p = 0.0006, n = 7). (C) Representative frequency of CD45RA⁺ Th1,

CD45RA⁻ Th1, and CD45RA⁻CCR6⁺ Th17 cells after stimulation with anti-CD3/CD28 beads and

anti-CTLA-4 or IgG control. (D) Relative change in frequencies of CD45RA⁺ Th1, CD45RA⁻

Th1, and CD45RA⁻CCR6⁺ Th17 cells after stimulation with anti-CD3/CD28 beads and anti-

CTLA-4 or IgG control (CD45RA⁺ Th1/Th17 p = 0.0152, CD45RA⁻ Th1/Th17 p = 0.0152, n =

6).

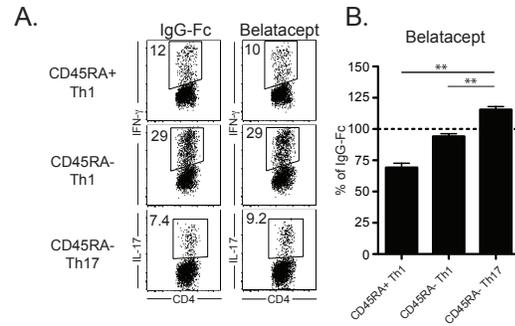


Figure 2.4. Renal Transplant Recipient Lymph Node CD45RA⁻ Th17 Cells are Resistant to Belatacept. T cells from explanted renal transplant recipient lymph nodes were stimulated with anti-CD3 for 3 d in the presence of belatacept or IgG-Fc control followed by brief restimulation with PMA/Iono (n = 5/group). CD4⁺ T cell subsets were defined by the following gating strategy: CD45RA⁺ Th1, CD3⁺CD4⁺CD45RA⁺IFN- γ ⁺; CD45RA⁻ Th1, CD3⁺CD4⁺CD45RA⁻IFN- γ ⁺; CD45RA⁻ Th17, CD3⁺CD4⁺CD45RA⁻IL-17⁺ (A) Representative frequency and (B) summary data of CD45RA⁺ Th1, CD45RA⁻ Th1, and CD45RA⁻ Th17 cells after proliferation and brief restimulation (CD45RA⁺ Th1/Th17 p = 0.0049, CD45RA⁻ Th1/Th17 p = 0.0049).

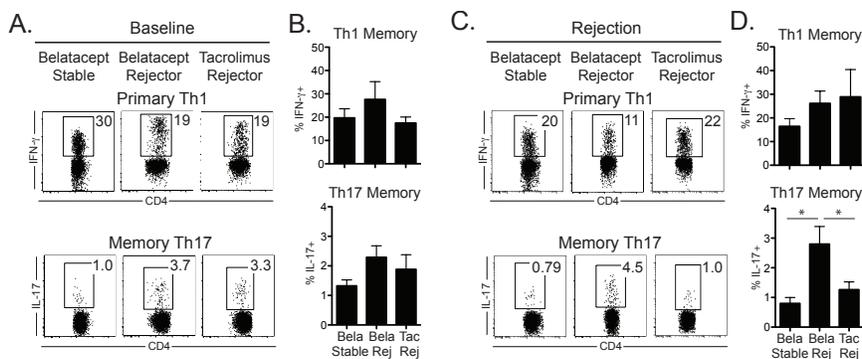
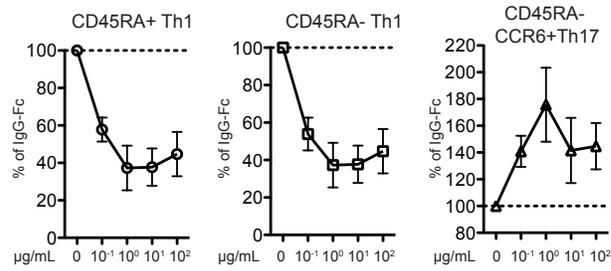


Figure 2.5. Renal Transplant Patients Experiencing Acute Rejection on Belatacept-based Regimens Have Elevated Frequencies of Th17 Memory Cells. Peripheral T cells from renal transplant patients classified as Belatacept Stable, Belatacept Rejectors, or Tacrolimus Rejectors (as described in the Methods) were stimulated for 4 h with PMA/Iono ($n = 4-8/\text{group}$). CD4^+ T cell subsets were defined by the following gating strategy: Th1 Memory, $\text{CD4}^+\text{CD45RA}^-\text{CCR6}^-$ $\text{IFN-}\gamma^+$, Th17 Memory, $\text{CD4}^+\text{CD45RA}^-\text{CCR6}^+\text{IL-17}^+$ (A) Representative frequencies from a single patient in each group and (B) summary frequencies of Th1 memory cells (Bela Stable/Bela Rejector $p = 0.755$, Bela Rejector/Tac Rejector $p = 0.612$) and Th17 memory cells (Bela Stable/Bela Rejector $p = 0.106$, Bela Rejector/Tac Rejector $p = 0.400$) collected at baseline. (C) Representative frequencies of a single patient in each group and (D) summary frequencies of Th1 memory cells (Bela Stable/Bela Rejector $p = 0.200$, Bela Rejector/Tac Rejector $p = 0.886$) and Th17 memory cells (Bela Stable/Bela Rejector $p = 0.0286$, Bela Rejector/Tac Rejector $p = 0.0286$) collected at 1 month following transplantation in Bela Stable or at the time of rejection in Belatacept and Tacrolimus Rejectors.



Supplemental Figure 2.1. Th1 cells are inhibited and CD45RA- Th17 cells are resistant to a range of belatacept concentrations. Peripheral blood T cells from healthy donors were stimulated with anti-CD3 in the presence of belatacept or IgG-Fc for 3 d followed by brief restimulation with PMA/Iono (n = 4). CD4⁺ T cell subsets were defined by the following gating strategy: CD45RA⁺ Th1, CD4⁺CD45RA⁺IFN- γ ⁺, CD45RA⁻ Th1, CD4⁺CD45RA⁻ IFN- γ ⁺, CD45RA⁻CCR6⁺ Th17, CD4⁺CD45RA⁻CCR6⁺IL-17⁺.

Chapter 3. *Candida*-Elicited Murine Th17 Cells Express High CTLA-4 Compared to Th1 Cells and Are Resistant to Costimulation Blockade

Introduction

T cells differentiate into distinct phenotypes based on the surrounding environment present during their initial interaction with cognate antigen, and largely maintain this phenotype during challenge with recall or cross-reactive antigen (3, 48-50). There is considerable evidence that pathogen primed memory cells can cross-react with allogeneic antigen and mediate graft rejection, a process termed allogeneic heterologous immunity (12-16). Recent work has demonstrated that alloreactive T cells are inherently polyspecific (42-44, 47), further elevating the potential importance of cross-reactive T cell responses in mediating alloreactivity. Heterologous T cell responses have been directly demonstrated to be a barrier to tolerance induction strategies such as costimulation blockade, highlighting the importance of understanding phenotypic diversity among pathogen elicited T cell subsets (41, 205).

The heterogeneity of T cell memory responses and their requirements for recall responses are critically important to the success of immunomodulatory therapy to prevent T cell mediated rejection following transplantation of solid organs or bone marrow (13, 206, 207). The CD28/CTLA-4 costimulation blocker CTLA-4 Ig is efficacious for the treatment of rheumatoid arthritis (abatacept) and the derivative belatacept was recently approved for renal transplantation. However, belatacept has been associated with an increased incidence of acute cellular rejection early after transplantation (186), spurring efforts to identify T cell populations that mediate breakthrough allograft rejection episodes.

CD4⁺ memory Th cells are regarded as dependent on CD28 signals for recall responses (41, 129). However, these seminal studies were conducted using Th1 phenotype cells, and the subsequent discovery of the Th17 lineage, which can mediate both autoimmune pathology and

GVHD, has complicated this understanding. Recent studies have suggested that the costimulatory signals that mediate differentiation of naïve Th0 cells into Th17 cells differ from those of Th1 cells, but exactly how they differ remains controversial. For example, reports have indicated that either CD28 or CTLA-4 can suppress Th17 differentiation (83-85, 99), that human Th17 clones are uniquely sensitive to CD28 stimulation (87), and that alternate costimulatory molecules are required to optimally differentiate Th17 cells (83, 84, 88, 89, 208). Despite these reports, which rely heavily on the use of in vitro polarization with exogenous cytokines, little is known about the costimulation requirements of microbe-elicited effector and memory Th17 cells.

Interestingly, the CTLA-4 Ig derivative abatacept has shown mixed results in the treatment of the Th17-mediated diseases MS and IBD (102). In the murine MS model EAE, a single dose of CTLA-4 Ig was unable to prevent relapsing disease and, remarkably, repeated prophylactic CD28/CTLA-4 blockade actually exacerbated disease (175). A recent clinical trial in IBD demonstrated minimal efficacy and disease exacerbation in some treatment groups (202). A case report detailed the development of IBD in a patient treated with CTLA-4 Ig for RA (209). Given the variable efficacy of CTLA-4 Ig in Th17-mediated autoimmunity, the early severe rejection observed in renal transplant recipients, and newly emerging appreciation of alternative costimulatory molecules required for optimal Th17 differentiation, we hypothesized that Th17 cells might be uniquely resistant to CD28/CTLA-4 blockade.

In this study, we investigated the phenotype of pathogen-elicited Th17 cells in an antigen-specific model of graft rejection. Th1 and Th17 cells were elicited via *Mycobacterium tuberculosis* (M.Tb) and *Candida albicans* (Candida) immunization. Candida immunization elicited a higher frequency of Th17 cells and correlated with resistance to costimulation blockade. Compared to the M.Tb group, Candida-elicited Th17 cells had several features of more pathogenic Th17 cells, including a greater frequency of IL-17⁺IFN- γ ⁺ producers, lower CCR6 expression, and a lower frequency of IL-10/IL-17 co-producers. Strikingly, Th17 cells differentially regulated the CD28/CTLA-4 pathway, expressing significantly greater amounts of

CTLA-4 compared to Th1 cells. Ex vivo blockade experiments demonstrate that Th17 cells are significantly less inhibited by CD28/CTLA-4 blockade with CTLA-4 Ig and were more sensitive to CTLA-4 coinhibition. These data demonstrate phenotypic features of pathogen-elicited Th17 cell populations that shed new light on strategies for modulating pathologic T cell responses in transplantation and autoimmunity.

Materials & Methods

Mice. B6-Ly5.2/Cr (H2-K^b, CD45.1) and C57BL/6 (H2-K^b, CD45.2) were obtained from the National Cancer Institute. OT-I and OT-II transgenic mice (purchased from Taconic Farms) were bred to Thy1.1⁺ background at Emory University. Membrane bound-OVA (mOVA) mice were a gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN) and were maintained in accordance with Emory University's Institutional Animal Care and Use Committee guidelines. All animals were housed in specific pathogen-free animal facilities at Emory University.

Adoptive Transfers and Pathogen Immunization. Spleens from Thy1.1⁺ OT-I and OT-II mice were processed to single-cell suspension and stained with mAbs for CD4 (RM4-5), CD8 (3B5), Thy1.1 (OX-7), V α 2 (B20.1), V β 5 (MR9-4) for flow cytometric analysis of T cell frequency. Cells were resuspended in PBS, and 1x10⁶ OT-I and 1x10⁶ OT-II were injected i.v. into naïve B6 recipients. For *Candida* immunization, *Candida albicans* were grown as yeast for 18 h overnight at 30 °C in YPD broth (Teknova, CA), then washed in PBS and diluted 1:50 in RPMI with 10% FBS. Transition to hyphae was induced for 4-6 h at 37°C and monitored by light microscopy. Mice were immunized with 1x10⁶ hyphae in Incomplete Freund's Adjuvant (Difco Laboratories, MI) mixed 1:1 in PBS and 100 μ g OVA₃₂₃₋₃₃₉ peptide (ISQAVHAAHAEINEAGR, Genscript, NJ) in each hind footpad. M.Tb mice were immunized with Complete Freund's Adjuvant (Difco Laboratories) containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* diluted 1:1 in PBS and 100 μ g OVA₃₂₃₋₃₃₉ peptide. Immunizations were performed 24-48 h after adoptive transfer to B6 recipients.

Skin Transplantation and Costimulation Blockade. Full thickness tail and ear skins were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages. Where

indicated, mice were treated with 500 µg of CTLA-4 Ig (Bristol-Myers Squibb, Princeton, NJ) and 500 µg hamster monoclonal anti-mouse CD154 (MR-1; BioXCell, West Lebanon, NH) on days 0, 2, 4, and 6 post transplantation.

Surface and Intracellular Staining and Flow Cytometry. Draining popliteal lymph nodes (LN) were processed to single-cell suspension. LN were restimulated with 10 µM OVA₃₂₃₋₃₃₉ peptide for 6 h, with 10 µg/mL GolgiStop added for the final 5 h. Cells were surface stained with the following antibodies: CD4 (RM4-5), CD8 (3B5), and CD28 (E18) or IgG2bκ. Intracellular cytokine staining was performed following the manufacturer's instructions (BD Biosciences) with the following antibodies: IFN-γ (XMG1.2), IL-17 (eBio17B7), CTLA-4 (UC10-4B9) or IgG. Flow cytometric analysis was performed on an LSRII flow cytometer and analyzed using FlowJo. Where indicated, OT-II cells were identified by FACS as CD4⁺CD8⁻B220⁻Thy1.1⁺, while OT-I cells were identified as CD8⁺CD4⁻B220⁻Thy1.1⁺.

Ex vivo Th1/Th17 costimulation blockade. CD4⁺ T cells from the draining popliteal LN of Candida and M.Tb OVA immunized mice were harvested and purified with the CD4⁺ T Cell Isolation Kit II (Miltenyi) according the manufacturer's protocol using LS columns and autoMACS Running Buffer (Miltenyi). CD11c⁺ cells were enriched from B6-Ly5.2/Cr (H2-K^b, CD45.1) splenocytes. Splenocytes were macerated in HBSS containing 100 U/mL Collagenase III. Remaining splenic fragments were then incubated in HBSS containing 400 U/mL Collagenase III at 37°C for 30 min. Cells were then washed in HBSS with 10 mM EDTA. CD19⁺ and Thy1.2⁺ cells were depleted using magnetic microbeads and LD columns (Miltenyi). CD11c⁺ cells were positively selected using magnetic microbeads and LS columns (Miltenyi). The frequency of CD11c⁺ cells was assessed at >85% by FACS. Between 750,000-1,000,000 CD4⁺ T cells were co-cultured with 100,000 CD11c⁺ cells for 4 d in the presence of 5 µM OVA₃₂₃₋₃₃₉

peptide and 100 µg/mL of either CTLA-4 Ig, human Ig1-Fc, anti-CTLA-4 (9H10), anti-CD154 (MR-1), or hamster IgG (all from BioXCell) in RPMI with 10% FBS. After 4 d, cells were washed 2x in media and restimulated for 4 h with 30 ng/mL PMA and 400 ng/mL Ionomycin in the presence of GolgiStop for the final 3 h. Intracellular cytokine staining for IFN-γ and IL-17 was assessed as described above. Absolute cell counts were determined by adding CountBrite (Invitrogen) beads prior to PMA/Iono restimulation. In some experiments, 10⁶ naïve OT-II splenocytes were activated with 5 µM OVA₃₂₃₋₃₃₉ peptide in the presence of 100 µg/mL of either CTLA-4 Ig, Ig-Fc control, anti-CTLA-4 Ab, or IgG for 5 days and restimulated with PMA/Iono prior to intracellular cytokine staining as described.

OVA Antibody Detection. Mice immunized with M.Tb OVA or Candida OVA as described and grafted with an mOVA skin graft and treated with CTLA-4 Ig and anti-CD154 were bled on day 10 post-transplant. Serum was prepared by centrifugation of blood samples at 12,000 rpm for 30 minutes at 4 °C. 96-well plates (Nunc) were coated with 50 µL per well of 10 µg/mL OVA antigen (Sigma A-2512) overnight at 4°C, washed three times with PBS with 0.5% Tween-20, and blocked at room temperature for 2 hours with PBS with 0.2% Tween-20 and 10% FBS. Anti-OVA mAb clone 14 (Sigma A6075) was used as a positive control and was incubated at decreasing concentrations starting at 2.5ug/ml. Samples were plated at 1:10 dilutions in PBS to a final volume of 100 µL per well. Plates were then incubated at 37 °C for two hours. Following washing, 100 µL of 2.75 µg/mL goat anti-mouse IgG-HRP was added incubated at room temperature for 1.5 h. 100uL per well of TMB substrate (BD 555214) was used following manufacturer instructions and plates were read at OD₄₅₀ on an ELISA plate reader.

Histology. Skin grafts were removed and frozen in cryomolds with OCT Embedding Compound (Tissue-Tek) on day 9 post transplantation. Longitudinal sections of grafts were cut into 5 µm-

thick sections with a cryostat (CM 1850; Leica Microsystems) and mounted on SuperFrost Plus microscope slides, fixed with 100% acetone, and stained with anti-mouse Thy1.1 or anti-GR-1. Six mice were used for immunohistochemical detection by 3,3 diaminobenzidine peroxidation and counterstained with hematoxylin. Representative images are shown at 40x. At least 20 image fields were analyzed per group.

Statistical Analysis. OT-II frequencies, surface marker expression, and cytokine production between M.Tb OVA and Candida OVA groups were analyzed with unpaired Student's t-test (two-tailed). Cellular infiltration of skin grafts and anti-OVA antibody responses were analyzed using one-way ANOVA with Bonferroni post test. In text statistics are average \pm SEM.

Results

Candida antigen immunization yields costimulation blockade resistant graft rejection.

Pathogen elicited memory cells can cross-react with allogeneic antigen and mediate graft rejection (15, 41). While the costimulation requirements of CD8⁺ memory have been well studied by our lab and others, the differential susceptibility of CD4⁺ Th memory subsets to immunomodulation is less well understood. We hypothesized that different classes of microbes can yield memory that has variable potency in graft rejection.

We activated antigen-specific CD4⁺ (OT-II) and CD8⁺ (OT-I) T cells using cognate OVA₃₂₃₋₃₃₉ peptide in the presence of two well-studied microbes, intracellular *Mycobacterium tuberculosis* (M.Tb OVA) or the ubiquitous fungus *Candida albicans* (Candida OVA; Figure 3.1A). In this experimental design, CD4⁺ cells are activated and differentially polarized in the presence of pathogen, but both CD4⁺ and CD8⁺ cells can respond to OVA expressed in mOVA skin graft. Both the M.Tb OVA and Candida OVA immunization strategies similarly expanded OVA-specific CD4⁺ cells compared to unimmunized controls (Figure 3.1B). Candida and M.Tb OVA immunization yielded similar frequencies of OVA-specific CD4⁺ T cells at day 9, but absolute numbers of Candida-OVA elicited cells were slightly decreased relative M.Tb-elicited cells (Figure 3.1B). OVA-specific CD8⁺ T cells were not activated by this immunization strategy and persisted at similar frequencies in both groups (Supplemental Figure 3.1A). At day 14 post immunization, OT-II populations contracted to similar frequencies and absolute numbers (Figure 3.1C). When OVA-expressing skin grafts were transplanted to the M.Tb OVA and Candida OVA groups, both groups of mice rejected mOVA skin grafts with similar kinetics in the absence of any immunosuppression (Figure 3.1D).

We next investigated the susceptibility of both types of pathogen elicited CD4⁺ cells to mediate graft rejection in the presence of costimulation blockade. We chose to use CTLA-4 Ig in the presence of anti-CD154 Ab because in this antigen-specific model of graft rejection CTLA-4

Ig is ineffective at providing long-term graft survival (210). In the presence of costimulation blockade, M.Tb OVA immunized mice were protected from graft rejection for > 60 days post transplantation (Figure 3.1D). In contrast, the majority of Candida OVA immunized mice rejected their grafts by 20 days post transplant (Figure 3.1D). These results indicate that Th polarization in the presence of Candida generates a population of CD4⁺ T cells that are resistant to costimulation blockade. By the time of grafting, the Candida hyphae were cleared, indicating that persistent infection does not account for the differences in rejection kinetics (Supplemental Figure 3.1B). To determine the potential role of anti-OVA Ab in graft model, we measured the serum levels of anti-OVA Abs at 9 days post-immunization. We found that anti-OVA Abs were similar between M.Tb and Candida groups (Supplemental Figure 3.1C). Costimulation blockade following transplantation did not affect the levels of pre-existing anti-OVA serum antibody in either the M.Tb OVA or the Candida OVA group (Supplemental Figure 3.1C). Together, these data suggest that Candida immunization elicits a population of CD4⁺ Th cells that mediate costimulation blockade-resistant rejection, and that this result is not due to differential elicitation of anti-OVA Abs.

Candida immunization elicits a Th17 skewed phenotype compared to M.Tb.

M.Tb and Candida each elicit Th1 and Th17 responses (69, 78). Therefore, we investigated the phenotype of antigen-specific CD4⁺ T cells during the peak of the effector response. Restimulation of draining lymph node cells on day 9 post immunization yielded a significant population of IFN- γ ⁺ Th1 cells in both groups. The frequencies of Th1 cells were not different between M.Tb and Candida immunized mice (Figure 3.2A). IL-17 producing Th17 cells were also found in both groups; however Candida immunization yielded a significantly greater frequency of Th17 cells than M.Tb immunization (M.Tb OVA 6.69 \pm 0.262%, Candida OVA 13.6 \pm 1.46%, p = 0.0005, Figure 3.2B). The frequency of Th17 cells were significantly greater in

pathogen immunized mice compared to mice immunized with OVA₃₂₃₋₃₃₉ peptide alone (Figure 3.2A-B), demonstrating that the balance of Th1 and Th17 cells in both M.Tb OVA and Candida OVA immunized mice is due to the pathogen-induced differentiation of naïve antigen-specific cells. These antigen-specific CD4⁺ IL-17 producing cells were bona fide Th17 cells as they expressed high levels of the lineage-defining transcription factor ROR γ T (Figure 3.2C) and low levels of the Th1 transcriptional factor T-bet compared to antigen-specific CD4⁺ IFN- γ producing cells (Supplemental Figure 3.2A). These data suggested that a greater frequency of Th17 cells might underlie the differential susceptibility of M.Tb and Candida immunized mice to graft rejection.

M.Tb and Candida elicit similar frequencies of effector memory cells

We further investigated the phenotype of antigen-specific cells polarized by M.Tb and Candida immunization. We found that both immunizations yielded similar frequencies of CD44^{high} cells, indicating that cells in both of these immunization groups effectively encountered cognate antigen (M.Tb OVA 84.9 \pm 6.3% and Candida OVA 88.5 \pm 6.4%, Figure 3.3A). In contrast to naïve antigen-specific CD4⁺ T cells, M.Tb and Candida OVA immunization both yielded a majority of CD44^{high}CCR7⁻ effector memory cells (T_{EM}) (M.Tb OVA 63.9 \pm 10.3% and Candida OVA 57.5 \pm 6.7%, Figure 3.3B).

M.Tb and Candida immunization has been associated with induction of additional cytokines, such as IL-2 and TNF (211, 212). To assess the potential role of these cytokines, we compared the frequencies of IL-2 and TNF in the draining lymph node at the peak of the response following immunization. We found that M.Tb and Candida immunization yielded similar frequencies of antigen-specific IL-2⁺ and TNF⁺ CD4⁺ T cells (Figure 3.2C-D). Both immunization strategies elicited similar frequencies of Th1 and Th17 cells that co-produced either IL-2 or TNF (Supplemental Figure 3.2B-C). Together, these data suggest that a high frequency of

Th17 cells are elicited by Candida immunization and correlate with costimulation blockade resistant graft rejection

Candida elicited Th17 cells have a more pathogenic phenotype compared to M.Tb elicited Th17 cells.

Th17 cells have been described that have either protective or pathogenic roles in mediating inflammation (67). Since Th17 cells were elicited by both M.Tb and Candida, we investigated differences in the phenotype of Th17 cells from both groups. Th17 cells that co-express IFN- γ have been described at sites of autoimmune inflammation (67). We found a greater frequency of IL-17⁺IFN- γ ⁺ cells in the draining LN of Candida OVA mice compared to M.Tb OVA mice (M.Tb OVA 2.01 \pm 0.315%, Candida OVA 4.15 \pm 0.564%, $p = 0.0042$, Figure 3.4A). The chemokine receptor CCR6 is associated with the production of IL-17 by CD4⁺ cells (78, 213), but loss of its expression is characteristic of more pathogenic Th17 cells in multiple models (78, 80). Consistent with previous reports (78, 213), CCR6 expression was high on Th17 cells compared to Th1 cells ($p < 0.001$, Supplemental Figure 3.3A). However, we found that CCR6 expression levels were significantly lower in Candida elicited Th17 cells compared to M.Tb polarized Th17 cells (M.Tb OVA 626 \pm 19.46 MFI, Candida OVA 492.3 \pm 52.45 MFI, $p = 0.0422$, Figure 3.4B). CCR6 expression on Th1 cells was not different between immunization groups (Supplemental Figure 3.3B). Together, these findings suggest that Candida-elicited Th17 cells are skewed towards a pathogenic phenotype.

Conversely, production of the anti-inflammatory cytokine IL-10 has been associated with protective Th17 cells (69, 80). M.Tb OVA yielded a significantly greater frequency of IL-10⁺ CD4⁺ T cells than Candida OVA immunization (M.Tb OVA 3.51 \pm 0.433%, Candida OVA 1.61 \pm 0.345%, $p = 0.0040$, Figure 3.4C). Additionally, M.Tb OVA mice had a significantly greater frequency of Th17 cells that co-produced IL-10 (M.Tb OVA 8.17 \pm 1.32%, Candida OVA

3.31±0.844%, $p = 0.0059$, Figure 3.4D). These data demonstrate that M.Tb elicited Th17 cells display features that are less pathogenic, and suggest that the inflammatory profile of Candida and M.Tb elicited Th17 cells might contribute to costimulation blockade-resistant graft rejection.

Th17 cells express greater amounts of CTLA-4 than Th1 cells.

We reasoned that because a greater frequency of Th17 cells in the Candida OVA group is associated with CTLA-4 Ig resistant graft rejection (Figure 3.1D), the CD28/CTLA-4 pathway might be differentially expressed on Th1 and Th17 cells. We evaluated the expression of these molecules in M.Tb and Candida polarized draining popliteal LN OT-II cells. We found that CD28 was constitutively expressed on endogenous CD4⁺ cells and Th1 and Th17 OT-II restimulated cells from both M.Tb and Candida OVA groups (Figure 3.5A-B, Supplemental Figure 3C). Th1 and Th17 cells expressed similar amounts of CD28 on the cell surface (Figure 3.5B).

CTLA-4 is not expressed on the surface of resting CD4⁺ effector or memory cells, but is released rapidly from intracellular vesicles following TCR stimulation. Using intracellular staining, we found that endogenous CD4⁺ T cells express low levels of CTLA-4 (22.7±1.74% CTLA-4⁺, 1557±99.2 MFI, Figure 3.5C-D), consistent with a low frequency of activation. Th1 cells upregulated CTLA-4 expression (61.5±2.41% CTLA-4⁺, Figure 3.5C-D). In contrast, a significantly greater fraction of Th17 cells were CTLA-4⁺ (74.8±2.51% CTLA-4⁺, $p = 0.0006$, Figure 3.5C), and Th17 cells also expressed a greater amount of CTLA-4 than Th1 cells (3870±312 MFI Th1 vs 5665±508 MFI Th17, $p = 0.0044$, Figure 3.5D). CTLA-4 expression levels were similar between Th1 and Th17 cells elicited from M.Tb or Candida (Supplemental Figure 3.3D-E). These data demonstrate that Th1 and Th17 cells have dramatically different regulation of the CD28/CTLA-4 pathway and suggest that Th17 cells might be resistant to CTLA-4 Ig due to greater reliance on coinhibitory CTLA-4.

Th17 cells are less susceptible to CTLA-4 Ig and more sensitive to CTLA-4 coinhibition than Th1 cells.

We hypothesized that greater expression of coinhibitory CTLA-4 would result in less suppression of Th17 cells compared to Th1 cells in the context of blockade of CD80/CD86 signals by CTLA-4 Ig. To investigate the differential functionality of physiologically relevant Th1 and Th17 cells, we purified Candida and M.Tb polarized CD4⁺ T cells and restimulated them with OVA₃₂₃₋₃₃₉ peptide in the presence of CTLA-4 Ig or control Ig-Fc. In order to robustly assess the effect of CTLA-4 Ig, which binds to CD80 and CD86 on APCs, we stimulated M.Tb OVA and Candida OVA CD4⁺ cells in the presence of purified CD11c⁺ DCs, which express high levels of MHC Class II allele I-A^b as well as cosignaling ligands CD80 and CD86 (Figure 3.5E). After 4 days, the absolute number of Th1 cells was significantly diminished by CTLA-4 Ig compared to Ig control (37.7±5.4% of Ig-Fc, Figure 3.5F-G). In contrast, the number of Th17 cells was slightly inhibited by CTLA-4 Ig (83.3±18.0% of Ig-Fc, Figure 3.5F-G). When compared to Th1 cell inhibition, Th17 cells were significantly less inhibited by CD28/CTLA-4 blockade (p = 0.0015, Figure 3.5F). As suggested by the similar expression of CD28 and CTLA-4 between M.Tb and Candida groups, there was no difference in the level of inhibition of either Th1 or Th17 cells in the Candida and M.Tb groups (Supplemental Figure 3.4A).

The coinhibitory function of CTLA-4 has been attributed to several cell intrinsic functions, such as enhanced phosphatase signaling, competition with CD28 for CD80/CD86, and exclusion of CD28 from the immunological synapse (139, 214). Recently, cell extrinsic functions have also been attributed to CTLA-4 on CD4⁺ and CD8⁺ T cells (164). We hypothesized that greater expression of coinhibitory CTLA-4 rendered Th17 cells more resistant to CD28/CTLA-4 blockade than Th1 cells owing to greater cell intrinsic coinhibitory function. In order to test this hypothesis, we specifically blocked CTLA-4 function using a monoclonal Ab that augments naïve OT-II differentiation into Th1 cells during activation (135) (Supplemental Figure 3.4B).

Anti-CTLA-4 mAb very minimally augmented the number of Th1 cells that were co-cultured with CD11c⁺ DCs (112±9.6% of IgG, Figure 3.6A-B). In contrast, the number of Th17 cells were augmented to a significantly greater degree compared to Th1 cells (140±9.2% of IgG, $p = 0.0387$ Figure 3.6A-B), demonstrating that Th17 cells are more sensitive to CTLA-4 cell intrinsic coinhibition. There was no difference in the effect of Th1 or Th17 cells between M.Tb OVA and Candida OVA groups (Supplemental Figure 3.4C). Together, these results suggest that higher expression of CTLA-4 on Th17 cells correlates to enhanced resistance to CTLA-4 Ig and greater sensitivity to CTLA-4 coinhibition, findings that suggest CTLA-4 functions as a potent cell intrinsic coinhibitor on Th17 cells.

Pathogen elicited Th1 and Th17 cells similarly regulate CD154 expression

The differential expression of CTLA-4 on Th17 cells suggests that this pathway plays a role in the costimulation blockade resistant rejection observed in Candida OVA immunized mice. To investigate whether the CD40/CD154 pathway, which was also blocked in our skin graft experiments, played a role in costimulation-blockade resistant rejection, we first investigated the expression of CD154 on antigen-specific CD4⁺ T cells. CD154 is expressed on activated but not naïve CD4⁺ T cells (Figure 3.7A) (215, 216). We found that overall M.Tb or Candida immunization resulted in similar expression of CD154 on antigen-specific Th1 and Th17 cells on day 9 (Figure 3.7A-B). By day 14, the expression levels of CD154 were diminished, but were similar among Th1 and Th17 cells from M.Tb OVA and Candida OVA mice (Figure 3.7C).

To determine the ability of CD154 blockade to diminish CD4⁺ T cell responses, we treated antigen-specific CD4⁺ T cells from the draining LNs M.Tb OVA and Candida OVA mice on day 9 post immunization with dendritic cells in the presence of anti-CD154 mAbs or control IgG. We found that the proliferation of Th1 and Th17 cells from Candida OVA and M.Tb OVA mice were inhibited by CD154 blockade (M.Tb Th1 44.7±7.1%, Candida Th1 63.6±5.4%, M.Tb

Th1 $52.8 \pm 11.5\%$, Candida Th17 $31.5 \pm 5.3\%$ of IgG, Figure 3.7C). Interestingly, Candida Th17 cells were more inhibited by anti-CD154 than Candida Th1 cells. Together, these results demonstrate that CD154 is not differentially expressed following M.Tb or Candida OVA immunization, and strongly suggest that the function of the CD154 pathway is not responsible for the costimulation blockade-resistant rejection observed in Candida OVA mice.

Neutrophils are recruited to skin grafts in a costimulation blockade independent manner by Candida-elicited T cell responses.

CD4⁺ Th cells coordinate effector responses via recruitment of pro-inflammatory cells, such as inflammatory macrophages and neutrophils. We investigated the recruitment of these populations into skin grafts in M.Tb and Candida immunized mice in the presence of costimulation blockade. Macrophages are associated with a Th1 type inflammation, while neutrophils are potently recruited to sites of inflammation by IL-17.

Macrophages were recruited into the grafts at similar frequencies between M.Tb and Candida groups (Figure 3.8A-B). Treatment with costimulation blockade diminished these populations in both groups (Figure 8A-B), suggesting that macrophages are not involved in effecting rejection. Neutrophils were also recruited into the graft to similar degrees in M.Tb and Candida mice (Figure 3.8C-D). In M.Tb OVA mice, however, costimulation blockade inhibited the population of neutrophils to a significant degree ($p = 0.0263$, Figure 3.8C-D). Candida OVA mice treated with costimulation blockade did not diminish the recruitment of neutrophils (Figure 3.8C-D). These data suggest that a Th17 type inflammation mediates costimulation blockade resistant graft rejection in Candida OVA mice.

Discussion

In this study, we investigated the capacity of pathogen elicited graft-specific CD4⁺ T cells to induce graft rejection. We found that while Th1 frequencies were similarly elicited by M.Tb and Candida immunization, a Th17 skewed response correlated with costimulation blockade resistant graft rejection in Candida immunized mice. Our results demonstrate that both the degree of Th17 pathogenicity and the frequency of CTLA-4 Ig resistant Th17 cells contribute to the observed costimulation blockade resistance in Candida OVA mice.

Distinct Th17 phenotypes correlated with pathogenicity in models of autoimmunity (67). We found that Candida-elicited effector cells contained more IL-17⁺IFN- γ ⁺ double-producers, a finding that has been associated with expression of the IFN- γ /Th1 transcription factor T-bet and associated with pathogenic Th17 cells at sites of autoimmune inflammation (68, 69, 71, 73). Reduced expression of CCR6 in these mice is also congruent with previous reports demonstrating that the loss of CCR6 correlates with IL-17⁺IFN- γ ⁺ pathogenic Th17 cells (78, 80). M.Tb elicited Th17 cells, on the other hand, displayed a more “classical” protective phenotype, typified by a greater proportion of IL-17 single producers, higher CCR6 expression, and greater production of the anti-inflammatory cytokine IL-10. In particular, IL-10 production by Th17 cells has been identified as a key feature of less pathogenic cells (69, 80). M.Tb mice contained a greater frequency of IL-17⁺IL-10⁺ cells, a finding that has been associated with bystander suppression of CD8⁺ effector cells in a model of autoimmunity (69). Recent reports have demonstrated that a pathogenic Th17 phenotype is dependent on IL-23 that is induced TGF- β 3 production (80, 212, 217-219). Our results extend previous reports using in vitro polarized cells by establishing that the degree of pathogenicity of microbe-elicited Th17 cells in vivo can vary depending on the specific microbe.

Importantly, this study also establishes that pathogen elicited Th17 cells have a CD28/CTLA-4 expression profile that is distinct from Th1 cells. Th17 cells expressed significantly more CTLA-4 upon restimulation compared to Th1 cells, suggesting that Th17 cells might be more susceptible to CTLA-4 coinhibition. In ex vivo blockade experiments, we observed a modest effect of CTLA-4 mAb on Th1 responses, which may be explained by some degree of mAb cross-linking induced negative signaling in our cultures. However, the significantly enhanced augmentation of Th17 responses relative to Th1 responses strongly suggests both a potent cell intrinsic role for coinhibitory CTLA-4 on Th17 cells and greater sensitivity to CTLA-4 blockade, both findings that correlate with high expression of CTLA-4 on Th17 cells. Several recent studies have demonstrated a cell extrinsic role for CTLA-4 in CD8⁺ and CD4⁺ T cells (154-157, 168). While our results demonstrate cell intrinsic effects on Th1 and Th17 cells, we cannot rule out an additional cell extrinsic role for CTLA-4 based on our experiments. The relative rarity of Th17 cells in healthy and inflamed tissues has been described in mouse models and human disease (67, 87, 220). High CTLA-4 expression on Th17 cells presents another contributing mechanism to the low frequency at which these cells are found. Thus, our study modifies previous understanding of coinhibitory CTLA-4 on an inflammatory CD4⁺ lineage and demonstrates a novel role for CTLA-4 on Th17 cells.

Blockade of the CD28/CTLA-4 pathway has proven to be effective at the modulation of pathogenic T cell responses in the context of rheumatoid arthritis and renal transplantation (207). However, the relative resistance of Th17 cells to CD28/CTLA-4 blockade has important implications for the further development of this reagent, as Th17 cells play a role in autoimmunity and GVHD, and have been reportedly involved in renal, lung, and liver allograft rejection (91-94). Furthermore, reports of the ineffectiveness of CTLA-4 Ig compounds in treating autoimmune diseases with Th17 components (IBD, MS, and SLE) further suggests that our findings have clinical relevance. These data imply that conceivably, CTLA-4 Ig therapy for following renal or bone marrow transplantation could be more selectively administered to

individuals for whom alloreactive Th17 populations are not prominent. In addition, this study provides a compelling mechanistic explanation to justify identifying new molecular targets to inhibit pathogenic Th17 cells.

Figures

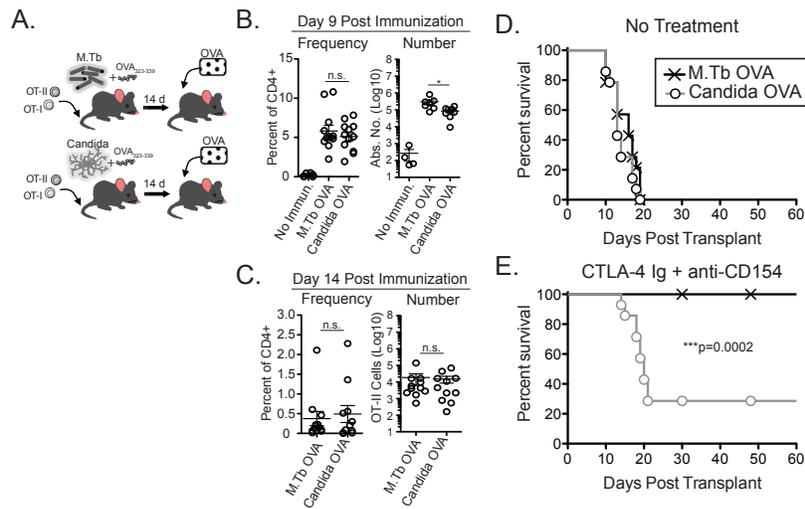


Figure 3.1. Candida elicited CD4⁺ T cells can mediate costimulation blockade resistant graft rejection. (A) Mice were adoptively transferred with 10^6 OT-I and OT-II cells 24 h before immunization with M.Tb or Candida and OVA peptide. (B-C) Draining popliteal LNs were isolated on (B) day 9 or (C) day 14 post-immunization and the frequency and absolute number of Thy1.1⁺ OT-II cells were determined ($p = n.s.$, $n = 10-12$ /group, 2 experiments). (D-E) At 14 d post-immunization, mice were transplanted with mOVA skin and monitored for graft survival ($n = 13-14$ /group, 3 experiments). (D) Mice were left untreated ($p = n.s.$) or (E) treated with 500 μ g each CTLA-4 Ig and anti-CD154 ($p = 0.0002$). Statistical comparisons performed using unpaired two-tailed Student's t-test (B) and log rank test (D-E), * $p < 0.05$, *** $p < 0.0005$. No Immun., no immunization control.

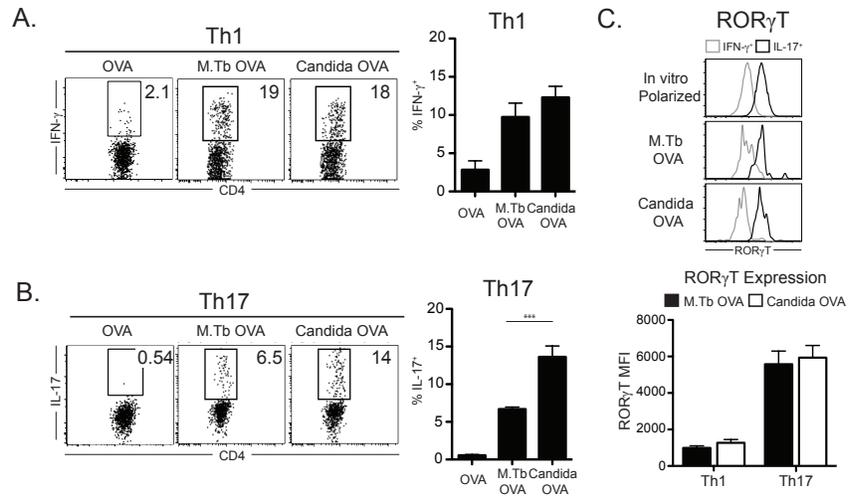


Figure 3.2. Candida elicits a higher frequency of Th17 cells than M.Tb. Draining popliteal LNs from M.Tb OVA and Candida OVA mice were collected on day 9 post-immunization and restimulated for 4 h with PMA/Iono ($n = 10-13/\text{group}$, 2 experiments). The frequencies of (A) IFN- γ^+ Th1 and (B) IL-17 $^+$ Th17 cells among antigen-specific CD4 $^+$ T cells ($p = 0.0005$). (C) Expression of ROR γ T expression on Th1 and Th17 cells. Statistical comparisons performed using unpaired two-tailed Student's t-test, *** $p < 0.0005$.

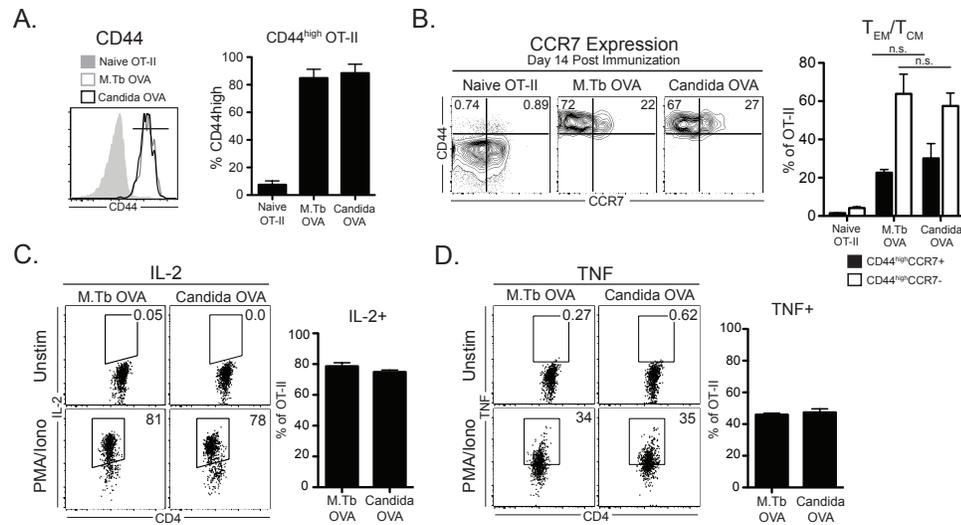


Figure 3.3. M.Tb and Candida elicit a similar CD4⁺ T_{EM} phenotype. (A-B) Draining popliteal LNs from M.Tb OVA and Candida OVA were collected on day 14 post immunization (n = 7-8/group, 2 experiments). (A) The frequency of antigen-specific OT-II CD44^{high} cells in both groups. (B) The frequency of OT-II T_{EM} and T_{CM} cells by CD44 and CCR7 expression. (C-D) Draining popliteal LNs from M.Tb OVA and Candida OVA were collected on day 9 post immunization and restimulated for 4 h with PMA/Iono. Frequency of (C) IL-2 and (D) TNF expression among OT-II CD4⁺ T cells (n = 10-13/group, 2 experiments). Statistical comparisons performed using unpaired two-tailed Student's t-test, *p<0.05.

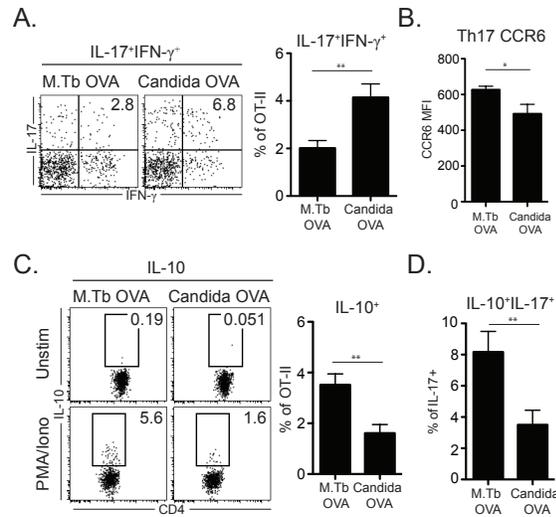


Figure 3.4. Candida elicits more pathogenic Th17 cells than M.Tb. Draining popliteal LNs from M.Tb OVA and Candida OVA mice were collected on day 9 post-immunization and restimulated for 4 h with PMA/Iono (n = 7-12/group, 2 experiments). (A) The frequency of IFN- γ ⁺IL-17⁺ cells among OT-II cells (p = 0.0042). (B) CCR6 expression was assessed on OT-II Th1 and Th17 cells (p = 0.0422). (C) The frequency of IL-10⁺ cells among OT-II cells (p = 0.0040). (D) The frequency of IL-10⁺IL-17⁺ cells among OT-II IL-17⁺ cells was assessed (p = 0.0035) Bar graphs show average \pm SEM. Statistical comparisons performed using unpaired two-tailed Student's t-test, *p<0.05, **p<0.005.

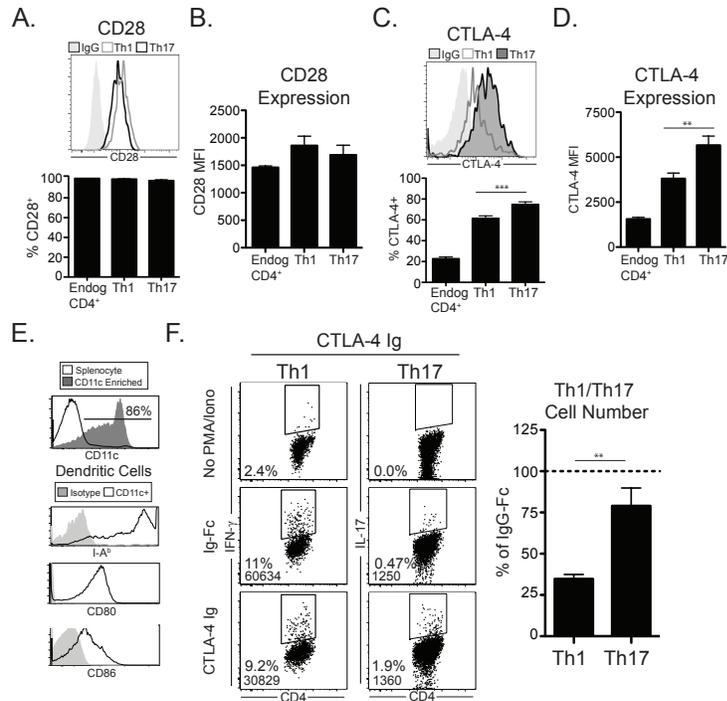


Figure 3.5. Th17 cells express higher levels of CTLA-4 and are less susceptible to CTLA-4 Ig than Th1 cells. Draining popliteal LNs from M.Tb OVA and Candida OVA mice were collected on day 9 post-immunization and (A-D) restimulated for 4 h with PMA/Iono or (E-F) co-cultured with OVA₃₂₃₋₃₃₉ and DCs in the presence of CTLA-4 Ig or IgG-Fc for 4 d before brief PMA/Iono restimulation. Data shown depict Th1 and Th7 OT-II cells from both immunization groups (n = 16-21/group, 3-4 experiments). (A) The frequency of CD28⁺ and (B) CD28 expression level on antigen-specific Th1 and Th17 cells (p = n.s.). (C) The frequency of CTLA-4⁺ (p = 0.0006) and (D) CTLA-4 expression level (p = 0.0044) on OT-II Th1 and Th17 cells. (E) CD11c⁺ DCs were purified from naïve splenocytes and analyzed for MHC Class II I-A^b, CD80, and CD86 expression. (F) M.Tb OVA and Candida OVA CD4⁺ T cells were co-cultured with DCs for 4 d in the presence of CTLA-4 Ig IgG-Fc control followed by brief PMA/Iono restimulation. (F) Representative frequencies (top number) and absolute numbers (bottom number) of OT-II Th1 and Th17 cells without PMA/Iono restimulation (first row) or with restimulation (middle/bottom rows, p = 0.0015). Bar graphs include average ± SEM. Statistical

comparison performed using (A-D) unpaired or (F) paired two-tailed Student's t-test. ** $p < 0.005$.

Endog. CD4⁺, endogenous CD4⁺ T cells. DCs, CD11c⁺ splenic dendritic cells. Isotype, IgG isotype control antibody.

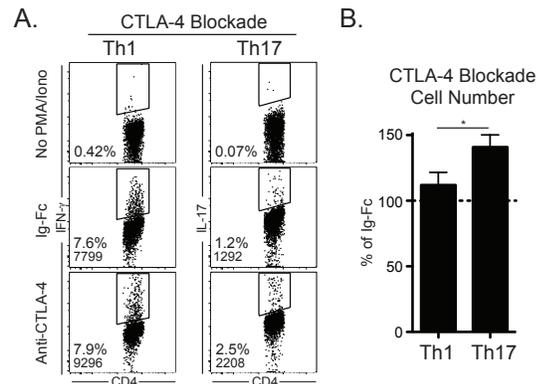


Figure 3.6. Th17 Cells are more susceptible to CTLA-4 coinhibition than Th1 cells. Draining popliteal LNs from M.Tb OVA and Candida OVA mice were collected on day 9 post-immunization and co-cultured with OVA₃₂₃₋₃₃₉ and DCs in the presence of anti-CTLA-4 or IgG for 4 d before brief PMA/Iono restimulation. Data shown depict OT-II Th1 and Th7 cells from both immunization groups (n = 13-15/group, 3 experiments). (A) Representative frequencies (top number) and absolute numbers (bottom number) of OT-II Th1 and Th7 cells without PMA/Iono restimulation (first row) or with restimulation (middle/bottom rows) (B) Th1 and Th7 cell numbers from M.Tb OVA and Candida OVA groups following anti-CTLA-4 blockade (p = 0.0387). Bar graphs depict average \pm SEM. Statistical comparison performed using unpaired two-tailed Student's t-test *p<0.05. Endog. CD4⁺, endogenous CD4⁺ T cells. DCs, CD11c⁺ splenic dendritic cells. Isotype, IgG isotype control antibody.

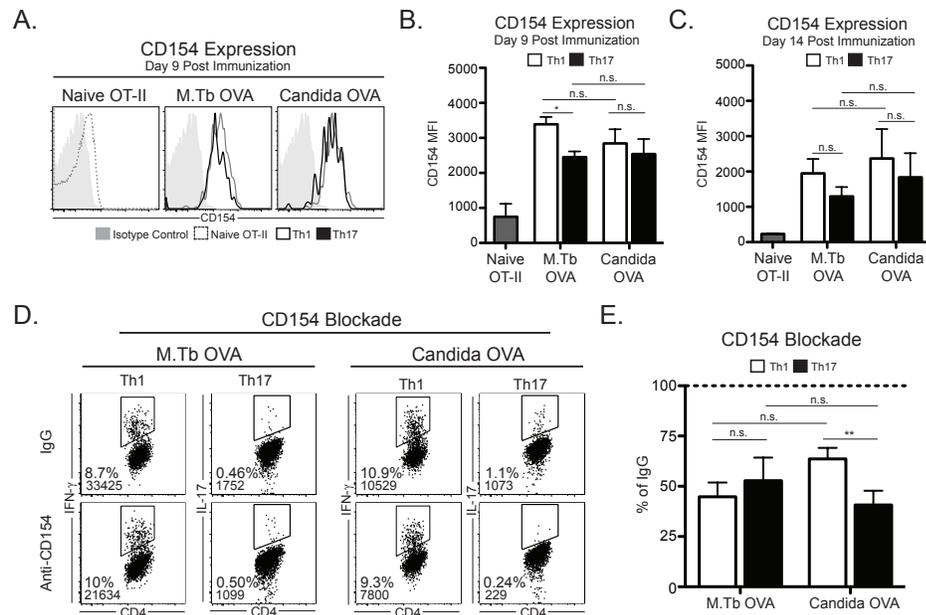


Figure 3.7. Th1 and Th17 cells express similar levels of CD154 and are inhibited by CD154 blockade. Draining popliteal LNs from M.Tb OVA and Candida OVA mice were collected on day 9 or day 14 post-immunization and restimulated for 4 h with PMA/Iono ($n = 5-7/\text{group}$, 2 experiments) or (D-E) co-cultured with OVA₃₂₃₋₃₃₉ and DCs in the presence of anti-CD154 or IgG for 4 d before brief PMA/Iono restimulation ($n = 8-9/\text{group}$, 2 experiments). (A) Representative histograms and (B) CD154 expression of naïve OT-II, M.Tb OVA, or Candida OVA LN cells on day 9 post immunization. (C) CD154 expression of naïve OT-II, M.Tb OVA, or Candida OVA LN cells on day 14 post immunization. (D) Representative frequencies (top number) and absolute numbers (bottom number) of OT-II Th1 and Th17 cells without PMA/Iono restimulation (first row) or with restimulation (middle/bottom rows). (E) Th1 and Th17 cell numbers from M.Tb OVA and Candida OVA groups following anti-CD154 blockade (Candida Th1/Th17 $p = 0.0008$). Bar graphs depict average \pm SEM. Statistical comparison performed using unpaired two-tailed Student's t-test * $p < 0.05$, ** $p < 0.005$. IgG, isotype control antibody.

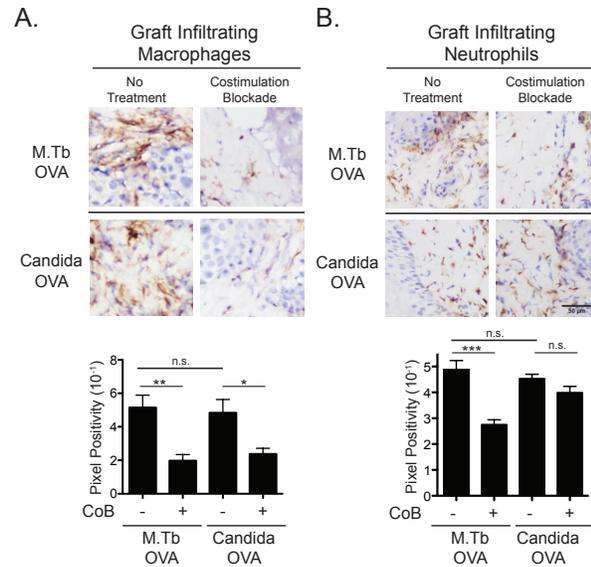
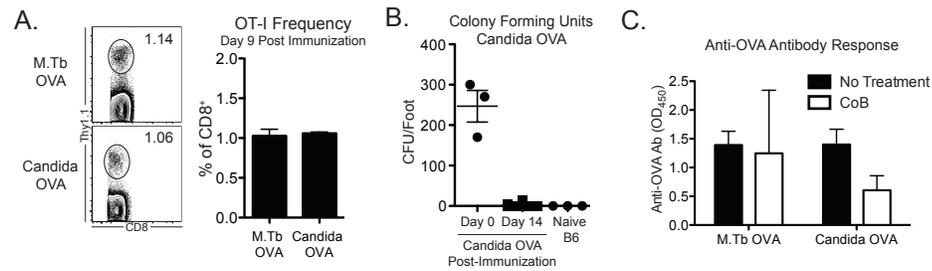
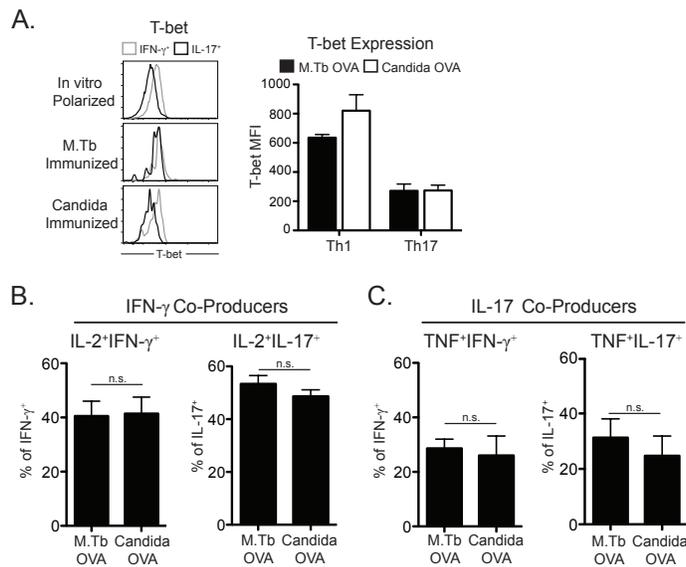


Figure 3.8. Candida polarized mice have greater neutrophil recruitment to skin grafts in the presence of CTLA-4 Ig. M.Tb OVA and Candida OVA mice received an OVA skin transplant on day 14 post-immunization. Mice were left untreated or were treated with 500 μ g each CTLA-4 Ig and anti-CD154. On day 6 post-graft skin grafts were harvested and snap frozen for histological analysis of (A) macrophage or (B) neutrophil infiltration. Cellular infiltration was quantified using Aperio ImageScope software (n = 20-22 image fields/group). Bar graphs show average \pm SEM. Statistical analysis was performed using one-way ANOVA with Bonferroni post test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

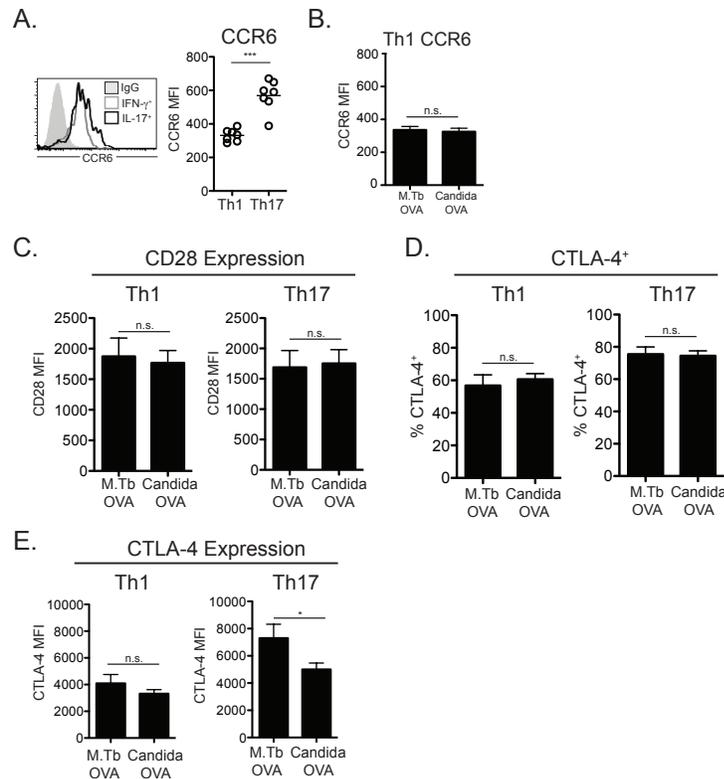


Supplemental Figure 3.1. M.Tb and Candida immunization and costimulation blockade

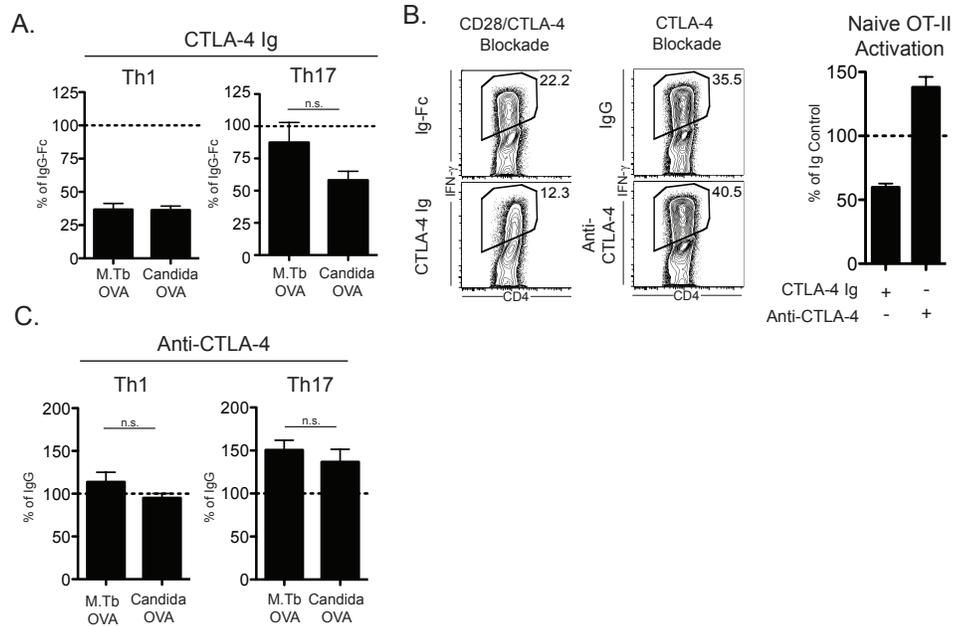
treatment yields similar levels of CD8⁺ OT-I cells and anti-OVA antibodies. Mice were adoptively transferred with 10^6 OT-I and 10^6 OT-II cells and immunized with M.Tb or Candida with OVA₃₂₃₋₃₃₉ peptide. (A) At 9 days post immunization OT-I cell frequencies were determined in the spleen ($p = n.s.$, $n = 5/\text{group}$). (B) Positive control Candida hyphae cultures, foot pads from day 14 post immunized Candida OVA mice ($n = 15$, 3 independent experiments) or foot pads from naïve B6 mice were minced and cultured overnight and colony forming units were quantified. (C) Mice received an OVA skin graft and were untreated or treated with CTLA-4 Ig and anti-CD154, 10 days post transplant anti-OVA antibodies were measured in the blood using ELISA ($p = n.s.$ all groups, $n = 2-3/\text{group}$). CFU, colony forming units.



Supplemental Figure 3.2. M.Tb and Candida elicited Th1 and Th17 cells express similar levels of T-bet and contain similar frequencies of IL-2 and TNF co-producers. Draining popliteal LNs from M.Tb OVA and Candida OVA mice were collected on day 9 post-immunization and restimulated for 4 h with PMA/Iono to identify Th1 and Th17 cells. (A) T-bet expression among OT-II Th1 and Th17 cells or in vitro Th1 polarized OT-II cells. (B) Frequency of IL-2⁺ cells among OT-II Th1 (left) or Th17 (right) cells. (C) Frequency of TNF⁺ cells among OT-II Th1 (left) or Th17 (right) cells. Bar graphs depict average \pm SEM. Statistical comparison performed using unpaired two-tailed Student's t-test, * $p < 0.05$.



Supplemental Figure 3.3. M.Tb and Candida elicited Th17 cells express more CCR6 and CTLA-4. Draining popliteal LNs from M.Tb OVA and Candida OVA mice were collected on day 9 post-immunization and restimulated for 4 h with PMA/Iono to identify Th1 and Th17 cells. (A) CCR6 expression on OT-II Th1 and Th17 cells populations ($p < 0.0001$, $n = 7-10/\text{group}$). (B) CCR6 expression on OT-II Th1 cells from M.Tb OVA and Candida OVA mice ($p = \text{n.s.}$, $n = 3-4/\text{group}$). Frequency of (C) CD28⁺ cells, (D) CTLA-4⁺ cells, and (E) CTLA-4 expression among M.Tb OVA and Candida OVA Th1 and Th17 cells. Bar graphs depict average \pm SEM. Statistical comparison performed using unpaired two-tailed Student's t-test, * $p < 0.05$.



Supplemental Figure 3.4. Th1 and Th17 cells from M.Tb and Candida are similarly inhibited by CTLA-4 Ig and augmented by anti-CTLA-4. Purified CD4⁺ T cells from the draining popliteal LNs of M.Tb OVA and Candida OVA mice were co-cultured with CD11c⁺ DCs and OVA₃₂₃₋₃₃₉ peptide for 4 days in the presence costimulation blockade or control molecules followed by brief PMA/Iono restimulation. (A) Effect of CTLA-4 Ig treatment of M.Tb OVA or Candida OVA mice relative to Ig-Fc control (9-13/group, 3 experiments). (B) Naïve OT-II splenocytes were stimulated with OVA₃₂₃₋₃₃₉ peptide in the presence of CTLA-4 Ig, anti-CTLA-4 or control molecules for 4 days followed by brief PMA/Iono restimulation. Left, representative frequencies of OT-II Th1 cells. Right, relative effect of CTLA-4 Ig or anti-CTLA-4 on Th1 cell frequency normalized to Ig control molecule (n = 5-8/group, 3 experiments). (C) Effect of anti-CTLA-4 treatment of M.Tb OVA or Candida OVA mice relative to IgG control. Bar graphs depict average \pm SEM. Statistical comparison performed using unpaired two-tailed Student's t-test, *p<0.05.

Chapter 4. High CTLA-4 Expression Corresponds with Diminished FOXO3 Expression on Human Th17 Memory Cells

Introduction

During a recall response, memory T cells maintain the functional and phenotypic properties that reflect their priming conditions (48). Recent studies have shown that pathogen-primed memory T cells can cross-react with alloantigen (16, 41) and that alloreactive T cells are inherently more polyspecific for peptide:MHC than conventional T cells (42, 44), suggesting that the alloreactive memory T cell pool reflects the pathogen-specific stimulation history of an individual.

The heterogeneity of T cell memory recall responses is critically important for transplant recipients receiving lifelong immunosuppression to prevent T cell mediated graft rejection. Th17 cells, which provide protective immunity to fungi and extracellular bacteria and can be pathogenic in autoimmune diseases such as multiple sclerosis (MS), inflammatory bowel disease (IBD), and systemic lupus erythematosus (SLE) (48, 197, 221, 222). Recently, our lab showed that Th17 cells were associated with acute cellular rejection in renal transplant recipients treated with the CTLA-4 Ig derivative belatacept. Th17 cells were also uniquely resistant to CD28/CTLA-4 blockade with belatacept in vitro (138, 171). Interestingly, CTLA-4 Ig and its derivatives have shown limited efficiency in clinical trials of MS, IBD and SLE (102, 201, 202). Together, these results demonstrate that Th17 cells might be ineffectual against Th17 populations in vivo. However, the mechanism of this resistance remains unclear.

There are conflicting reports about the costimulation requirements of murine and human Th17 cells. Recent studies independently suggesting that either CD28 or CTLA-4 signals can suppress Th17 differentiation (83-85), that human Th17 clones are uniquely sensitive to CD28 stimulation (87), and that alternate costimulatory molecules are required to optimally differentiate

Th17 cells (84, 86, 88, 89). Our lab showed that human and murine Th17 cells express similar levels of CD28 but significantly higher levels of coinhibitory CTLA-4 (138, 171).

Here we show that human Th17 memory cells are resistant to selective CD28 blockade. High CTLA-4 expression on Th17 cells was dependent on CD28 signals, and did not reflect an overall difference in activation status of Th1 and Th17 cells. Overall, Th17 cells expressed less of the transcription factor FOXO3, which is known to bind upstream of the *CTLA-4* gene, suggesting that this molecule controls high CTLA-4 expression in Th17 memory cells. This study demonstrates important differences in the cosignaling pathways of CD4⁺ T cells, which has important implications for immunomodulation of memory T cells in transplantation and autoimmunity.

Results & Discussion

Human Th17 cells are resistant to selective CD28 blockade

Previously, our lab has shown that Th17 cells express high levels of CTLA-4 in response to PMA/Ionomycin stimulation (138). Human Th17 cells are resistant to the CD28/CTLA-4 blocker CTLA-4 Ig (belatacept, (138)). We questioned whether CD28 or CTLA-4 signals were primarily responsible for this observation. Using an anti-CD28 domain antibody (dAb), which contains a single antigen binding site and thus is unable to be cross-linked via Fc receptors (223), we selectively inhibited CD28 signals during polyclonal stimulation with anti-CD3. We found that primary and memory Th1 cells were effectively inhibited (Figure 4.1A). Th17 memory cells, in contrast, were not inhibited by CD28 blockade (Figure 4.1A). To rule out unanticipated activation of CD28 on Th17 cells, we performed this assay in the presence of CD28 ligation. We found that Th1 populations were augmented by CD28 signals, while Th17 memory cells were inhibited by CD28 signals (Figure 4.1B). Together, these results demonstrate that CD28 signaling has remarkably different outcomes on Th1 and Th17 populations.

Th1 and Th17 cells are similarly activated by CD28 signals

Recent work has demonstrated that human Th17 clones are hyporesponsive to TCR signals and simultaneously uniquely sensitive to CD28 signals (87). We questioned whether high CTLA-4 expression on Th17 cells was indicative of greater global cellular activation by CD28 ligation compared to Th1 cells. To minimize engagement of other costimulation pathways, we purified CD4⁺ T cells and activated them in the presence of CD3 or CD3/CD28 beads. We found that primary Th1, memory Th1, and memory Th17 cells expressed similar levels of CD69 following TCR stimulation alone (Figure 4.2A). The addition of CD28 signals upregulated CD69 expression to similar degree on all three populations (Figure 4.2A). The expression of CD25 is also associated with activation of T cells. We found that Th1 and Th17 populations expressed similar levels of CD25 following activation and that CD28 signals did not affect CD25 expression (Figure 4.2B). However, in the presence of strong TCR ligation, the expression of CTLA-4 was significantly upregulated in Th17 cells ($p < 0.05$, Figure 4.2C). The addition of CD28 signals further upregulated CTLA-4 on Th17 cells ($p < 0.01$, Figure 4.2C). Together, these results demonstrate that Th1 and Th17 cells express similar levels of the global activation markers CD69 and CD25 following TCR or TCR and CD28 signals. Th17 cells are programmed to express significantly higher levels of CTLA-4, and a significant portion of this expression is mediated by CD28 signaling.

De novo *Ctla4* gene expression is induced by both CD3 and CD28 signaling pathways (139, 140). However, several putative transcription factor binding sites have been identified upstream of CTLA-4, and the relative importance of these signals is not known (139, 224). However, experimental evidence suggests that a significant portion of CTLA-4 expression has been shown to be induced by CD28 signals. Indeed, early studies and our work have demonstrated that CD28 signals are required for optimal CTLA-4 expression (135, 137, 138). These results demonstrate that Th17 cells are programmed to express high levels of CTLA-4 in

response to strong TCR stimulation, but that a significant portion of CTLA-4 expression in these Th17 memory cells is dependent on CD28 signals.

FOXO3 expression correlates inversely with CTLA-4 expression

Intracellular signals downstream of CD28 are transmitted through PI3K-Akt-mTOR axis, which terminate in the transcription factors FOXO1 and FOXO3 in T cells (225, 226). Mouse models of FOXO1 and FOXO1/FOXO3 deletion have diminished CTLA-4 expression on CD4⁺ populations (141, 227, 228). Recent work has demonstrated that IL-17 production by human Th17 cells is uniquely reliant on Akt, PI3K, and FOXO1 (229), and that Akt is constitutively activated in human Th17 cells (228). However, a specific connection between FOXO and CTLA-4 expression has not been shown.

We found that in resting CD4⁺ T cells, FOXO1 and FOXO3 were expressed at high levels (Figure 4.3A-B). Following stimulation, FOXO1 and FOXO3 expression was downregulated, consistent with their known targeted degradation (Figure 4.3A-B). We next questioned whether FOXO expression correlated with CTLA-4 expression. Gating on high, medium, and low expression of FOXO1 revealed that all three of these populations expressed similar levels of CTLA-4 (Figure 4.3C). Stratification of FOXO3 with similar gates, however, revealed that the loss of FOXO3 expression correlated with higher CTLA-4 expression (Figure 4.3D). Together, these results demonstrate that FOXO is dynamically controlled following T cell stimulation, and suggests that lower FOXO3 expression correlates inversely with CTLA-4 expression.

FOXO3 expression is diminished in Th17 cells

We questioned whether the loss of FOXO3 expression correlated with high CTLA-4 expression in Th17 memory cells. We investigated the expression of FOXO1 and FOXO3 on Th1 populations. Th1 and Th17 populations expressed similar levels of FOXO1 (Figure 4.4A).

However, FOXO3 expression was significantly lower on Th17 cells compared to both Th1 primary and memory populations ($p < 0.01$, Figure 4.4B). This is the first description of differential regulation of FOXO expression on human CD4⁺ T cell subsets. Together, these data strongly suggest that lower FOXO3 correlates with the high expression of CTLA-4 on Th17 memory cells.

In sum, this study demonstrates that the resistance of Th17 cells to belatacept is mediated through differences in CD28 signaling that result in high CTLA-4 expression. These results demonstrate that cosignaling differences can have profound implications in cellular function and has implications for immunomodulation of pathogenic Th17 populations.

Materials & Methods

Cells and Stimulations. Fresh peripheral blood mononuclear cells (PBMC) were isolated from normal healthy donors using protocols approved by the Emory University Institutional Review Board (IRB #00006248) using BD Vacutainer CPT tubes (BD Diagnostics). In some experiments, cells were frozen in 10% DMSO and 50% FBS and stored in liquid nitrogen. Cells were thawed and rested overnight at 37°C before stimulations. Cells were cultured in a 96 well plate in RPMI supplemented with 10% FBS (Mediatech, VA), 2.4 mM L-glutamine, and 10 μ M 2-mercaptoethanol (Sigma). For PMA/Iono stimulations, cells were stimulated with 30 ng/mL PMA and 400 ng/mL Ionomycin (Sigma) for 4 h, 10 μ g/mL GolgiStop (BD Biosciences) was added for the final 3 h. For anti-CD3 stimulations, fresh or frozen PBMC were stimulated with 1 μ g/mL functional grade anti-CD3 (OKT3; eBiosciences) for 4 hours, with 10 μ g/mL GolgiStop (BD Biosciences) added for the final 3 h. For detection of CTLA-4 upregulation, 5×10^5 /mL PBMC were cultured in media with 0.5 μ g/mL anti-CD3 (OKT3, eBiosciences) and 5 μ g/mL CTLA-4 Ig

(Bristol-Meyers Squibb) or IgG-Fc (BioXCell) for 5 h in 96 well U-bottom plates. For the final 4 h, anti-CTLA-4 PE and 10 µg/mL GolgiStop (BD Biosciences) was added.

Proliferation Assay. 3×10^5 /mL PBMC were cultured in media with 1 µg/mL anti-CD3 (OKT3, eBiosciences) and either 10 µg/mL anti-CD28 mAb (clone CD28.2, BD Biosciences), 10 µg/mL anti-CD28 mAb (clone 9.3, BioXCell), or 10 µg/mL anti-CD28 dAb (Bristol-Myers Squibb) in 96-well flat bottomed plates for 3 d at 37 °C. After 3 d, CountBrite beads were added (Invitrogen) and cells were transferred to 96 well U-bottom plates and stimulated with PMA/Ion as described above. Some cultures were restimulated with 30 ng/mL PMA and 400 ng/mL Ionomycin (Sigma) for 4 h, and 10 µg/mL GolgiStop (BD Biosciences) was added for the final 3 h.

Anti-CD28 bead stimulations. Fresh or frozen PBMC were isolated as described. In some experiments, CD4⁺ T cells were isolated using CD4⁺ T cell isolation kit II (Miltenyi) according to manufacturer's instructions. CD4⁺ T cells were stimulated using CD3/Ig or CD3/CD28 stimulation M450 beads. Beads were prepared using M450 Dynal polystyrene beads (Invitrogen) and conjugating 5 µg each of anti-CD3 (OKT3) with anti-CD28 (9.3) or human IgG-Fc (BioXCell) according to manufacturer's instructions. Cells were stimulated with beads at a 1:3 ratio for 5 h, with 10 µg/mL GolgiStop (BD Biosciences) and anti-CTLA-4 PE for the final 4 h.

Human Surface and Intracellular Staining for Flow Cytometry. Surface anti-CTLA-4 was detected by adding anti-CTLA-4 PE (BN13) during stimulations as described above (230). Surface antigen staining was performed for 15 min at 23 °C using the following antibodies: CD3 (UCHT-1), CD4 (L200 or RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD19 (HIB19), CD25 (BC96), CD28 (CD28.2), CD69 (FN50), CD45RA (HI100). For cytokine analysis, cells were prepared for intracellular staining following manufacturer's protocol (BD Biosciences Fix/Perm

Kit) and stained with the following antibodies: IFN- γ (45.B3), and IL-17A (eBio64DEC17).

Detection of FOXO1 (clone C29H4) and FOXO3a (clone 75D8) was performed using 500 ng mAb per test with PE Anti-rabbit F(ab')₂ secondary (all from Cell Signalling Technology) and the FOXP3 Fix/Perm kit (eBiosciences). Samples were analyzed using an LSR II flow cytometer (BD Biosciences), and data was analyzed using FlowJo software (Treestar, San Carlos, CA).

Statistical Analysis. Frequencies of cytokine production and relative protein expression levels were assessed using Student's t-test (two-tailed) or one-way ANOVA with Bonferroni post test. Comparisons of anti-CD3/Ig vs anti-CD3/anti-CD28 beads were performed using two-way ANOVA with Bonferroni post test. Significance was determined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figures

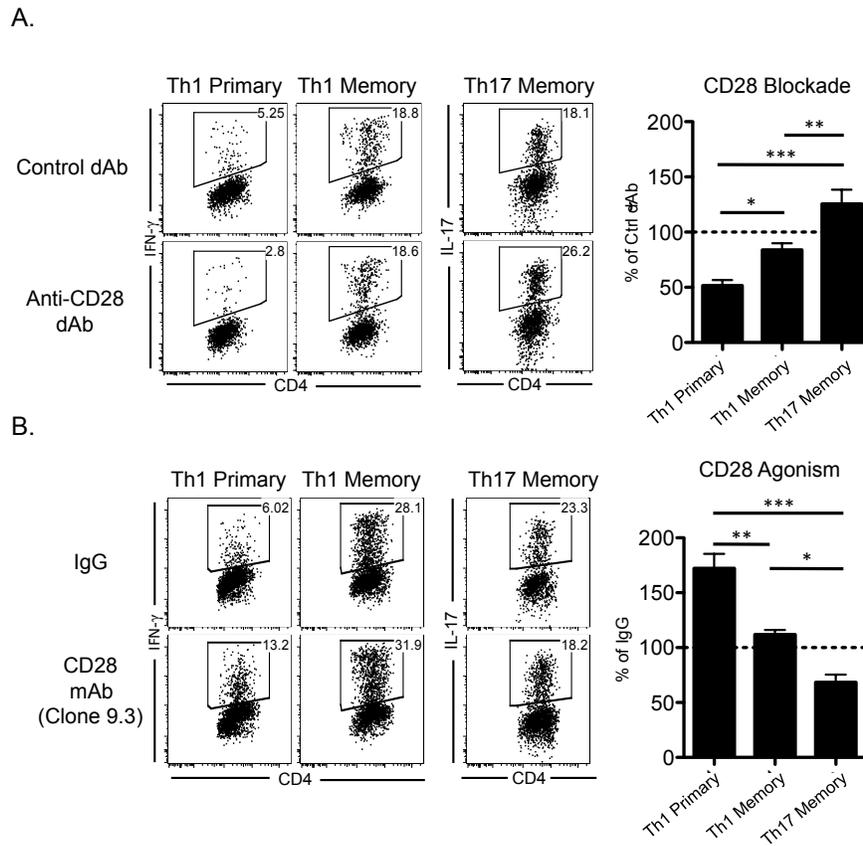


Figure 4.1. CD28 mediates divergent signaling outcomes on Th1 and Th17 memory cells.)

Health donor PBMC were stimulated with anti-CD3 in the presence of CD28 blockade or ligation for 3 d, followed by brief restimulation with PMA/Iono to assess the frequency of Th1 primary, Th1 memory, and Th17 memory populations. (A) Cells were stimulated in the presence of anti-CD28 dAbs or control dAb (Th1 primary vs Th1 memory $p < 0.05$, Th1 primary vs Th17 memory $p < 0.001$, Th1 memory vs Th17 memory, $p < 0.001$). (B) Cells were stimulated in the presence of anti-CD28 mAb or control IgG (Th1 primary vs Th1 memory $p < 0.01$, Th1 primary vs Th17 memory $p < 0.001$, Th1 memory vs Th17 memory, $p < 0.05$). All experiments depict > 6 donors from > 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PBMC, peripheral blood mononuclear cells. dAbs, domain antibodies.

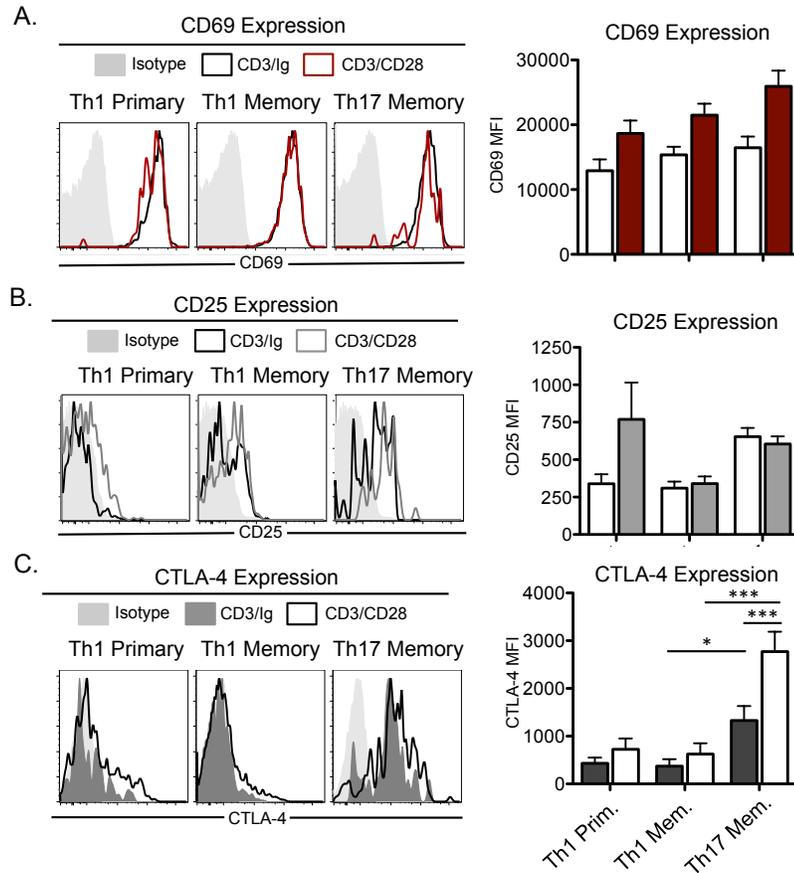


Figure 4.2. Th1 and Th17 cells are similarly activated by TCR and CD28 signals. Purified CD4⁺ T cells were stimulated with beads coated with anti-CD3 with IgG, or anti-CD3 and anti-CD28 for 5 h and expression of surface molecules was assessed on primary Th1, memory Th1, and memory Th17 populations. (A) CD69 expression, (B) CD25 expression or (C) surface CTLA-4 expression (CD3/Ig Th1 memory vs Th17 memory p<0.05, CD3/CD28 Th1 memory vs Th17 memory p<0.001, Th17 CD3/Ig vs CD3/CD28 p<0.001. Experiments depict >8 donors from >3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

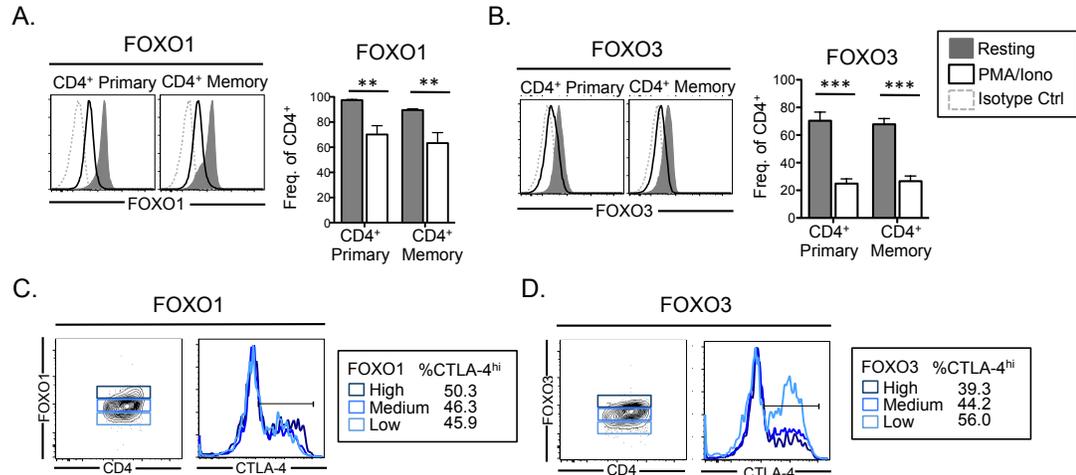


Figure 4.3. CTLA-4 expression correlates inversely with FOXO3 expression. PBMC were stimulated with PMA/Ion for 4 h and intracellular expression of FOXO1 and FOXO3 were assessed. Resting and stimulated CD4⁺ primary and CD4⁺ memory population expression of (A) FOXO1 (CD4⁺ primary resting vs stimulated $p < 0.01$, CD4⁺ memory resting vs stimulated $p < 0.01$) and (B) FOXO3 (CD4⁺ primary resting vs stimulated $p < 0.001$, CD4⁺ memory resting vs stimulated $p < 0.001$). (C) Expression of CTLA-4 within high, medium, and low FOXO1 populations on CD4⁺ T cells. (D) Expression of CTLA-4 within high, medium, and low FOXO3 populations on FOXO3 CD4⁺ T cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PBMC, peripheral blood mononuclear cells.

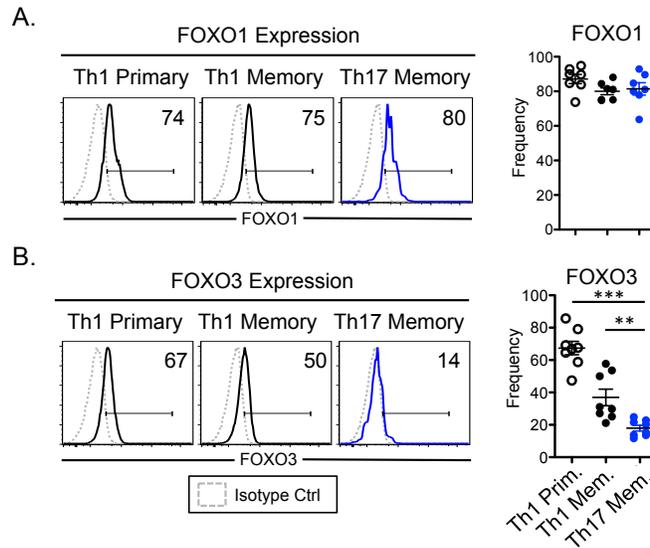


Figure 4.4. Th17 memory cells express less of the transcription factor FOXO3. PBMC were stimulated with PMA/Ion for 4 h and intracellular expression of FOXO1 and FOXO3 were assessed. Primary Th1, memory Th1, and memory Th17 expression of (A) FOXO1 and (B) FOXO3 (Primary Th1 vs memory Th17 $p < 0.001$, memory Th1 vs memory Th17 $p < 0.01$). Experiments depict >6 donors from >3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PBMC, peripheral blood mononuclear cells.

Chapter 5. Low Affinity Memory CD8⁺ T Cells Mediate Robust Heterologous Immunity

Introduction

Following encounter with microbial antigen, T cells can differentiate into memory cells with a multitude of phenotypic profiles in order to provide long-lasting protection against subsequent encounters with pathogens (3, 231). In settings of transplantation, microbe-elicited T cell memory can also cross-react with allogeneic antigen and mediate graft rejection, a process termed allogeneic heterologous immunity (12, 41). Memory T cells are recognized as a barrier to many immunomodulation strategies aimed at limiting alloreactive T cell responses (4).

The strength of TCR interaction with peptide:MHC is known to have a critical impact on the subsequent differentiation of the T cell (189-191, 195). Several studies have investigated the effects of limited or chronic antigen exposure (232-236) and of TCR priming affinity (191, 194, 196, 237) on the differentiation of effector and memory CD8⁺ T cells. Recently, it has been shown that low affinity priming can lead to stable memory T cell responses capable of secondary recall and that low affinity cells can drive autoimmune responses (191, 196, 238), highlighting the importance of understanding the role of low affinity T cells during pathologic immune responses.

The affinity of the TCR for antigen is also likely a critical facet of allogeneic heterologous immunity. In this scenario, memory CD8⁺ T cells that were primed with microbial antigen can recognize a unique cross-reactive allogeneic antigen, and each of these interactions is likely have a distinct affinity. Indeed, memory T cells recognizing allogeneic antigen have been measured to have a similar range of affinities as those against tumor and microbial antigens (12). Recent work has also demonstrated that allogeneic T cells are suited to recognize a greater number of peptide:MHCs than conventional T cells, providing strong evidence that allogeneic T cell interactions occur over a range of high and low TCR affinities (42-44).

In the context of allogeneic heterologous immunity, however, the contribution of TCR priming affinity is poorly understood. In particular, the functional consequences of distinct priming and secondary restimulation affinities on memory CD8⁺ T cell populations are not well defined. Here we found that low affinity priming affinity dictates a distinct differentiation program compared to high affinity priming that is characterized by high CD45RB and CD62L expression. Low affinity primed CD45RB^{hi} cells were capable of robust secondary responses and, strikingly, mediated faster graft rejection kinetics. High CD45RB expression tuned low affinity primed memory CD8⁺ T cells to better respond upon heterologous rechallenge, as sorted CD45RB^{hi} cells proliferated better than CD45RB^{lo} cells, and blockade of CD45RB prolonged graft rejection after low affinity priming. The secondary effector phenotype of low affinity primed cells was associated with concurrent downregulation of CD45RB and enhanced IL-2 production. This study establishes a novel connection between the affinity of CD8⁺ T cell priming and CD45-mediated T cell tuning, and provides mechanistic insight into the functionality low affinity T cells in transplantation, protective immunity, and autoimmunity.

Results

Low affinity CD8⁺ T cell priming efficiently generates memory cells

The affinity of TCR interactions during priming impacts CD8⁺ T cell programming during effector and memory phases (3, 189, 190). We utilized the OVA-based TCR transgenic system in which congenically labeled OT-I T cells are primed during infection with an acutely cleared *Listeria monocytogenes* strain engineered to express single amino acid substitutions of the OT-I epitope SIINFEKL (N4 OVA), thus creating lower altered peptide ligands (APLs) that are recognized by OT-I T cells (191). Each of these APLs has a lower functional avidity relative to N4 OVA as follows: SIIQFEKL (Q4 OVA) 18.3 fold, SIITFEKL (T4 OVA) 70.7 fold, and SIIVFEKL (V4 OVA) 680 fold (191).

We found that the peak of proliferative response in the blood was proportional to the strength of priming TCR stimulation (Figure 1A). Previous studies have demonstrated that the low affinity V4 OVA APL is capable of generating stable memory populations (191). However, the relative efficiency of differentiation into memory precursor cells, which are detectable among primary effector cells, is not known. Using the markers KLRG-1 and CD127 to distinguish short-lived effector CD8⁺ T cells (KLRG-1^{hi} CD127^{lo}) and memory precursor CD8⁺ T cells (KLRG-1^{lo} CD127^{hi}, (3)) we found that high affinity N4 or low affinity V4 OVA priming generated similar frequencies of both of these populations (Figure 5.1B).

Using an enrichment technique to reliably detect and quantify very low frequency OT-I memory T cells (239), we found that high affinity N4 and low affinity V4 OVA primed cells formed memory populations in secondary lymphoid organs (Figure 5.1C). Low affinity V4 OVA cells were found at ~10-fold lower frequency than N4 OVA (N4 OVA $1.5 \times 10^5 \pm 4.2 \times 10^4$, V4 OVA $3.4 \times 10^4 \pm 2.0 \times 10^4$ Figure 5.1C). Consistent with the phenotype of high quality CD8⁺ memory T cells, both N4 OVA and V4 OVA primed memory cells expressed high levels of CD44 and CD127, and low levels of CD69 and Granzyme B (Supplemental Figure 5.1A). Together, these

results demonstrate that while primary effector proliferation correlates with TCR signal strength, low affinity priming efficiently leads to memory cell formation.

Low affinity memory CD8⁺ T cells have a distinct central memory phenotype

Memory CD8⁺ T cells can differentiate into many phenotypic subsets with distinct characteristics (3, 231). While the phenotype of low affinity primed CD8⁺ T cells has been examined during the primary effector phase (191, 195, 196, 237), relatively little is known about the effect of TCR priming affinity on CD8⁺ memory phenotype. Murine memory CD8⁺ T cells are most commonly classified as CCR7^{hi}CD62L^{hi} central or CCR7^{lo}CD62L^{lo} effector memory cells (3, 231). While the relative expression of these markers has been reported in model of limited antigen or TCR signaling (232, 233, 240), the phenotype of low affinity primed memory CD8⁺ T cells has not been investigated. We investigated the expression of CCR7 and CD62L on high and low affinity OVA primed CD44^{hi} memory CD8⁺ T cells in secondary lymphoid organs. We found that while CCR7 expression was similar between these populations (Supplemental Figure 5.1B), low affinity V4 OVA memory cells expressed significantly higher levels of CD62L compared to high affinity cells (Figure 5.1D). We found that CD62L expression correlated with TCR priming affinity in low affinity V4 OVA and intermediate affinity Q4 OVA memory cells (Supplemental Figure 1C). This increased CD62L expression on low affinity primed cells was present on in both spleen and LN (Supplemental Figure 5.1C). Together, these data demonstrate that low affinity priming leads to more T_{CM} CD62L expression of memory cells in both the spleen and lymph nodes.

Low affinity memory CD8⁺ T cells are tuned to generate robust secondary recall responses

Alloreactive T cells have been demonstrated to have a range of affinities for cognate antigen and are more polyspecific than conventional T cells (12, 42, 43). In the setting of allogeneic heterologous immunity, CD8⁺ T cells activated over a range of affinities cross-react

with allogeneic antigen of a different, potentially higher, affinity. To model a scenario in which low and high affinity pathogen primed memory T cells cross-react with an allograft, we challenged mice possessing high affinity N4 OVA and low affinity V4 OVA memory CD8⁺ T cells with a skin graft expressing high affinity N4 OVA (Figure 5.2A). High affinity N4 OVA memory elicited rejection of grafts with an MST = 17 days. Surprisingly, despite a lower precursor frequency, low affinity V4 OVA memory cross-reacting with graft-expressed N4 OVA mediated faster graft rejection (MST 11 days, $p < 0.0001$, Figure 5.2A). Challenge of mice with memory against either of two additional intermediate-affinity OVA APLs Q4 and T4 resulted in graft rejection kinetics that were faster than high affinity N4 OVA (Q4 OVA MST = 15.5 days, T4 OVA MST = 14.0 days, Figure 5.2B). The mean survival time of high affinity N4 OVA skin graft challenge correlated with the relative avidity of priming ((191), Figure 5.2B). These results could not be attributed to different frequencies of OVA-specific endogenous CD8⁺ populations, as similar frequency of endogenous CD8⁺ T cells were OVA-specific (Figure 5.2C). Thus, despite the lower precursor frequency of V4 OVA memory, low affinity memory CD8⁺ T cells were able to elicit faster graft rejection kinetics compared to high affinity N4 OVA memory cells.

We quantified the relative ability of high and low affinity memory cells to respond to high affinity antigen following an *in vivo* rechallenge immunization. Despite a lower precursor frequency (Figure 5.1C), low affinity V4 OVA primed memory cells proliferated to a similar absolute number as high affinity N4 OVA memory cells (Figure 5.2D). When normalized to pre-rechallenge precursor frequency, low affinity V4 OVA cells were calculated to have undergone a greater fold expansion in response to high affinity N4 antigen rechallenge (Figure 5.2E). These data demonstrate that low affinity primed memory CD8⁺ T cells are tuned to become potent secondary effectors when challenged with high affinity antigen.

CD45RB is a stable marker of the affinity experience of memory CD8⁺ T cells

CD45 is a transmembrane phosphatase that is critical for the development of T cells (241). The relative abundance of CD45 isoforms has been used to distinguish naïve from effector and memory T cells. In mice, the expression of CD45RB is used as a marker for large CD45 isoforms, with naïve CD8⁺ T cells defined as CD45RB^{hi}, while effector and memory cells downregulate CD45RB expression, becoming predominantly CD45RB^{lo} (241). As CD45 is a molecule that is critical for TCR signaling and is a surface marker of T cell differentiation, we investigated the expression of CD45RB on OVA APL primed cells. During the primary effector response, we found that high affinity primed N4 OVA effectors downregulated CD45RB (48.9±2.2% CD45RB^{hi}, Figure 5.3A). Surprisingly, V4 primed OT-I cells in the blood maintained a predominantly CD45RB^{hi} status compared to high affinity primed cells (V4 OVA 68.7±3.6% CD45RB^{hi}, p<0.001, Figure 5.3A). At memory, the divergent expression of CD45RB was maintained in high affinity and low affinity primed OT-I cells residing in secondary lymphoid tissue (N4 OVA 54.7±6.76%, V4 OVA 79.3±3.4%, p=0.0064, Figure 5.3B). We also investigated the expression of several other molecules that have been shown to modulate TCR signaling. We found no significant difference in expression between Vα2, CD8, or CD5 expression on high and low affinity primed OT-I cells (Supplemental Figure 5.2A). Recent reports have identified CD69 and Nur77, respectively, as measures of cumulative TCR signaling strength early following stimulation (242, 243). We found that high and low affinity memory CD8⁺ T cells upregulated similar levels of both CD69 and Nur77 in response to high affinity N4 OVA rechallenge (Supplemental Figure 5.2B-C), demonstrating that these molecules do not account for the observed differences in secondary effector potency following heterologous rechallenge.

In order to determine if CD45RB expression on low affinity primed CD8⁺ memory T cells was functionally important in mediating graft rejection, we treated N4 OVA and V4 OVA memory mice with anti-CD45RB mAb during skin graft rechallenge. Blockade of CD45RB during N4 OVA skin graft challenge resulted in no significant prolongation of graft survival on mice containing N4 OVA primed cells compared to untreated mice (Untreated MST 17 d, Figure

5.2A; anti-CD45RB MST 17 d, Figure 5.3C). However, treatment of mice containing V4 OVA primed memory CD8⁺ T cells with anti-CD45RB led to significant prolongation of graft survival (Untreated MST 11 d, Figure 5.2A; anti-CD45RB MST 21 d, Figure 5.3C), demonstrating that CD45RB is functionally important specifically in the secondary recall response mediated by low affinity primed memory cells. These data are the first demonstration that CD45RB expression can be used to stratify the priming affinity of naïve CD8⁺ T cells, and that long-lived CD44^{hi} memory T cells can remain predominantly CD45RB^{hi}.

High CD45RB expression tunes CD8⁺ T cells to respond to heterologous rechallenge antigen

Following *in vitro* priming of OT-I cells with OVA APLs, we found that Tm cells similarly upregulated CD44 expression but that CD45RB expression correlated with decreasing TCR avidity ($R^2=0.95$, Supplemental Figure 5.3A-B). Previous *in vitro* work has demonstrated that larger CD45 isoforms tunes T cells for stronger proximal TCR signaling compared to smaller isoforms (241). However, the mechanisms underlying the induction of different CD45 isoforms on memory T cells *in vivo* are unknown. We hypothesized that the affinity of TCR ligation during priming may control CD45RB expression and hence the magnitude of recall response in these cells. To investigate whether high CD45RB expression is sufficient to confer enhanced recall potential during secondary challenge, we sorted Q4 OVA primed OT-I cells, which have intermediate CD45RB expression (Supplemental Figure 5.3A), into CD45RB^{hi} and CD45RB^{lo} populations and rechallenged them with N4 OVA peptide in naïve congenic hosts (Figure 5.3C). CD45RB^{hi} cells proliferated to a significantly greater number than CD45RB^{lo} cells ($p=0.0012$, Figure 5.3D). Together, these data demonstrate that low affinity primed memory CD8⁺ T cells are tuned by CD45 to robustly respond to heterologous rechallenge.

Low affinity primed secondary effector cells have a distinct phenotype

In light of the finding that low affinity primed CD8⁺ memory T cells mount robust secondary recall responses against high affinity antigen, we investigated the expression of CD45RB following rechallenge. High affinity N4 OVA peptide rechallenge of N4 OVA memory cells slightly downregulated CD45RB expression (30.2±3.39% CD45RB^{hi}, Figure 5.4A). Strikingly, V4 OVA primed memory cells significantly downregulated CD45RB expression during the secondary proliferative response to high affinity antigen (19.6±1.96% CD45RB^{hi}, Figure 5.4A). The downregulation of CD45RB was significantly greater in V4 OVA primed secondary effectors compared to N4 OVA primed secondary effectors (p<0.001, Figure 5.4B). While V4 OVA primed cells significantly downregulated CD62L expression, they retained higher CD62L expression compared to high affinity primed secondary effectors (p<0.001, Figure 5.4C). These data demonstrate that the affinity of priming conveys a distinct phenotype on CD8⁺ T cell even during recall with antigen of the same TCR affinity.

The cosignaling receptor PD-1 has been shown to be a marker of cumulative effector signal strength on effector CD8⁺ T cells and to correlate with CD62L expression (232). PD-1 was significantly upregulated in CD45RB^{lo} fraction of V4 OVA primed cells (Figure 5.4D), demonstrating that this population perceives strong TCR signals. Taken together, these data demonstrate that CD45RB expression can be used to describe the affinity experience of memory and secondary effector cells, and that low affinity primed memory CD8⁺ T cells have a distinct phenotype during secondary response to high affinity antigen.

Low affinity primed secondary effector CD8⁺ T cell produce high levels of IL-2 and upregulate IL-2R α

Production of cytokine during proliferative responses is important for functional immunity against pathogens (231). We investigated the ability of high and low affinity primed secondary effectors to produce cytokines. Following high affinity N4 OVA rechallenge, a similar frequency of high affinity N4 OVA and low affinity V4 OVA primed secondary effectors

produced IFN- γ , and the frequency of IFN- γ producers did not vary with degree of CD45RB expression (Supplemental Figure 5.4A-B). Expression of TNF was slightly lower in V4 OVA primed secondary effectors, while both high and low affinity primed CD45RB^{lo} populations expressed more TNF than CD45RB^{hi} populations (Supplemental Figure 5.4A-B).

IL-2 is a critical cytokine for CD8⁺ T cell secondary responses (244). Consistent with previous published results (2, 245), a low frequency of N4 OVA primed secondary effectors produced IL-2 (Figure 5.5A). In contrast, we found that a significantly greater proportion of V4 OVA primed CD45RB^{lo} cells produced IL-2 (Figure 5.5A). High IL-2 was specific to low affinity primed CD45RB^{lo} secondary effectors during N4 OVA rechallenge, as high and low affinity primary effectors produced similarly low levels of IL-2 (Figure 5.5B).

IL-2 signaling can induce the expression of the high affinity IL-2R α chain (CD25, (246)), and high CD25 expression is associated with differentiated effector cells (246-248). We found that V4 OVA primed CD45RB^{lo} cells expressed significantly higher levels of CD25 compared to both CD45RB^{hi} cells and N4 OVA primed CD45RB^{lo} cells (N4 OVA CD45RB^{hi} vs CD45RB^{lo} p<0.001, CD45RB^{lo} N4 OVA vs V4 OVA p<0.01, Figure 5.5C). High expression of the IL-2R β chain, CD122, can confer sensitivity to IL-15 signaling in memory T cells, while lower expression is associated with IL-2 sensitivity and effector T cells (248, 249). We found that high affinity N4 OVA primed secondary effectors expressed similar levels of CD122 (Figure 5.5D). Among low affinity V4 OVA primed secondary effectors, however, CD45RB^{lo} cells expressed significantly lower CD122 levels than CD45RB^{hi} cells (p<0.01, Figure 5.5D). These data suggest that low affinity primed secondary effector cells that downregulate CD45RB expression also downregulate CD122 to effector cell levels. Together, these results demonstrate that low affinity V4 OVA primed secondary effectors effectively switch into an effector-like phenotype characterized by high IL-2 production, high expression of CD25, and effector CD122 expression.

Low affinity polyclonal CD8⁺ T cells express high levels of CD45RB

To determine if these findings in TCR transgenic cells apply to polyclonal CD8⁺ T cell populations, we investigated the ability of CD45RB to distinguish the affinity experience of polyclonal CD8⁺ T cells. We infected mice with LM-OVA and investigated the expression of CD45RB on the endogenous CD8⁺ T cell population. We found a range of CD45RB expression among OVA-specific polyclonal CD8⁺ T cells (Figure 5.6A). Similar to secondary effector V4 OVA primed OT-I cells (Figure 5.4C), OVA-specific CD45RB^{hi} CD8⁺ T cells maintained higher expression of CD62L compared to CD45RB^{lo} cells (Figure 5.6B).

Low affinity cells do not efficiently bind tetramer compared to high affinity cells (238). We found that compared to CD8⁺CD44^{hi}CD45RB^{hi} cells, CD44^{hi}CD45RB^{lo} cells more efficiently bound tetramer at lower concentrations, demonstrating that the CD44^{hi}CD45RB^{lo} compartment contains a greater frequency of high affinity cells (Figure 5.6C). Thus, in a polyclonal population of CD8⁺ T cells, antigen experienced CD45RB^{hi} cells express greater levels of CD62L and bind tetramer less efficiently than CD45RB^{lo} cells.

Discussion

Here we establish a novel mechanism by which low affinity primed memory CD8⁺ T cells can become potent secondary effectors during heterologous rechallenge. Compared to high affinity priming, low affinity priming elicited a unique differentiation program that enabled potent secondary responses *in vivo*, as demonstrated by faster graft rejection kinetics and larger proliferative responses. These findings have direct implications for allogeneic heterologous immunity, which can be a significant barrier to transplantation tolerance (13, 16). While it is appreciated that high affinity memory CD8⁺ T cells can mediate graft rejection (250, 251), this study demonstrates the surprising finding that low affinity priming is sufficient to generate memory cells that, despite a lower precursor frequency, mediate potent secondary responses against a cross-reactive graft challenge.

We found that low affinity primed cells maintain high expression CD45RB, which has previously been described only on naïve CD8⁺ T cells. CD45RB was stably expressed at high levels on fully differentiated CD44^{hi} TCR transgenic and polyclonal memory CD8⁺ T cells following low affinity priming, but was significantly downregulated on secondary effectors. Expression of other molecules that are known to tune T cell signaling, including CD8 and CD5, were similarly expressed on high and low affinity memory cells. We also found that expression of CD69 and Nur77, two markers of cumulative TCR signal strength, did not reflect the increased secondary effector potency of low affinity primed memory CD8⁺ T cells (242, 243). Expression of these markers, as well as functional avidity measured by IFN- γ , rapidly change following stimulation (191, 242, 243). Thus, in conjunction with CD44 expression, high CD45RB expression is a novel and stable marker for low affinity primed CD8⁺ memory T cells.

Previous *in vitro* work has demonstrated that large CD45 isoforms tune T cells for stronger proximal TCR signaling by undergoing less inhibitory dimerization than smaller isoforms (241, 252). However, the mechanisms underlying the induction of different CD45

isoforms on effector memory T cells *in vivo* are poorly described. Here, we provide a novel physiologic context for CD45 mediated tuning by linking CD8⁺ TCR priming affinity and CD45RB expression on memory and secondary effector T cells. Consistent with previous *in vitro* and biochemical work, we demonstrate that low affinity primed memory and CD45RB^{hi} sorted T cells are poised to undergo greater proliferative and effector responses compared to high affinity cells. Many investigations of the consequences of altered T cell receptor signaling potency have focused on antigen density or genetically attenuated TCR signaling (189, 240, 253-256). However, a recent study elegantly demonstrated that TCR binding affinity and antigen density induce broadly distinct gene expression profiles (237). Thus, the role of CD45 tuning represents a previously unappreciated mechanism by which TCR priming affinity dictates the differentiation program of memory CD8⁺ T cells during heterologous rechallenge.

These data suggest a model in which CD45RB defines an affinity-based differentiation switch on CD8⁺ T cells, as low affinity priming enables proliferation and effector functions along with high CD45RB expression levels similar to those observed on naïve T cells. Following a subsequent high affinity rechallenge, CD45RB^{hi} cells are tuned to undergo robust proliferative responses and CD45RB downregulation. Thus, this study supports a progressive differentiation model of CD8⁺ T cell memory development in which low affinity priming leads to a CD44^{hi}CD45RB^{hi}CD62L^{hi} differentiated memory cell, which is distinct from high affinity primed memory populations.

We found that low affinity primed memory CD8⁺ T cells also displayed a more CD62L^{hi} T_{CM} phenotype, and retained higher CD62L expression following secondary rechallenge. Several groups have described increased CD62L expression in T cell populations exposed to limited antigen or inhibited TCR signaling (232, 233, 240). A recent report found that low affinity priming led to enhanced CD62L expression in the blood until day 30 post infection (257). Following high affinity rechallenge, low affinity primed CD45RB^{hi} cells differentiate into a potent effector phenotype characterized by loss of CD45RB and increased PD-1 expression.

While PD-1 is a marker of exhaustion on CD8⁺ memory T cells following chronic antigen exposure, its expression is also associated with recent antigen experience in effector CD8⁺ T cells (232). It remains to be seen whether PD-1 is functioning in a costimulatory or coinhibitory capacity on secondary effectors under these conditions.

We found that low affinity primed memory CD8⁺ T cells differentiate into secondary effectors characterized by IL-2 production, high CD25 and lower CD122 expression. This phenotype was not a general characteristic of high affinity priming, as N4 OVA and V4 OVA primary effectors expressed similarly low levels of IL-2. Recent work revealed that IL-2 signals are critical for secondary effector CD8⁺ responses (244, 258) and that CD25 as a marker of terminally differentiated effectors (246, 247). These data are also in line with earlier work demonstrating that CD62L^{hi} T_{CM} produce more IL-2 than T_{EM} (2, 245, 259). However, this study is the first to identify TCR affinity as a driver of an IL-2 dependent memory CD8⁺ phenotype and extends previous work establishing the requirement of IL-2 for secondary recall responses by revealing that low affinity primed CD8⁺ secondary effectors produce significant amounts of IL-2.

In the context of protective immunity, the ability of low affinity priming to form memory and undergo robust secondary responses to heterologous rechallenge represents a means of maintaining clonal diversity and protection against encounter with diverse pathogens. However, following transplantation this phenomenon represents a potentially potent driver of allogeneic T cell responses and a barrier to successful immunomodulation. The contribution of low affinity memory T cells to heterologous immunity provides new rationale for the therapeutic targeting of CD45RB to prevent pathogenic T cell responses, a strategy that has shown promise in pre-clinical murine (149, 260, 261) and non-human primate transplantation models (262-264). Together, these data highlight the importance of the pathogen priming history of an individual in shaping the potentially pathogenic memory T cell repertoire in settings of allogeneic heterologous immunity and autoimmunity.

Materials & Methods

Mice

C57BL/6 Ly5.2-Cr (CD45.1, H-2^b) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (265) transgenic mice, purchased from Taconic Farms, were bred to Thy1.1⁺ background at Emory University. mOVA mice (C57BL/6 background, H-2^b; (266) were a gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN). All animals were maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines. All animals were housed in pathogen-free animal facilities at Emory University.

Generation of OT-I memory and secondary effectors

For adoptive transfers of donor-reactive T cells, Thy1.1⁺ OT-I mice were processed to single cell suspension and stained with monoclonal antibodies for CD8, Thy1.1, and V α 2 (BD) for flow cytometry analysis. Cells were resuspended in PBS and 1.0×10^4 cells were transferred intravenously. *Listeria monocytogenes* strains engineered to express the OVA APL epitope (LM-OVA APLs) were provided by Dr. Michael Bevan (University of Washington, Seattle, WA; (191)). Mice were infected with 10^4 CFU of LM-OVA APL strains intraperitoneally 24 hours after adoptive transfer.

Assessment of in vivo populations

At primary effector time point day 7 post infection, Thy1.1⁺ OT-I cells were identified in the peripheral blood following collection in heparinized capillary tubes and red blood cells lysis. Splenic primary effector Thy1.1⁺ OT-I cells were identified from single cell suspensions. At memory time point week 4 post infection (day 28-35), spleen and lymph nodes (popliteal, inguinal, mesenteric, brachial, axial, and cervical) were pooled and enriched for Thy1.1 cells using magnetic beads (239). Briefly, single cell suspensions were incubated with anti-Thy1.1 PE

and anti-PE microbeads (Miltenyi), following by enrichment over LS columns. The unbound column flow-through and wash fraction was routinely absent of OT-I cells. Absolute cell numbers were determined using AccuCheck beads (Invitrogen).

In vitro OVA APL OT-I stimulations

Spleen and mesenteric lymph node cells from OT-I mice were processed to single cell suspension and 3×10^6 splenocytes were plated in 24 well plates in complete RPMI supplemented with 0.1 μ M OVA APL peptide, 0.1 μ g/mL anti-CD28 (37.51, Biolegend), and 10 ng/mL IL-2 (Biolegend) for 3 days. Dead cells were removed using Lymphocyte Separation Medium (CellGro) and cells were cultured in media containing 10 ng/mL IL-15 (Biolegend) overnight, followed by flow cytometry. For CD45RB cell sorting, Q4 OVA primed cells were isolated using Lymphocyte Separation Medium and stained with Live/Dead Aqua (Invitrogen), gated on Aqua⁻ CD8⁺CD44^{hi}Thy1.1⁺, and sorted as CD45RB^{hi} and CD45RB^{lo} using a FACS Aria II (BD).

Skin transplantation

Full-thickness tail and ear skins were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages as previously described (267). In some experiments, mice were treated with 500 μ g each hamster monoclonal anti-mouse CD154 (MR-1, BioXCell) and CTLA-4 Ig, or 250 μ g anti-CD45RB (HB-220, BioXCell) on days 0, 2, 4, and 6 post transplant.

N4 OVA immunization restimulation assay

At 4 weeks following infection, mice were immunized with 10 μ g SIINFEKL peptide (GenScript, Inc) in each hind foot pad. Five days later, OT-I T cells were assessed in the single cell suspensions of the draining popliteal lymph nodes. Absolute cell counts were obtained using AccuCheck beads (Invitrogen).

Flow cytometry and intracellular cytokine staining

Single cell suspensions were stained with anti-CD3, anti-CD8, anti-CD19, anti-CD25, anti-CD44, anti-CD45RB, anti-CD62L, anti-CD69, anti-CD122, anti-CD127, anti-CD11c, anti-PD-1, anti-Thy1.1, and anti-KLRG-1 or appropriate isotype control (BD Biosciences or Biolegend) for 15' at room temperature. Anti-CCR7 was stained 1:25 for 1 h at room temperature. N4 OVA tetramer staining was performed at room temperature for 30' at 1:100 dilution or as described. For intracellular cytokine staining, cells were incubated for 5 h at 37 C in the presence of 1 μ M SIINFEKL peptide and 10 μ g/ml GolgiPlug (BD Biosciences) and stained for intracellular IL-2, TNF, and IFN- γ following manufacturer's instructions (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

Statistical analysis

Survival data were plotted on Kaplan-Meier curves and log-rank tests were performed. For analysis of absolute numbers and expression levels, paired or unpaired Student's t-tests (two-tailed) were performed, where appropriate. Analysis of expression of markers on naïve and memory cells was performed using one-way ANOVA with Bonferroni post test. Analysis of expression of markers by CD45RB expression was performed using 2-way repeated measures ANOVA with Bonferroni post test. Fold expansion in proliferation of N4 OVA rechallenged secondary effectors was calculated as (Absolute number OT-I N4 OVA rechallenge/Average number resting OT-I). Fold change of CD45RB expression on resting memory vs secondary effectors was calculated as $(100 - (\%CD45RB^{hi} \text{ secondary effector} / \text{Average } \%CD45RB^{hi} \text{ resting memory}))$. Percent maximum of N4 OVA tetramer binding was calculated as $(\text{Frequency N4 OVA Tet}^+) / (\text{Frequency N4 OVA Tet}^+ \text{ 1:50 dilution}) \times 100$. Correlations of MST and CD45RB expression were performed using relative EC₅₀ values (191). Results were considered significant

if $p < 0.05$. All analyses were done using GraphPad Prism software (GraphPad Software Inc).

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

Figures

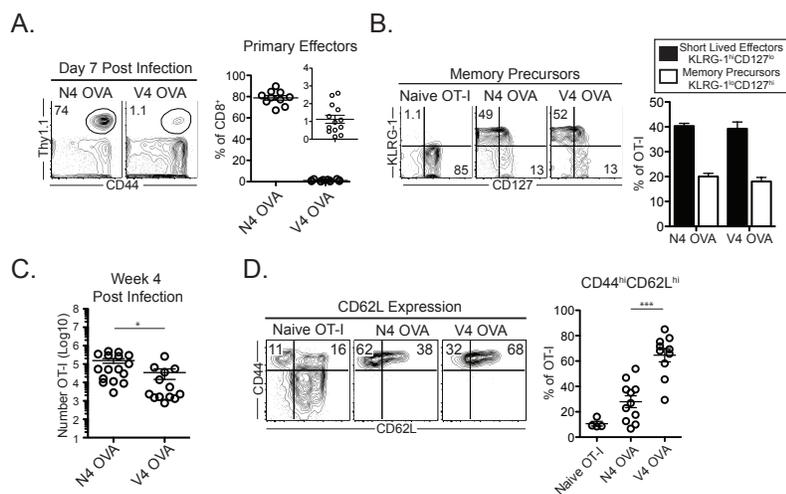


Figure 5.1. Low affinity priming efficiently generates central memory CD8⁺ T cells. Naïve mice adoptively transferred with 10⁴ OT-I T cells were infected the following day with either LM-N4 OVA or LM-V4 OVA and Thy1.1⁺ OT-I T cells were assessed (A-B) in the blood on day 7 post infection, or (C-D) in secondary lymphoid organs 4 weeks post infection. (A) Frequency of OT-I cells among CD8⁺ T cells (11-13 mice/group, representative of >10 experiments). Inset depicts LM-V4 OVA frequencies. (B) CD127 and KLRG-1 expression (p>0.05 N4 OVA vs V4 OVA SLEC and MPEC groups, respectively; 20-24 mice/group, 3 experiments.) (C) Absolute number of OT-I cells found in pooled spleen and lymph node tissue (p=0.026, n=13-16 mice/group, 4 experiments). (D) Frequency of CD44^{hi}CD62L^{hi} memory N4-OVA and V4 OVA primed memory OT-I cells in pooled secondary lymphoid organs (N4 OVA vs V4 OVA p<0.001, 10-11 mice/group, 4 experiments). Analysis by (B) 2-way ANOVA (Bonferroni post test), (C) unpaired Student's t-test (2-tailed), (D) 1-way ANOVA (Bonferroni post test). *p<0.05. LM, *Listeria monocytogenes*.

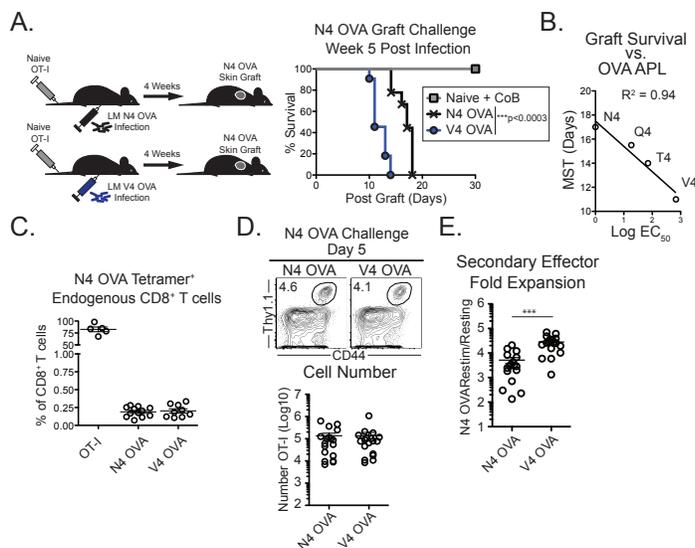


Figure 5.2. Low affinity primed CD8⁺ T cells mount potent secondary responses against

high affinity antigen. Naïve mice adoptively transferred with 10^4 OT-I T cells were infected the following day with either LM-N4 OVA or LM-V4 OVA. (A) Naïve mice or mice containing N4 OVA or V4 OVA primed memory OT-I cells were transplanted with N4 OVA skin grafts 5 weeks post infection. (N4 OVA MST=17 d, V4 OVA MST=11 d, $p < 0.0003$, 9-11 mice/group, 3 experiments). Naïve mice were treated with CTLA-4 Ig and anti-CD154 costimulation blockade. (B) Correlation between mean survival time and relative EC_{50} values of OT-I cells stimulated with OVA APL peptide ($R^2 = 0.94$). (C) Frequency of N4 OVA specific endogenous CD8⁺ T cells mice containing N4 OVA or V4 OVA primed OT-I memory cells 5 weeks post infection ($p > 0.05$ for all groups, 9-12 mice/group, 2 experiments). (D-E) Mice containing N4 OVA or V4 OVA memory OT-I cells were challenged with N4 OVA peptide in the foot pad and the draining popliteal lymph nodes were assessed 5 days later. (D) Frequency of secondary effector OT-I cells among CD8⁺ T cells ($p > 0.05$, $n = 18$ mice/group, 5 experiments). (E) Fold induction of N4 OVA and V4 OVA primed secondary effectors ($p < 0.001$, $n = 14$ mice/group, 5 experiments). Analysis by (A) log-rank test, (B) linear regression, (C-E) unpaired Student's t test (2-tailed). *** $p < 0.001$. CoB, costimulation blockade.

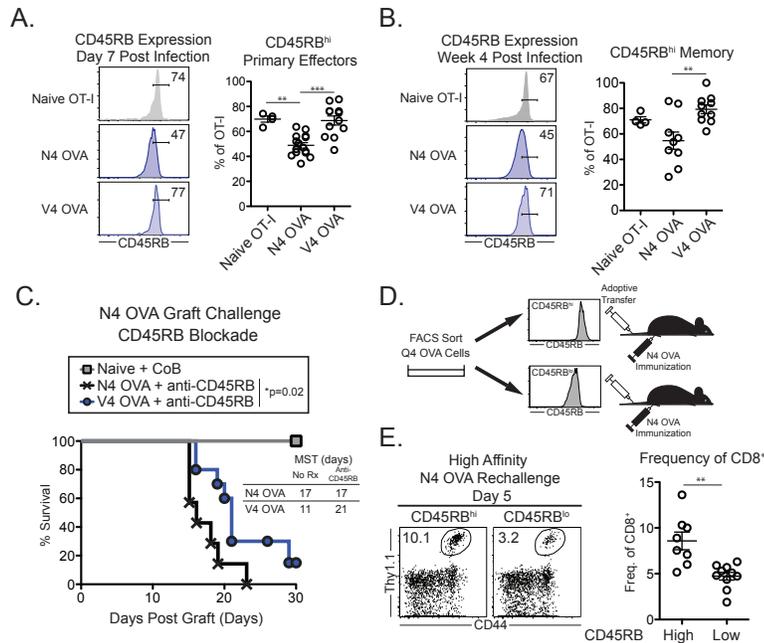


Figure 5.3. High CD45RB expression tunes low affinity primed memory CD8⁺ T cells for heterologous rechallenge responses. (A-C) Naïve mice adoptively transferred with 10⁴ OT-I T cells were infected the following day with either LM-N4 OVA or LM-V4 OVA and memory OT-I T cells and assessed (A) in the blood on day 7 or (B-C) in the secondary lymphoid organs 4 weeks post infection. (A) Frequency of CD45RB^{hi} cells on naïve or primary effector N4 OVA or V4 OVA T cells (Naïve OT-I vs N4 OVA $p < 0.01$, N4 OVA vs V4 OVA $p < 0.001$, 12-15/group, 3 experiments). (B) Frequency of CD45RB^{hi} cells among naïve OT-I or N4 OVA or V4 OVA memory OT-I cells (N4 OVA vs V4 OVA $p < 0.01$, 9-10/group, 3 experiments). (C) Naïve mice or mice containing N4 OVA or V4 OVA primed memory OT-I cells were transplanted with N4 OVA skin grafts 5 weeks post infection and treated with anti-CD45RB (N4 OVA MST=17 d, V4 OVA MST=21 d, $p = 0.02$, 7-10 mice/group, 2 experiments). Naïve mice were treated with CTLA-4 Ig and anti-CD154 costimulation blockade. (D) In vitro Q4 OVA primed OT-I cells were sorted into CD45RB^{hi} and CD45RB^{lo} populations and adoptively transferred into naïve congenic hosts and rechallenged with N4 OVA peptide. (E) Frequency of Q4 OVA cells 5 days following rechallenge ($p = 0.0012$, 2 experiments, 8-10 mice/group). Analysis by (A-B) 1-way ANOVA

(Bonferroni post test), (C) log-rank test, or (D) unpaired Student's t-test (2-tailed); ** $p < 0.01$,

*** $p < 0.001$.

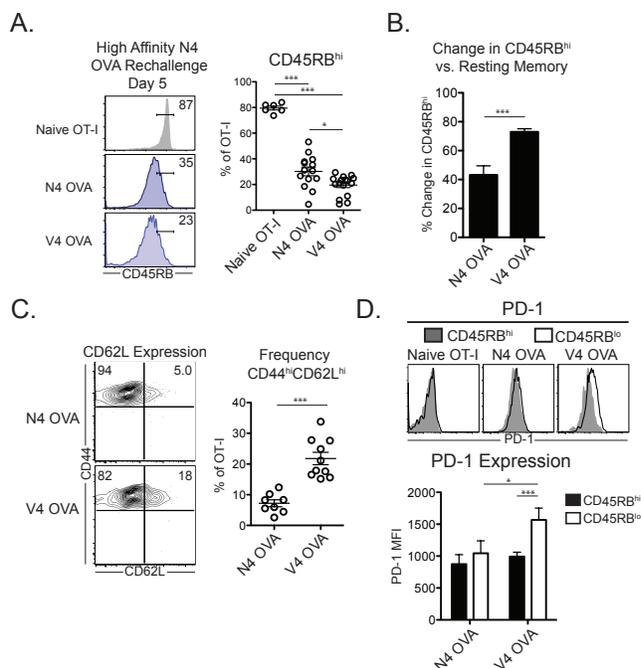


Figure 5.4. Low affinity primed secondary effectors downregulate CD45RB and have a distinct effector phenotype. Naïve mice adoptively transferred with 10^4 OT-I T cells were infected the following day with either LM-N4 OVA or LM-V4 OVA, and 4 weeks post infection were challenged with N4 OVA peptide in the foot pad and the draining popliteal lymph nodes were assessed 5 days later. (A) Frequency of CD45RB^{hi} cells among naïve and secondary effector OT-I T cells (naïve OT-I vs N4 OVA $p < 0.001$, naïve OT-I vs V4 OVA $p < 0.001$, N4 OVA vs V4 OVA $p < 0.05$, $n = 14-15$ /group, 5 experiments). (B) Relative reduction in CD45RB^{hi} frequency in secondary effector populations compared to resting memory cells ($p = 0.0002$, $14-15$ /group, 5 experiments). (C) Frequency of CD62L^{hi} cells among naïve or memory OT-I cells ($p < 0.001$, 8-9 mice/group, 3 experiments). (D) PD-1 expression among CD45RB^{hi} vs CD45RB^{lo} secondary effector OT-I cells (CD45RB^{hi} vs CD45RB^{lo} V4 OVA $p < 0.001$; N4 OVA vs V4 OVA CD45RB^{low} $p < 0.001$; $n = 8-10$ /group, 2 experiments). Analysis by (A) 1-way ANOVA (Bonferroni post test), (B-C) unpaired Student's t-test (2-tailed), (D) 2-way repeated measures ANOVA (Bonferroni post test); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

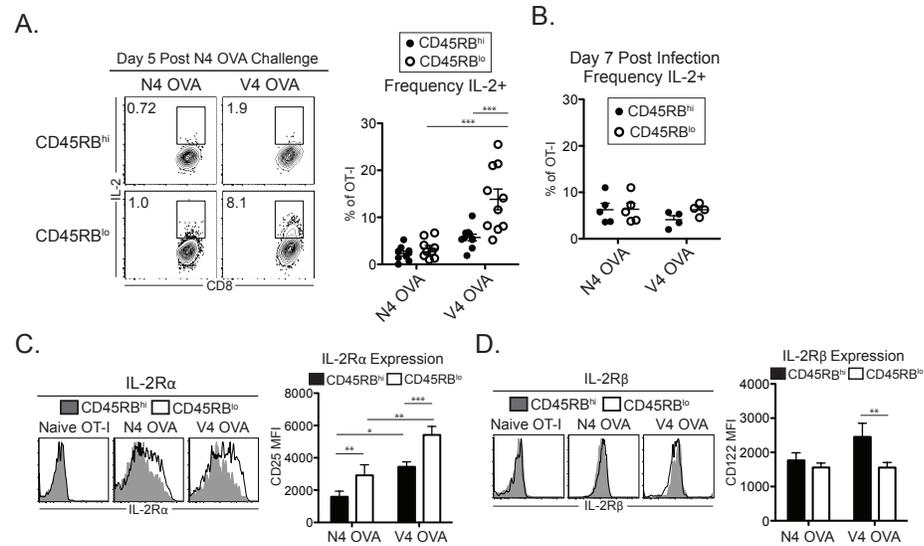


Figure 5.5. Low affinity primed secondary effectors have a distinct IL-2 dominant phenotype. Naïve mice were adoptively transferred with 10^4 OT-I T cells and were infected the following day with either LM-N4 OVA or LM-V4 OVA. (A, C-D) 4 weeks post infection mice were challenged with N4 OVA peptide in the foot pad and the draining popliteal lymph nodes were assessed 5 days later. (A) IL-2 expression by secondary effectors following brief N4 peptide restimulation in CD45RB^{hi} and CD45RB^{lo} fractions (IL-2: CD45RB^{hi} vs CD45RB^{lo} V4 OVA $p < 0.001$, N4 OVA vs V4 OVA CD45RB^{lo} $p < 0.001$; $n = 10-11$ /group, 3 experiments). (B) IL-2 production on day 7 post infection following brief N4 peptide restimulation in CD45RB^{hi} and CD45RB^{lo} fractions ($n = 4-5$ /group, 2 experiments). (C) IL-2R α (CD25) expression in CD45RB^{hi} and CD45RB^{lo} fractions of secondary effector OT-I cells (CD45RB^{hi} vs CD45RB^{lo} N4 OVA $p < 0.001$, V4 OVA $p < 0.001$; N4 OVA vs V4 OVA CD45RB^{hi} $p < 0.05$, CD45RB^{lo} $p < 0.001$; $n = 6-8$ /group, 2 experiments). (D) IL-2R β (CD122) expression in CD45RB^{hi} and CD45RB^{lo} fractions of secondary effector OT-I cells (CD45RB^{hi} vs CD45RB^{lo} V4 OVA $p < 0.01$; $n = 6-8$ /group, 2 experiments). Analysis by 2-way repeated measures ANOVA (Bonferroni post test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

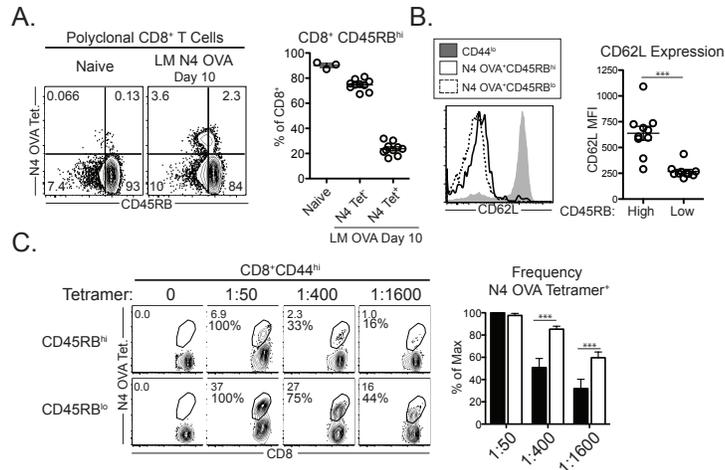
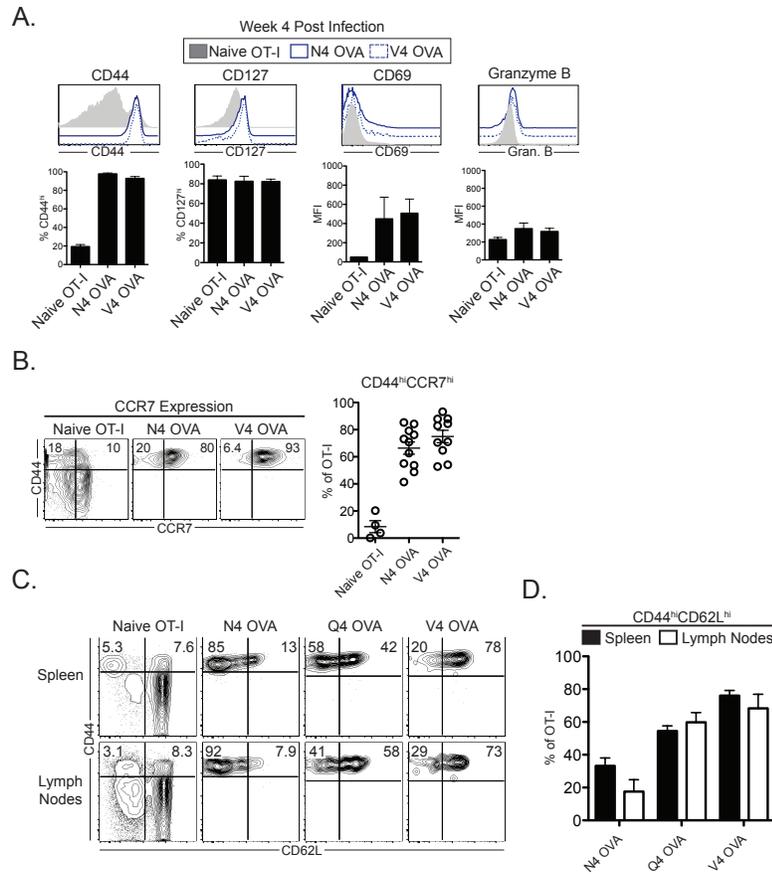
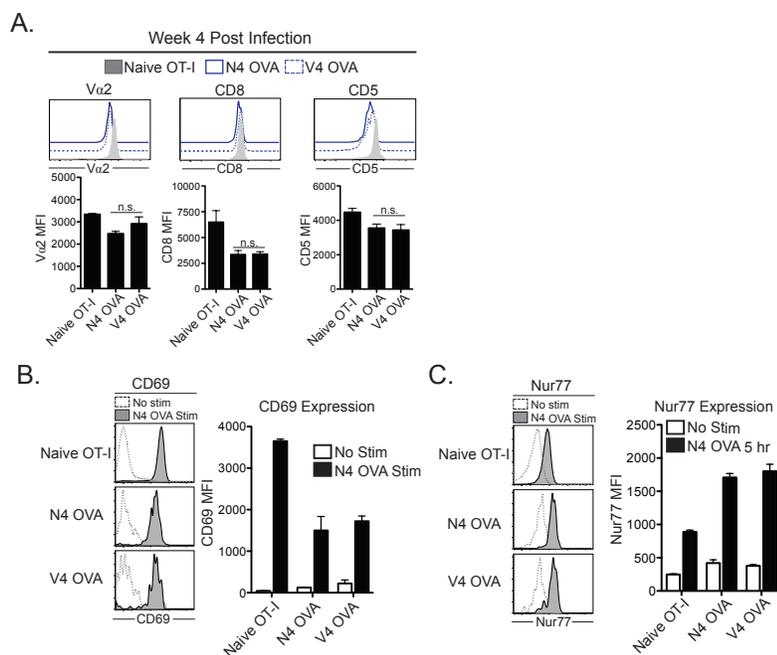


Figure 5.6. Low affinity polyclonal CD8⁺ T cells express high levels of CD45RB. Naïve B6 mice were infected with LM-N4 OVA and splenic CD8⁺ T cells were assessed 10 days later. (A) CD45RB expression and N4-OVA specific CD8⁺ T cells ($p < 0.001$). (B) CD62L expression on CD44^{lo}, CD44^{hi}CD45RB^{hi}, and CD44^{hi}CD45RB^{lo} CD8⁺ T cells ($p < 0.001$). (C) Frequency of tetramer staining among CD45RB^{hi} and CD45RB^{lo} fractions of CD8⁺ T cells. All experiments depict 10 mice/group, 2 experiments. Analysis by (A-B) paired Student's t-test (2 tailed) and (C) 2-way repeated measures ANOVA (Bonferroni post test), *** $p < 0.001$

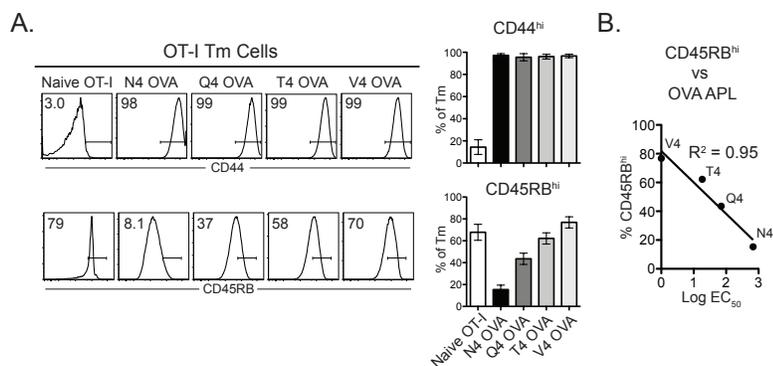


Supplemental Figure 5.1. Low affinity priming generates quality memory CD8⁺ T cells.

Naïve mice adoptively transferred with 10^4 OT-I T cells were infected the following day with either LM-N4 OVA or LM-V4 OVA and Thy1.1⁺ OT-I T cells were assessed in secondary lymphoid organs 4 weeks post infection. (A) Expression of CD44, CD127, CD69, and Granzyme B in OT-I T cells ($p > 0.05$ N4 OVA vs V4 OVA). (B) Frequency of CD44^{hi}CCR7^{hi} (N4 OVA vs V4 OVA $p > 0.05$, 10-11 mice/group, 4 experiments). (C) Representative expression and (D) summary frequencies of CD44^{hi}CD62L^{hi} N4 OVA, Q4 OVA, and V4 OVA primed memory OT-I cells in (top) spleen or (bottom) lymph nodes (Spleen vs LN $p > 0.05$ all groups; 4-7 mice/group, 3 experiments). Analysis by (A-C) 1-way ANOVA (Bonferroni post test) or (D) 2-way ANOVA (Bonferroni post test); * $p < 0.05$. LM, *Listeria monocytogenes*.

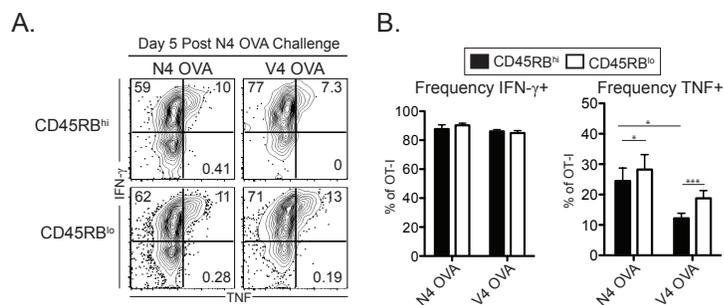


Supplemental Figure 5.2. High and low affinity primed memory CD8⁺ T cells express similar levels of TCR tuning molecules. Naïve mice adoptively transferred with 10⁴ OT-I T cells were infected the following day with either LM-N4 OVA or LM-V4 OVA and memory OT-I T cells and assessed in the secondary lymphoid organs 4 weeks post infection. (A) Expression of Vα2, CD8, and CD5 on naïve or memory OT-I T cells (N4 OVA vs V4 OVA p>0.05 all markers). (B-C) Naïve OT-I, N4 OVA memory, or V4 OVA memory cells were briefly restimulated with N4 OVA peptide and assessed for (B) surface CD69 or (C) intracellular Nur77 expression. Analysis by (A) 1-way ANOVA (Bonferroni post test) or (B-C) 2-way ANOVA (Bonferroni post test); *p<0.05.



Supplemental Figure 5.3. CD45RB expression correlates with T cell priming affinity *in vitro*.

OT-I T cells were activated *in vitro* with N4 OVA, Q4 OVA, T4 OVA, or V4 OVA peptide for 4 days. (A) Expression of CD44 and CD45RB on OVA APL Tm cells. (B) Correlation between frequency of CD45RB^{hi} cells and relative EC₅₀ values of OT-I cells stimulated with OVA APL peptide ($R^2=0.95$). Analysis by (C) linear regression; * $p<0.05$.



Supplemental Figure 5.4. High and low affinity primed secondary effectors produce similar levels of IFN- γ and TNF. Naïve mice were adoptively transferred with 10^4 OT-I T cells and were infected the following day with either LM-N4 OVA or LM-V4 OVA. Cells were assessed in the draining popliteal lymph node 5 days following N4 OVA peptide rechallenge (4 weeks post infection). (A) Representative IFN- γ and TNF expression and (B) summary data following brief N4 peptide restimulation in CD45RB^{hi} and CD45RB^{lo} fractions (TNF production: CD45RB^{hi} vs CD45RB^{lo} N4 OVA $p < 0.05$, V4 OVA $p < 0.001$; N4 OVA vs V4 OVA CD45RB^{hi} $p < 0.05$). Analysis by 2-way repeated measures ANOVA (Bonferroni post test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Chapter 6. Enhanced Requirement for TNF Signaling on Low Affinity Memory CD8⁺ T Cells During Heterologous Immunity

Introduction

Following encounter with microbial antigen, T cells can differentiate into memory cells with an array of phenotypic profiles in order to provide long-lasting protection against subsequent encounters with pathogens (3, 231). During transplantation, microbe-elicited T cell memory can also cross-react with allogeneic antigen and mediate graft rejection, a process termed allogeneic heterologous immunity (12, 41). Memory T cells are recognized as a barrier to many immunomodulation strategies aimed at limiting alloreactive T cell responses (4, 268).

The course of T cell differentiation is dependent on a number of environmental and cellular factors. The strength of the T cell receptor (TCR) interaction with peptide:MHC has a profound impact on T cell function (189, 269). Recent work has demonstrated that low affinity priming can lead to stable memory T cell generation and that low affinity primed T cells can drive autoimmune responses (189-191, 195). In transplantation, TCR affinity is hypothesized to be critically important in the context of allogeneic heterologous immunity (12, 41). In this scenario, TCR interactions with microbial priming antigen and allogeneic challenge antigen are likely to have different affinities (12). Recent work has also demonstrated that allogeneic T cells are suited to recognize a greater number of peptide:MHCs than conventional T cells, strongly suggesting that allogeneic T cell interactions occur over a range of high and low TCR affinities (42-44). However, the role that low affinity primed memory T cells play in graft rejection and the distinct effector mechanisms these cells employ relative to high affinity T cells remain undefined. Here, we sought to determine how altered memory T cell differentiation following encounter with low affinity antigen impacts allograft rejection.

Cosignaling through stimulatory and inhibitory receptors also plays a critical role in shaping the course of T cell differentiation (270), and have been shown to potently modulate T cell responses during the course of transplant rejection or acceptance. While many studies have correlated distinct profiles of receptor expression with T cell subsets, relatively few have elucidated unique functional cosignaling requirements for T cell populations. Furthermore, the distinct cosignaling requirements of low and high affinity primed T cells during heterologous immunity have not been explored. The TNF family of immune receptors is recognized to play critical roles in both T cell survival and apoptosis (271). TNFR2 is expressed on T cells and has been posited to provide costimulation to CD4 and CD8 T cells (272-274). Among the TNF family of receptors, TNFR2 is unique because of its constitutive expression on most T cell populations. However, little is known about the ability of TNF and TNFR2 signals to provide costimulatory signals *in vivo* during effector or memory T cell responses.

Here we report an important role for TNF signaling on low affinity primed secondary effector CD8⁺ T cells. Low affinity primed memory CD8⁺ T cell were capable of mediating graft rejection, and their response was characterized by uniquely high levels of TNF compared to high affinity primed CD8⁺ T cells. TNF-mediated costimulation on low affinity-primed memory T cells was further potentiated by the upregulation of surface TNFR2 relative to high-affinity primed memory T cells following high affinity rechallenge. Importantly, blockade of TNFR2 significantly prolonged skin graft survival in mice containing low but not high affinity primed memory T cells. Together, these results demonstrate the importance of TNF signaling in low affinity, cross-reactive CD8⁺ T cell responses during heterologous immunity and highlight the role of TCR affinity in dictating costimulation requirements of T cell responses.

Materials & Methods

Mice

C57BL/6 Ly5.2-Cr (CD45.1, H-2^b) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (265) transgenic mice, purchased from Taconic Farms, were bred to Thy1.1⁺ background at Emory University. mOVA mice (C57BL/6 background, H-2^b; (266) were a gift from M. Jenkins (University of Minnesota, Minneapolis, MN). All animals were maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines. All animals were housed in pathogen-free animal facilities at Emory University.

Generation of OT-I Memory and Secondary Effectors

For adoptive transfers of donor-reactive T cells, Thy1.1⁺ OT-I mice were processed to single cell suspension and stained with monoclonal antibodies for CD8 (both from Invitrogen), Thy1.1, and Va2 (BD) for flow cytometry analysis. Cells were resuspended in PBS and 1.0×10^4 cells were transferred intravenously. *Listeria monocytogenes* strains engineered to express the OVA APL epitope (LM-OVA APLs) were provided by Dr. Michael Bevan (University of Washington, Seattle, WA; (191)). Mice were infected with 10^4 CFU of LM-OVA APL strains intraperitoneally 24 hours after adoptive transfer. Primary effector Thy1.1⁺ OT-I cells were identified on day 7 post infection in the peripheral blood or spleen from single cell suspensions. Secondary effectors were generated in 4 week post-infection mice by immunizing with 50 μ g N4 OVA peptide (GenScript) emulsified in IFA (Gibco) in both hind foot pads. Five days later, draining popliteal LNs were collected and pooled for analysis.

Skin transplantation

Full-thickness tail and ear skins were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages as previously described (267). Mice were treated with

500 µg of CTLA-4 Ig on days 0, 2, 4, 6, or with 500 µg anti-TNFR2 (T75, BioXCell) on days 2, 4, 6, 8 post transplant.

Flow cytometry and intracellular cytokine staining

Splenic and lymph node cells were stained with anti-CD8, anti-Thy1.1, anti-CD44 (BD or Biolegend) for 15 min at room temperature. For TNFR2 expression, cells were stained with biotin anti-TNFR2 (CD120b, clone TR75-89) or Armenian hamster IgG for 15 min, washed, and stained for 15 minutes with streptavidin secondary antibody (Biolegend). For intracellular cytokine staining, cells were incubated for 5 h at 37 C in the presence of 1 µM SIINFEKL peptide and 10 µg/ml GolgiPlug (BD Biosciences), then stained for intracellular TNF, and IFN- γ following manufacturer's instructions (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

TNFR2 ELISA

Following OT-I adoptive transfer and infection with LM-OVA APLs as described above, or 5 days following secondary N4 OVA restimulation as described, serum was collected in non-heparinized capillary tubes. Serum was collected and analyzed using Ready-Set-Go ELISA kit according to manufacturer's instructions (eBiosciences).

Statistical analysis

Survival data were plotted on Kaplan-Meier curves and log-rank tests were performed. Analysis of cytokine expression on naïve and memory cells was performed using 1-way ANOVA with Bonferroni post test. Analysis of expression on primary effectors and soluble TNFR2 expression was performed using 2-way ANOVA with Bonferroni post test. Results were considered significant if $p < 0.05$. All analyses were done using GraphPad Prism software (GraphPad Software Inc). Error bars depict average \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Low affinity primed memory CD8⁺ T cells produce high levels of TNF

The affinity of TCR interactions during priming impacts CD8⁺ T cell programming during effector and memory phases (3, 189, 190). However, the functional consequences of low TCR affinity priming on subsequent CD8⁺ T cell memory responses are poorly understood. We utilized the OVA-based TCR transgenic system in which congenically labeled OT-I T cells are primed during infection with an acutely cleared *Listeria monocytogenes* strain engineered to express the high affinity OT-I epitope SIINFEKL (N4 OVA) or its altered peptide ligand (APL) variant SIIVFEKL (V4 OVA, (191)). Previous work has shown that despite a 680-fold lower functional avidity for the OT-I T cell receptor (TCR) than N4 OVA, V4 OVA priming of OT-I T cells can elicit memory cell differentiation (191). We found that both high and low affinity priming with *Listeria* generated primary effector populations (N4 OVA 40 ± 3.5%, V4 OVA 0.58 ± 0.12% of CD8⁺ T cells, Figure 6.1A) that expressed similarly high levels of CD44 (Figure 6.1B).

We first investigated whether low affinity primed CD8⁺ memory cells could mediate graft rejection to heterologous rechallenge. We challenged mice containing high or low affinity memory populations with a high affinity skin graft in the presence of CTLA-4 Ig (Figure 6.1C). Consistent with previous results, we found that high affinity primed memory CD8⁺ T cells were capable of mediating graft rejection in the presence of CTLA-4 Ig ((250), MST = 14, Figure 6.1D). Despite a dramatically lower clonal expansion (Figure 1A) and memory lower precursor frequency (data not shown), low affinity memory CD8⁺ T cells also mediated costimulation blockade-resistant rejection (MST = 13.5, Figure 6.1D). These results demonstrate that despite lower numbers, low affinity primed memory CD8⁺ T cells are programmed to potently respond to heterologous graft rechallenge.

Low affinity primed memory CD8⁺ T cells produce high levels of TNF

To investigate the functional characteristics of low affinity primed CD8⁺ T cells, we assessed the relative ability of low affinity primed memory cells to produce cytokine in response to heterologous rechallenge. In response to brief low affinity V4 OVA restimulation, a significantly higher frequency of low affinity V4 OVA memory cells produced both IFN- γ and TNF compared to high affinity N4 OVA memory (Figure 6.2A). These data demonstrate that low affinity primed T cells produce more potent cytokine responses to low affinity rechallenge than high affinity primed T cells. In order to determine the effect of low affinity priming on the recognition of high affinity antigen during heterologous rechallenge, low affinity V4 OVA and high affinity N4 OVA primed cells were restimulated *ex vivo* with high affinity antigen (Figure 6.2B). This resulted in a similar frequency of IFN- γ producers within low and high affinity memory T cell populations (Figure 6.2B), demonstrating that V4 OVA memory cells are competent to respond to high affinity antigen. Strikingly low affinity V4 OVA memory cells produced significantly higher levels of TNF compared to N4 OVA memory cells (Figure 6.2B). Overall, these results demonstrate low affinity memory CD8⁺ T cells are capable of robust effector responses in response to heterologous rechallenge with high affinity antigen, and that low affinity priming elicits a differentiation program that causes memory CD8⁺ T cells to produce high levels of TNF in response to rechallenge.

Low affinity primed secondary effectors upregulate TNFR2 expression

Given the higher expression of TNF produced by low affinity memory CD8⁺ T cells, we questioned whether T cell priming affinity affected the expression of TNF receptor on memory cells. We found that primary day 7 effectors exhibited increased TNFR2 expression compared to naïve OT-I T cells (Figure 6.3A). Although expression was slightly higher in the spleen, high and low affinity primed primary effector CD8⁺ T cells expressed similar levels of TNFR2 in the blood and the spleen (Figure 6.3A).

We reasoned that increased TNF production by low affinity primed memory might induce changes in TNFR2 expression in secondary effectors. To test this, we rechallenged mice with high affinity peptide and monitored expression of TNFR2. TNFR2 expression was maintained at high levels on high affinity primed cells (Figure 6.3B). Strikingly, low affinity primed day 5 secondary effectors exhibited increased TNFR2 expression relative to high affinity primed secondary effectors ($p < 0.001$, Figure 6.3B).

TNFR2 can provide costimulation to T cells as a cell surface receptor or can be shed by proteases and act as a decoy receptor (271). We questioned whether low affinity primed secondary effectors also increased the amount of soluble TNFR2. Prior to rechallenge, N4 OVA and V4 OVA primed mice contained similar levels of soluble TNFR2 in the serum (Figure 6.3C). Following rechallenge, the sera of mice containing low and high affinity primed secondary effectors contained similar levels of soluble TNFR2 (Figure 6.3C). This suggests that the increase in TNFR2 on low affinity primed secondary effectors is maintained on the cell surface. Together, these data demonstrate that low affinity primed secondary effectors upregulate TNFR2 surface expression, and raise the possibility that TNF signaling may be uniquely important for low affinity primed memory $CD8^+$ T cells to mediate optimum recall responses.

Low affinity primed memory $CD8^+$ T cells are dependent on TNFR2 signaling in order to optimally mediate graft rejection during heterologous rechallenge

We interrogated the functional importance of TNFR signaling on low affinity primed cells in mediating heterologous immunity to a skin graft challenge. Mice containing high or low affinity primed memory OT-I cells were challenge with N4 OVA expressing skin grafts and either left untreated or treated with anti-TNFR2. TNFR2 blockade had no effect on graft survival in mice containing high affinity primed memory populations (Untreated MST = 17, anti-TNFR2 MST = 17, Figure 6.4A). Surprisingly, low affinity primed memory cells without treatment mediated faster rejection than N4 OVA memory mice (MST = 11.5, Figure 6.4B). Importantly, in

recipients containing low affinity primed cross-reactive memory T cells, blockade of TNFR2 significantly prolonged graft survival (Figure 6.4B, MST = 21, $p = 0.0016$). Together, these results demonstrate that TNFR2 costimulation is critically important for low affinity primed CD8⁺ memory T cell recall responses during heterologous rechallenge.

Discussion

This study demonstrates a critical role for TNF signaling on low affinity primed memory CD8⁺ T cells during recall. We found that low affinity primed memory CD8⁺ T cells were tuned to produce increased TNF in response to high or low affinity rechallenge. In response to high affinity heterologous rechallenge, low affinity primed secondary effectors upregulated TNFR2 expression, and blockade of TNFR2 significantly prolonged graft survival. Many studies have described the production of TNF by CD8⁺ T cell populations. However, relatively few studies have demonstrated a costimulatory role for TNF signaling on CD8⁺ T cells. Recent work has elucidated roles for TNF family members in the survival of low affinity T cells (192, 275). Van Gisbergen et al. demonstrated that CD27:CD70 signals are required for the survival of low affinity primed CD8⁺ T cell memory during influenza infection (192). Recently, Mahmud et al. showed that expression of the TNFR family receptors GITR, OX40, and TNFR2 correlated to the affinity of CD4⁺ FoxP3⁺ Treg progenitors in the thymus (275). Our work is the first demonstration of a specific role for TNF production and TNFR2 costimulation on low affinity memory CD8⁺ T cells in order for them to optimally execute a response.

This study provides important insight into the functional costimulation requirements of low affinity primed CD8⁺ memory T cells. These results also highlight the concept that unique cosignaling pathways may be required during distinct phases of the adaptive immune response, as TNFR2 expression was significantly upregulated on secondary effectors – but not primary effectors – following encounter with low affinity antigen. Interestingly, many TNFR family members are induced on memory CD8⁺ T cells, and TNFR2 is considered the only TNFR family member to be constitutively expressed on T cells. However, we found a functional role for TNFR expression following low affinity CD8⁺ T cell priming. This increased TNFR2 expression may be responsible for tuning low affinity primed cells during secondary heterologous rechallenge responses. This finding is consistent with previous studies demonstrating that *in vitro* hypo-

proliferation of TNFR2^{-/-} T cells can be augmented with exogenous IL-2, suggesting that TNF signaling lowers the threshold of activation of T cells (272). However, this work is the first to link TCR priming affinity with TNFR2 mediated costimulation.

In the context of heterologous immunity, this study raises a novel avenue for immunomodulation of memory CD8⁺ T cell responses. Indeed, memory CD8⁺ T cells are considered to be a potent barrier to tolerance induction (4, 268). A number of TNF-based therapeutics are available for autoimmune disease, and this work suggests that blocking TNF signals might be a viable strategy for limiting cross-reactive heterologous T cell responses following transplantation.

Figures

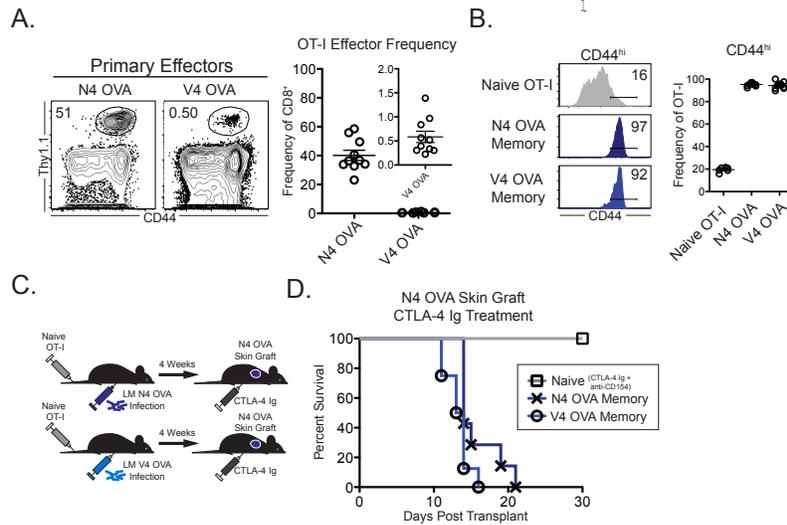


Figure 6.1. Low affinity primed memory $CD8^+$ T cells mediate graft rejection. Naïve mice adoptively transferred with 10^4 OT-I T cells were infected the following day with LM-N4 OVA or LM-V4 OVA. (A) Frequency of OT-I cells among $CD8^+$ T cells and (B) frequency of $CD44^{hi}$ N4 OVA or V4 OVA OT-I primary effector cells in the spleen on day 7 post infection (n=10 mice/group). (C-D) Four weeks post-infection, mice received an N4 OVA skin graft and CTLA-4 Ig (p = 0.040, n=7-8 mice/group). Analysis by log-rank test; *p<0.05. LM-OVA, *Listeria monocytogenes* expressing OVA.

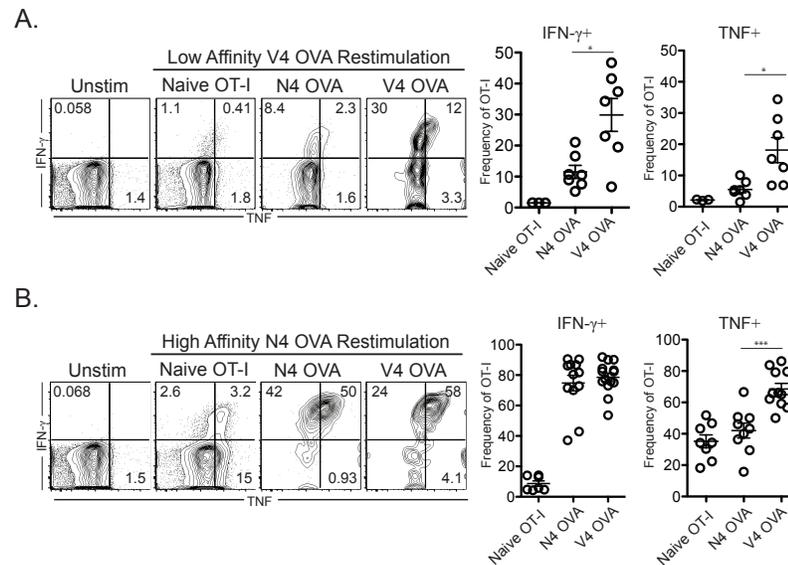


Figure 6.2. Low affinity primed memory CD8⁺ T cells produce high levels of TNF in response to heterologous rechallenge. Naïve mice adoptively transferred with 10⁴ OT-I T cells were infected the following day with LM-N4 OVA or LM-V4 OVA. Four weeks post infection, memory OT-I T cells were isolated and restimulated with peptide for 5 h. (A) Frequency of naïve or memory OT-I cells producing IFN- γ and TNF following restimulation with low affinity V4 OVA peptide (IFN- γ ⁺ N4 OVA vs V4 OVA p<0.05, TNF⁺ N4 OVA vs V4 OVA p<0.05, 7 mice/group). (B) Frequency of naïve or memory OT-I cells producing IFN- γ and TNF following restimulation with high affinity N4 OVA peptide (IFN- γ ⁺ N4 OVA vs V4 OVA p>0.05, TNF⁺ N4 OVA vs V4 OVA p<0.001, 12-14 mice/group). Analysis by 1-way ANOVA (Bonferroni post test); *p<0.05, ***p<0.001.

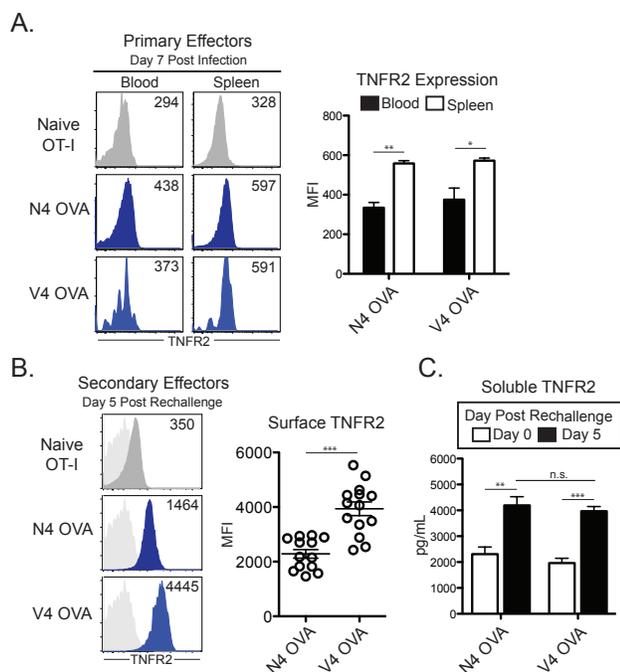


Figure 6.3. Low affinity primed CD8⁺ secondary effectors upregulate surface TNFR2 expression. Naïve mice were adoptively transferred with 10⁴ OT-I T cells were infected the following day with either LM-N4 OVA or LM-V4 OVA. (A) Expression of surface TNFR2 7 days post-infection in the blood and spleen (Blood vs Spleen, N4 OVA $p < 0.01$ and V4 OVA $p < 0.05$; $n = 5-10$ mice/group). (B) Four weeks post-infection, mice were challenged with N4 OVA in the foot pad and the draining popliteal lymph nodes were assessed 5 days later for surface TNFR2 expression ($p < 0.001$, 13-14 mice/group). (C) Soluble TNFR2 was measured by ELISA 4 weeks post-infection (day 0) or 5 days following secondary N4 OVA rechallenge (Day 0 vs. day 5, N4 OVA $p < 0.01$, V4 OVA $p < 0.001$). Analysis by (A, C) 2-way ANOVA (Bonferroni post test) or (B) unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$.

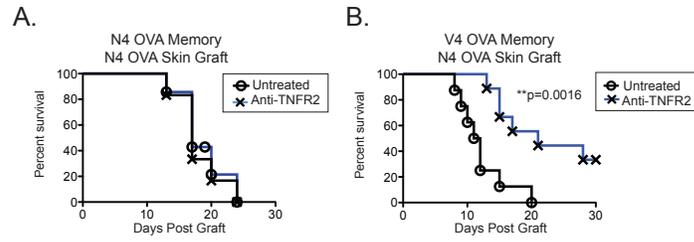


Figure 6.4. Low affinity primed memory CD8⁺ T cells rely on TNFR2 signaling. Naïve mice were adoptively transferred with 10^4 OT-I T cells were infected the following day with either (A) LM-N4 OVA or (B) LM-V4 OVA. Four weeks post-infection, mice received an N4 OVA skin graft and either left untreated or given anti-TNFR2 (n=6-8 mice/group). Analysis by log-rank test. ***p<0.001.

Chapter 7. Discussion

The Impact of CD8⁺ T Cell Affinity During Allogeneic Heterologous Immunity

Understanding the impact of low affinity T cell stimulation

Seminal work from Evavold et al over twenty years ago revealed that TCR priming affinity has a profound impact on T cell fate (276). Specifically, this work demonstrated that two T cell effector functions, cytokine production and proliferation, could be decoupled by differences in TCR ligand affinity using altered peptide ligands. Despite this compelling finding, understanding of the precise role that TCR priming affinity plays in dictating immune responses *in vivo* is poorly understood. Indeed, while a tremendous amount of work has elucidated the phenotypic and functional characteristics of CD4⁺ and CD8⁺ T cells in a variety of infectious, autoimmune, and transplantation models, isolating the specific differences between high and low affinity cells functionally has proven difficult. Adoptive transfer systems using monoclonal T cells have provided a wealth of phenotypic information about *in vivo* T cell function. Advances in tetramer technology have enabled the study of antigen-specific *in vivo* populations, providing another layer of understanding of T cell responses *in vivo*. However, neither approach is able to specifically assess the role that TCR priming affinity plays on the character of subsequent T cell responses.

Despite these technical difficulties, low affinity primed effector and memory cells are increasingly recognized as physiologically important for protective immunity and pathogenic T cell responses. Two recent studies have demonstrated the importance of low affinity T cells in physiologic T cell responses. Using a modified OT-I adoptive transfer model, Zehn et al showed that CD8⁺ T cells could be primed with a range of affinities following acutely cleared LM-OVA APL strains (191). Despite a 680-fold range in functional avidity, each of these infections were able to elicit stable memory populations capable of robust recall responses. Interestingly, Zehn et al reported very minor phenotypic differences among primary effectors primed across a range of

affinities. Elegant work from Sabatino et al demonstrated that low affinity CD4⁺ T cells are prevalent at frequencies similar to those of high affinity cells in EAE and following LCMV infection (238). The implications for this work are that T cells receiving low affinity priming interactions are programmed to generate T cell effectors and memory populations.

Compared to high affinity effector and memory T cells, low affinity priming is recognized to confer several key features of T cell phenotype and function. Our work and others has demonstrated a preferential T_{CM} phenotype that correlates inversely with TCR priming affinity, e.g. higher affinity priming leads to a less T_{CM} phenotype (232, 233, 240, 249, 257). Interestingly, we found that levels of CCR7 expression did not change with priming affinity in secondary lymphoid organs, which is the first demonstration that CD62L and CCR7 expression diverges on T cells due to priming affinity. This finding has potential implications for T cell trafficking, as the cumulative expression of these lymph node and spleen homing molecules likely dictates the kinetics and quality of recall responses. Beyond these two markers, relatively little is known about differences in expression of T cell trafficking molecules. Intriguingly, King et al demonstrate that lower affinity priming limits the upregulation of VLA-4 on primary effectors (195). Future work in this field should address trafficking differences of low vs high affinity primed CD8⁺ T_{EM} and T_{CM} cells. For example, are low affinity T_{CM} and high affinity T_{CM} capable of similar differentiation into secondary effectors and trafficking to distal sites on a per cell basis? Are low affinity primed memory T cells better at clearing systemic infectious rechallenges due to their greater T_{CM} phenotype? Do high or low affinity T cells reside in similar locations within secondary lymphoid organs? How does the expression of VLA-4 (and other integrins) on low affinity T cells affect the course of autoimmunity and transplant rejection?

Our work showed that low affinity primed memory CD8⁺ T cells are surprisingly poised to undergo secondary effector responses against high affinity antigen. This was evident in both the kinetics of graft rejection and proliferation in response to high affinity antigen.

Mechanistically, the ability of low affinity primed CD8⁺ T cells to respond to heterologous

rechallenge seemed dependent on enhanced proliferative potential conferred by high CD45RB expression. While previous work has demonstrated that CD45 isoforms confer important effects on T cell signaling, this is the first *in vivo* demonstration that high CD45RB isoforms confer a particular physiologically relevant proliferative advantage. Interestingly, Mehlhop-Williams demonstrated that on a per cell basis, naïve CD8⁺ T cells were capable of greater proliferative responses and stronger TCR signaling than CD8⁺ memory cells (277). Although this study did not specifically address the expression of CD45 isoforms, presumably the similar expression of CD45RB isoforms on naïve and low affinity memory CD8⁺ T cells has similar functional consequences.

Progress towards identifying surface markers of CD8⁺ T cell effectors and memory affinity

One particular technical difficulty in the study of TCR affinity among memory T cells has been the lack of a definitive stable surface markers that correlates with priming affinity. Phenotypic memory markers, such as CD44 are universally upregulated following memory T cell differentiation. Other acutely expressed activation markers that have been correlated with priming affinity, such as CD69, CTLA-4, and IFN- γ , are not maintained for more than a few days following activation (139, 191, 243). Recently, mice that report the expression of the orphan nuclear receptor Nur77 have been generated to study the *in vivo* affinity of T cell responses. Nur77 is one of the earliest transcripts to be dynamically upregulated following T cell activation, and presents an elegant way to study the role of TCR affinity during priming of naïve T cells *in vivo* (242). However, the role of Nur77 on memory and secondary effectors remains to be seen. We saw no difference in the expression of Nur77 on resting high and low affinity memory CD8⁺ T cells, and found no difference in the upregulation of Nur77 following high affinity rechallenge. The identification of high CD45RB expression on low affinity primed memory cells represents an important advance toward this aim, as CD44^{hi}CD45RB^{hi} cells can now be identified as by a stable surface phenotype as low affinity primed effector and memory T cells. This means that

subsequent studies will be able to study differences in high and low affinity polyclonal T cells in isolation in *in vivo* and *in vitro* assays. In particular, these studies will be able to assess the phenotypic and functional distinctions between high and low affinity T cells, and full transcriptional analysis.

Therapeutic implications of low affinity T cell reliance on TNF cosignaling

Cosignaling is another facet of T cell functionality that is influenced by T cell affinity. While costimulation has long been studied in the context of single receptors and pathways, recently it has been appreciated that T cell costimulation is a highly dynamic process, and depends on a constellation of costimulatory and inhibitory receptors. Termed the tidal model of cosignaling (270), this model describes how initial T cell interaction leads to a wave of costimulatory signals followed by the recession of these signals and the concomitant rise of expression and signaling of coinhibitory receptors, thereby providing a means of tuning T cell functionality and providing a feedback loop of regulation.

Recent work has provided evidence that TCR priming affinity dictates the specific character of cosignaling receptors, particularly the TNF receptor family members CD27, GITR, OX40, and TNFR2 (192, 275). In our model of antigen-specific CD8⁺ T cell memory, we found a specific role for TNFR2 signals on low affinity primed secondary effectors. This finding extends recent work demonstrating the importance of the TNFR family on low affinity CD8⁺ T cells, but also highlights the importance of the temporal dynamics of the interplay between costimulation and T cell affinity. Based on our findings, one can extend the tidal wave model of cosignaling to include the caveat that high and low affinity priming produce different types of cosignaling waves, and that primary waves are distinct from secondary waves of cosignaling. Dissecting critical intracellular signaling components could potentially lead to novel therapeutic targets, but is a daunting task. Indeed, the TNFR family is often treated as a redundant group of signaling

molecules in both original studies and literature reviews due to the overlap of the use of these intracellular signaling molecules (271, 275, 278, 279).

Our finding that low affinity primed secondary effectors has immediate therapeutic implications for transplantation, as several TNF-based therapeutics are clinically available for autoimmune disease (271, 280). This work suggests that TNF blocking agents might be an important companion to existing anti-rejection therapeutics because they might effectively inhibit memory T cell responses, particularly low affinity primed T cells that are cross-reactive with graft antigen. Despite the widely accepted ability of T cells to produce and respond to TNF (2, 191, 281), relatively few groups have published the results of anti-TNF therapy in animal models of solid organ or bone marrow transplantation (282-284). Nevertheless, particularly in the aim of inhibiting memory T cells, anti-TNF therapy remains an attractive target for transplantation.

A proposed model of polyclonal CD8⁺ T cell heterologous immunity

Despite the advances in understanding antigen-specific T cells in the context of heterologous immunity made possible by adoptive transfer TCR transgenic system, there is tremendous power in utilizing polyclonal models to investigate heterologous immunity. Recent advances in tetramer technology, particularly among class I MHC tetramers, have made it possible to track antigen-specific cells in naïve, effector, and memory states of differentiation. The major advantage of such a system in transplantation is the ability to study truly allogeneic T cell responses. One critique of the adoptive transfer OT-I system is that while this model allows for easy identification of antigen-specific cells and precise control of parameters such as priming kinetics, cell number, and TCR affinity, this model still represents a surrogate minor antigen mismatch versus a fully allogeneic model of graft rejection. This difference is manifest in two conceptual ways: first, the biochemistry of TCR:pMHC recognition as well as the nature of the antigen. While a great deal of work has demonstrated that allogeneic and self MHC-restricted recognition are equally peptide-dependent, there is considerable evidence that TCR binding to

allogeneic pMHC is structurally distinct from self MHC-restricted interactions (12, 36, 37, 285, 286). Second, although in reality there are likely many minor histocompatibility antigens that play an important role in graft rejection, the dogma in the field is that allogeneic interactions are driven by MHC-derived antigen.

Ideally, a model of heterologous immunity would involve a known TCR specificity that expands in response to an infection, and thus forms effector and memory cells. Subsequently these same cells are cross-reactive to an allogeneic antigen presented during grafting. Generation of such a model is primarily limited by the number of known allogeneic antigens, e.g. BALB/c H-2^d antigens that are recognized by C57BL/6 H-2^b restricted T cells. While some work has been done towards the goal of identifying such antigens, the tools for such a system were generated nearly two decades ago by Eisen and colleagues with the 2C transgenic TCR system (287-291). The 2C TCR was generated against BALB/c H-2^d splenocytes, and has been demonstrated to recognize a number of H-2L^d peptides derived from a citric acid cycle enzyme, oxaloglutarate dehydrogenase (OGDH). A remarkable amount of elegant work was done demonstrating that several OGDH peptides bind to H-2L^d and are recognized by 2C T cells with a range of affinities (287, 288, 291).

One would hypothesize that a portion of the naïve C57BL/6 T cell repertoire would recognize one or more of these allogeneic complexes, which could be identified by tetramer. If a model murine pathogen infection also expanded these alloreactive T cells, this would create a scenario in which a population of pathogen-specific CD8⁺ memory T cells is alloreactive for a known allogeneic pMHC complex, thus providing a model of polyclonal heterologous immunity. The pathogen antigen specificity of these alloreactive T cells would not be known, but would not be required for such a system, since the allogeneic tetramer could be used to identify the cells.

This particular model would provide several opportunities for investigation not afforded by adoptive transfer models or allogeneic models without a known allogeneic antigen. First, it would enable analysis of TCR repertoire, phenotype, and functionality of the entire allogeneic

population. Second, it would enable analysis of trafficking of T cells in a physiologic setting in naïve, memory, and post-graft timepoints. Finally, this model would have the added advantage of *in vitro* studies, as both 2C TCR transgenic mice and 2C TCR expressing hybridomas are available.

A model such as this would enable the assessment of very fundamental questions about the nature of allogeneic responses *in vivo* under various physiologic conditions. For example, does the overall TCR affinity or TCR repertoire narrow following pathogen infection compared to the naïve alloreactive repertoire? What cytokines are produced or necessary during an allogeneic secondary response? What is the kinetics and magnitude of the proliferative response to allogeneic antigen? What type of cells present allogeneic antigen during rechallenge? Answers to many of these questions have the potential to both uncover novel pathways that are particularly important to allogeneic T cell responses and to generate insights that are applicable to other types of pathogenic T cell responses.

Implications of TCR affinity for protective and heterologous immunity

Recent work from our lab and others has established that low affinity memory CD8⁺ T cells are maintained at low frequency following priming and are fit to undergo secondary clonal expansion. Overall, however, the protective impact of low frequency CD8⁺ T cells is less than that of higher frequency high affinity primed memory CD8⁺ T cells. What, then, is the evolutionary purpose of maintaining low affinity T cells? One compelling explanation is the maintenance of clonal diversity for protection against subsequent infections and cross-reactive challenges. This diversity can come in the form of effector function – greater cytokine production, for example – but also at the level of TCR cross-reactivity. Our work along with others suggests that low affinity priming generates cells that have a greater functional response against cross-reactive epitopes than high affinity primed CD8⁺ T cells (192, 257). Additionally, this idea is supported by the finding that “original antigenic sin” does not seem to apply to low affinity T

cells responses, as the presence of high affinity CD8⁺ T cell memory did not limit the secondary response to low affinity rechallenge (292). Thus, a teleological purpose of low affinity memory T cells seems to be to maintain a breadth of protective immunity (293).

This concept significantly departs from the “one T cell, one epitope” dogma that was initially proposed following the discovery of the TCR, and likely has important implications for the interplay between protective and pathogenic immunity. This work preliminarily suggests that low affinity CD8⁺ T cell memory is at once important for maintaining maximal protective immunity against pathogens, but could elicit the most pathogenic T cell responses in the setting of transplantation or autoimmunity. Future human T cell studies should determine if low affinity primed memory CD8⁺ T cells are associated with pathogenic responses. If so, efforts should be made to find existing immunomodulatory strategies or agents that effectively inhibit low affinity memory T cells. For example, one could hypothesize that TCR-signal inhibiting agents, such as CNIs, would effectively inhibit low affinity T cells. In sum, despite its lower frequency, low affinity memory T cell memory is increasingly recognized as a physiologically important population that has a profound impact on T cell responses.

Th17 Memory in Transplantation: Revisiting the Function of CTLA-4

New insight into the CD28/CTLA-4 pathway on Th17 cells

Our studies found that human Th17 memory cells are resistant to costimulation blockade with belatacept, and are associated with acute rejection episodes in renal transplant recipients (138, 171). Importantly, Th17 memory cells expressed significantly more of the coinhibitor CTLA-4 than Th1 populations. This suggests that Th17 cells receive more coinhibitory signals following activation than Th1 populations. We hypothesize that in the context of CD28/CTLA-4 blockade, therefore, Th1 cell costimulatory signals are largely inhibited, thereby limiting their proliferation. Th17 cells under CD28/CTLA-4 blockade, in contrast, perceive a relatively greater loss of coinhibitory CTLA-4 signals than costimulatory CD28 signals, thereby resulting in

augmentation of this population instead of inhibition. A great amount of work remains to be done to fully understand this observed *in vitro* and *in vivo* resistance of Th17 cells to CTLA-4 Ig. Future studies must investigate the mechanism by which Th17 cells are not inhibited by CD28/CTLA-4 blockade. There are two conceptual possibilities for this: first, that this mechanism is CD28/CTLA-4 intrinsic or, a second possibility is that another pathway or cell type is at play.

It is possible that CD28 blockade induces distinct secondary effects on Th1 and Th17 cells, such as the upregulation or downregulation of other cosignaling molecules. For example, a more comprehensive understanding of the signals that Th17 cells rely on is important. Recent work has suggested that Th17 cells rely on other cosignaling molecules, such as ICOS, SLAMF3, and SLAMF6 for optimal differentiation. It remains unknown whether these signals are also important for activation of Th17 memory cells, or whether signaling by these molecules becomes more prominent in the absence of CD28 signals. One important experiment will be to further investigate the expression profiles of Th1 and Th17 cells in the presence of CD28 blockade. This study has the potential to uncover important transcriptional, intracellular signaling, and cosignaling changes that occur in the absence of CD28 in an unbiased manner. The goal of this work would be to understand the relative similarities and differences of the CD28 pathway on Th1 and Th17 cells. Conceivably, this could also lead to the identification of novel or existing therapeutic targets that would be effective at inhibiting a costimulation blockade resistant population. Recently, one such target was identified by the bromo domain and extra terminal domain (BET) family of chromatin adaptors selectively control Th17 differentiation, and that their pharmacological inhibition can limit murine model of arthritis and EAE (294).

It is possible that the distinct effects of CD28 on Th1 and Th17 cells are in fact dependent on CTLA-4, as a significant portion of CTLA-4 expression has been shown to depend on CD28. Interestingly, we found that selective CD28 blockade with a domain antibody also augments Th17 populations while inhibiting Th1 populations, suggesting that the resistance of belatacept is

due to differences in CD28 signaling and not primarily to CTLA-4 signals. Our preliminary data suggests that a portion of high CTLA-4 expression on Th17 cells is dependent on TCR and CD28 signals, respectively. Future studies will have to address whether the magnitude of CD28-dependent CTLA-4 expression on Th17 cells is sufficient to confer resistance to CD28 blockade relative to Th1 cells.

Second, the resistance of Th17 cells to CD28/CTLA-4 and CD28 blockade could involve differentiation of or regulation by $CD4^+CD25^+FoxP3^+$ T reg (Treg). While the literature on Treg is fraught with contradictions, several groups have demonstrated that Th17 and Treg are reciprocally differentiated depending on the cytokine milieu present, and several recent studies have demonstrated that Th17 cells are augmented by the presence of Treg *in vitro* (198, 295-297). Furthermore, Treg have been shown to depend on CD28 signals and inhibited by CTLA-4 Ig based therapy (102, 177, 298). It is conceivable that in proliferation assays with CTLA-4 Ig or anti-CD28 dAbs, Treg could be functioning in way that confers a resistant phenotype of Th17 cells. Thus, it remains worth exploring whether these observed differences in susceptibility to CTLA-4 Ig and CD28 blockade are mediated through Treg. There are two experiments that can be used to elucidate that possibility. First, using a cytokine capture assay, the function of purified Th1 and Th17 cells can be assessed an *in vitro* proliferation assay. This experiment can definitively show that the observed differences in CD28 signaling are cell intrinsic. Second, Treg can be selectively removed from the cell culture prior to the assay by depleting $CD25^+$ T cells, which is a sensitive marker for Treg. Results of these experiments will reveal whether the effect of CD28 blockade on Th17 cells is cell intrinsic or Treg mediated.

CTLA-4 function on Th17 cells – future directions

Many fundamental questions remain about the specific role of CTLA-4 on $CD4^+$ T cell subsets. Currently, many distinct mechanisms of CTLA-4 coinhibition have been identified [Krummey et al AJT, in press]. However, not only are these mechanisms distinct but they are not

mutually exclusive. One particularly important area of investigation is likely to be CTLA-4 localization (139). While traditional model of CTLA-4 function maintains that it can compete with CD28 for ligation of CD80/CD86 and traffic through the immunological synapse, the relative prominence of this occurrence on T cell subsets has not been elucidated. In response to Lu et al (168), who found that Treg and Tcon have distinct trafficking patterns when cultured with APCs, does this phenomena correspond to distinct localization relative to the immunological synapse?

Additionally, it remains to be seen whether CTLA-4 on Th17 cells acts predominantly in a cell extrinsic or intrinsic manner (164). Our work has demonstrated a cell intrinsic role for CTLA-4, but it remains undetermined whether high CTLA-4 expression also leads to greater cell extrinsic function on Th17 cells compared to Th1 cells. If high CTLA-4 confers a suppressive function on Th17 memory cells, that would raise the questions of whether elevated Th17 cells observed during acute rejection in belatacept-treated patients are mediating or suppressing rejection. It is entirely possible that CTLA-4 might function to inhibit certain aspects of the allogeneic immune response (e.g. the proliferation of other CD4⁺ and CD8⁺ T cells) while promoting others (e.g. IL-17-mediated recruitment of neutrophils and innate cells). Devising an experimental system with which to interrogate the cell-intrinsic or -extrinsic function of CTLA-4 on Th17 cells would be technically challenging, as it would require Th1, Th17, and Treg cell lines or clones with stable silencing of CTLA-4 in combination with a Treg-style suppression assay. While challenging, this avenue of investigation would potentially provide important information about the role of high CTLA-4 in pro-inflammatory Th17 cells.

Therapeutic implications of costimulation blockade resistance

Beyond understanding the precise mechanism underlying the resistance of Th17 cells to belatacept, our work highlights the idea that differences in the expression of cosignaling molecules can have a profound impact on the outcome of immunomodulatory strategies. It also

suggests that the effects of immunomodulatory therapies should be, to the greatest degree possible, examined in a subset-specific manner. This idea likely extends beyond CD28/CTLA-4, as other cosignaling molecules are differentially expressed on distinct T cell subsets.

This work provides an important caveat to the clinical use of CTLA-4 Ig-based therapeutics (abatacept and belatacept) in transplantation and autoimmunity. In early clinical studies, abatacept has a mixed clinical record in IBD, SLE, and MS (but has worked in the treatment of rheumatoid arthritis) (102, 201, 202). While CTLA-4 Ig offers a major conceptual advance by selectively targeting T cell costimulation, this mixed record highlights the difficulty in developing more selective (and thus less toxic) immunomodulatory agents: the specific context and timing matter a great deal. While the clinical picture of belatacept following renal transplantation invokes the idea that pre-formed memory that is resistant to the agent can mediate pathogenic responses early following transplantation, this likely informs factors that contribute to the limited efficacy of CTLA-4 Ig in managing Th17-mediated autoimmune disease. *In vitro* human studies and animal models have demonstrated that CTLA-4 Ig effectively inhibits *de novo* T cell priming events (138, 299). Thus, one could hypothesize that in the context of autoimmunity or transplantation, administration of the drug at times when there is a high antigen burden and high occurrence of naïve T cell priming, perhaps termed “naïve-biased T cell state,” CTLA-4 Ig would be particularly efficacious. In contrast, during instances when there is a “memory-biased T cell state,” defined by a particularly low antigen burden (and thus less naïve T cell priming) or a recent history of microbial exposure that has generated a high frequency of Th17 polarized cells – the conditions are stacked against CTLA-4 Ig treatment. The problem is that clinically these two scenarios are largely indistinguishable, and markers that could distinguish these two divergent immunological states are not yet defined.

With these new caveats what, then, is the way forward for CTLA-4 Ig based therapies? Unfortunately, the nuances in these basic science findings likely signal a need for nuance in the clinical use of CTLA-4 Ig. First, it is possible that CTLA-4 Ig should be accompanied by drugs

that can cover Th17 memory responses. A number of Th17-targeted therapies are undergoing clinical development for autoimmune disease, particularly IBD. The outcome of this cohort of agents will certainly be relevant to transplantation, as Th17 memory cells are increasingly recognized as a component of the pathogen-elicited alloreactive repertoire. Studies in our lab are underway to investigate the role of Th17 cells in liver transplant patients, and the relative presence of Th17 cells compared to Th1 cells in these patients. As well, our group is working to add on a Th17-targeting therapy, anti-IL-12/23, to belatacept in non-human primate model of renal transplantation. In the case of transplantation, this strategy is being deployed with calcineurin inhibitor (CNI) induction therapy following renal transplant for 6 months, followed by a CNI taper to long-term belatacept monotherapy.

Second, studies should investigate the efficacy of different kinetics and dosing of belatacept around the time of transplantation. For example, would pre-transplant use of belatacept (logistically difficult in transplantation with the exception of perhaps living related kidney donation) improve outcomes by enabling the Th17-biased response early following initiation of therapy to run its course before the exposure of an allograft? Furthermore, early studies in EAE demonstrated that low dose CTLA-4 Ig treatment exacerbated disease in a relapsing-remitting model of EAE (175). Thus, it is conceivable that higher dosing of CTLA-4 Ig in the setting of autoimmune disease or transplantation might lead to improved outcomes. It is worth noting, however, that in clinical development of belatacept for renal transplantation, the incidence of acute rejection was lower in the low dose than the high dose arm (300). Nevertheless, the dose effect of belatacept on T cell responses has been relatively understudied and might inform an improvement in clinical outcome.

What is the Future of Immunosuppression?

Collectively these data demonstrate that the character of effector and memory T cells is highly dynamic and dependent on a number of environmental, cellular, biochemical, genetic, and

kinetic cues. Indeed, the majority of basic immunology research over the past twenty-five years (perhaps since the discovery of Th1/Th2 cells) has focused on defining properties of distinct T cell populations. Indeed, we are quite likely only beginning to grasp the range of T cell “subsets” that exist and the factors that govern their generation and maintenance. Current therapeutics targeting T cells were designed with the goal of broadly inhibiting T cells, with kinetics being the largest source of specificity for pathogenic versus non-pathogenic cells. Indeed, many of these drugs (CNIs, anti-TNF agents, T cell depleting therapies) were first conceptualized in the relative dawn of the study of T cells.

However, this model of immunosuppression is seemingly at odds with much of what we understand about the strengths of protective immunity, mainly the ability to maintain long-term protection to a broad array of pathogens. In patients with active autoimmune disease or transplant patients at risk of rejection, the collective immune system – including T cells – is still providing a great deal of effective and dynamic protection against microbes. Thus, inhibiting or eliminating random subsets of this protective repertoire, thus leaving holes in protective immunity, is a clumsy strategy for inhibiting pathogenic T cell responses. Clearly, a more refined and selective approach to limit pathological immunological responses is needed.

It appears that next-generation therapeutics will capitalize on disease-causing factors or characteristics of T cells that are specific to individuals, with the goal of most efficiently inhibiting only pathogenic T cells while sparing non-pathogenic populations. This strategy presumes that the immunological variation between individuals is a determining factor in the progression of disease. For example, if a renal transplant recipient has a high frequency of Th17 memory cells, they might receive drugs that specifically target Th17 cells. Alternatively, a heavy Th1 bias of the CD4⁺ compartment coupled with a paucity of Treg might be driving rheumatoid arthritis in a group of patients. This concept of personalized medicine requires careful analysis of patients and matching the specific therapeutic regimens to particular immune characteristics to identify therapeutic regimens that are most likely to be effective. In many ways, this is a logical

extension of traditional therapeutic strategies, where treatment timing is a major source of specificity. However, this represents an advance over existing strategies because it takes into account the heterogeneity of immunologic disease and variation within individuals: more specifically, the notion that across an entire population with a given disease – or who receive a transplant – distinct T cell subsets might be driving pathological responses in subsets of patients but not others.

Through careful human immune monitoring studies, this scenario is occurring and is identifying parameters that stratify patients likely to benefit from particular immunotherapeutics. In the case of belatacept and transplantation, for instance, work is already well underway to stratify patients based on the risk of rejection on belatacept versus CNIs. Overall, the next step is to actually use these types of studies to develop improved therapies based on targets identified through the unbiased study of patients. Thus, careful investigation of human T cell function in healthy humans and patients is critical for progress towards improve therapeutics. Ultimately, major advancements in immunomodulatory therapy over the next decades are likely to come from experimental investigation of human T cells.

References

1. Zhu, J., Yamane, H., and Paul, W.E. 2010. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 28:445-489.
2. Wherry, E.J., Teichgraber, V., Becker, T.C., Masopust, D., Kaech, S.M., Antia, R., von Andrian, U.H., and Ahmed, R. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4:225-234.
3. Kaech, S.M., and Cui, W. 2012. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat Rev Immunol* 12:749-761.
4. Ford, M.L., and Larsen, C.P. 2010. Overcoming the memory barrier in tolerance induction: molecular mimicry and functional heterogeneity among pathogen-specific T-cell populations. *Curr Opin Organ Transplant* 15:405-410.
5. Lanzavecchia, A., and Sallusto, F. 2000. From synapses to immunological memory: the role of sustained T cell stimulation. *Curr Opin Immunol* 12:92-98.
6. Lanzavecchia, A., and Sallusto, F. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 290:92-97.
7. Ahmed, R., and Gray, D. 1996. Immunological memory and protective immunity: understanding their relation. *Science* 272:54-60.
8. Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.
9. Brehm, M.A., Pinto, A.K., Daniels, K.A., Schneck, J.P., Welsh, R.M., and Selin, L.K. 2002. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. *Nat Immunol* 3:627-634.
10. Brehm, M.A., Selin, L.K., and Welsh, R.M. 2004. CD8 T cell responses to viral infections in sequence. *Cell Microbiol* 6:411-421.

11. Selin, L.K., Brehm, M.A., Naumov, Y.N., Cornberg, M., Kim, S.K., Clute, S.C., and Welsh, R.M. 2006. Memory of mice and men: CD8+ T-cell cross-reactivity and heterologous immunity. *Immunol Rev* 211:164-181.
12. Smith, C., Miles, J.J., and Khanna, R. 2012. Advances in direct T-cell alloreactivity: function, avidity, biophysics and structure. *Am J Transplant* 12:15-26.
13. Adams, A.B., Pearson, T.C., and Larsen, C.P. 2003. Heterologous immunity: an overlooked barrier to tolerance. *Immunol Rev* 196:147-160.
14. Brehm, M.A., Markees, T.G., Daniels, K.A., Greiner, D.L., Rossini, A.A., and Welsh, R.M. 2003. Direct visualization of cross-reactive effector and memory allo-specific CD8 T cells generated in response to viral infections. *J Immunol* 170:4077-4086.
15. Brehm, M.A., Daniels, K.A., Priyadharshini, B., Thornley, T.B., Greiner, D.L., Rossini, A.A., and Welsh, R.M. 2010. Allografts stimulate cross-reactive virus-specific memory CD8 T cells with private specificity. *Am J Transplant* 10:1738-1748.
16. Amir, A.L., D'Orsogna, L.J., Roelen, D.L., van Loenen, M.M., Hagedoorn, R.S., de Boer, R., van der Hoorn, M.A., Kester, M.G., Doxiadis, II, Falkenburg, J.H., et al. 2010. Allo-HLA reactivity of virus-specific memory T cells is common. *Blood* 115:3146-3157.
17. Lechler, R., Batchelor, R., and Lombardi, G. 1991. The relationship between MHC restricted and allospecific T cell recognition. *Immunol Lett* 29:41-50.
18. Lombardi, G., Sidhu, S., Daly, M., Batchelor, J.R., Makgoba, W., and Lechler, R.I. 1990. Are primary alloresponses truly primary? *Int Immunol* 2:9-13.
19. Macedo, C., Orkis, E.A., Popescu, I., Elinoff, B.D., Zeevi, A., Shapiro, R., Lakkis, F.G., and Metes, D. 2009. Contribution of naive and memory T-cell populations to the human alloimmune response. *Am J Transplant* 9:2057-2066.
20. Valujskikh, A., Pantenburg, B., and Heeger, P.S. 2002. Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. *Am J Transplant* 2:501-509.

21. Zhai, Y., Meng, L., Gao, F., Busuttil, R.W., and Kupiec-Weglinski, J.W. 2002. Allograft rejection by primed/memory CD8+ T cells is CD154 blockade resistant: therapeutic implications for sensitized transplant recipients. *J Immunol* 169:4667-4673.
22. Heeger, P.S., Greenspan, N.S., Kuhlenschmidt, S., Dejelo, C., Hricik, D.E., Schulak, J.A., and Tary-Lehmann, M. 1999. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol* 163:2267-2275.
23. van Halteren, A.G., Jankowska-Gan, E., Joosten, A., Blokland, E., Pool, J., Brand, A., Burlingham, W.J., and Goulmy, E. 2009. Naturally acquired tolerance and sensitization to minor histocompatibility antigens in healthy family members. *Blood* 114:2263-2272.
24. Patel, S.R., Cadwell, C.M., Medford, A., and Zimring, J.C. 2009. Transfusion of minor histocompatibility antigen-mismatched platelets induces rejection of bone marrow transplants in mice. *J Clin Invest* 119:2787-2794.
25. Goldrath, A.W., Bogatzki, L.Y., and Bevan, M.J. 2000. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J Exp Med* 192:557-564.
26. Murali-Krishna, K., and Ahmed, R. 2000. Cutting edge: naive T cells masquerading as memory cells. *J Immunol* 165:1733-1737.
27. Wu, Z., Bensinger, S.J., Zhang, J., Chen, C., Yuan, X., Huang, X., Markmann, J.F., Kassaee, A., Rosengard, B.R., Hancock, W.W., et al. 2004. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med* 10:87-92.
28. Sener, A., Tang, A.L., and Farber, D.L. 2009. Memory T-cell predominance following T-cell depletion therapy derives from homeostatic expansion of naive T cells. *Am J Transplant* 9:2615-2623.

29. Hislop, A.D., Annels, N.E., Gudgeon, N.H., Leese, A.M., and Rickinson, A.B. 2002. Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. *J Exp Med* 195:893-905.
30. Burrows, S.R., Silins, S.L., Moss, D.J., Khanna, R., Misko, I.S., and Argat, V.P. 1995. T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen. *J Exp Med* 182:1703-1715.
31. Morice, A., Charreau, B., Neveu, B., Brouard, S., Soullillou, J.P., Bonneville, M., Houssaint, E., and Degauque, N. 2010. Cross-reactivity of herpesvirus-specific CD8 T cell lines toward allogeneic class I MHC molecules. *PLoS One* 5:e12120.
32. Burrows, S.R., Silins, S.L., Khanna, R., Burrows, J.M., Rischmueller, M., McCluskey, J., and Moss, D.J. 1997. Cross-reactive memory T cells for Epstein-Barr virus augment the alloresponse to common human leukocyte antigens: degenerate recognition of major histocompatibility complex-bound peptide by T cells and its role in alloreactivity. *Eur J Immunol* 27:1726-1736.
33. Burrows, S.R., Khanna, R., Burrows, J.M., and Moss, D.J. 1994. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for graft-versus-host disease. *J Exp Med* 179:1155-1161.
34. Umetsu, D.T., Yunis, E.J., Matsui, Y., Jabara, H.H., and Geha, R.S. 1985. HLA-DR-4-associated alloreactivity of an HLA-DR-3-restricted human tetanus toxoid-specific T cell clone: inhibition of both reactivities by an alloantiserum. *Eur J Immunol* 15:356-361.
35. Koelle, D.M., Chen, H.B., McClurkan, C.M., and Petersdorf, E.W. 2002. Herpes simplex virus type 2-specific CD8 cytotoxic T lymphocyte cross-reactivity against prevalent HLA class I alleles. *Blood* 99:3844-3847.

36. Colf, L.A., Bankovich, A.J., Hanick, N.A., Bowerman, N.A., Jones, L.L., Kranz, D.M., and Garcia, K.C. 2007. How a single T cell receptor recognizes both self and foreign MHC. *Cell* 129:135-146.
37. Macdonald, W.A., Chen, Z., Gras, S., Archbold, J.K., Tynan, F.E., Clements, C.S., Bharadwaj, M., Kjer-Nielsen, L., Saunders, P.M., Wilce, M.C., et al. 2009. T cell allorecognition via molecular mimicry. *Immunity* 31:897-908.
38. Yin, L., Huseby, E., Scott-Browne, J., Rubtsova, K., Pinilla, C., Crawford, F., Marrack, P., Dai, S., and Kappler, J.W. 2011. A single T cell receptor bound to major histocompatibility complex class I and class II glycoproteins reveals switchable TCR conformers. *Immunity* 35:23-33.
39. Landais, E., Morice, A., Long, H.M., Haigh, T.A., Charreau, B., Bonneville, M., Taylor, G.S., and Houssaint, E. 2006. EBV-specific CD4⁺ T cell clones exhibit vigorous allogeneic responses. *J Immunol* 177:1427-1433.
40. Elkington, R., and Khanna, R. 2005. Cross-recognition of human alloantigen by cytomegalovirus glycoprotein-specific CD4⁺ cytotoxic T lymphocytes: implications for graft-versus-host disease. *Blood* 105:1362-1364.
41. Adams, A.B., Williams, M.A., Jones, T.R., Shirasugi, N., Durham, M.M., Kaech, S.M., Wherry, E.J., Onami, T., Lanier, J.G., Kokko, K.E., et al. 2003. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* 111:1887-1895.
42. Felix, N.J., Donermeyer, D.L., Horvath, S., Walters, J.J., Gross, M.L., Suri, A., and Allen, P.M. 2007. Alloreactive T cells respond specifically to multiple distinct peptide-MHC complexes. *Nat Immunol* 8:388-397.
43. Morris, G.P., Ni, P.P., and Allen, P.M. 2011. Alloreactivity is limited by the endogenous peptide repertoire. *Proc Natl Acad Sci U S A* 108:3695-3700.
44. Falkenburg, W.J., Melenhorst, J.J., van de Meent, M., Kester, M.G., Hombrink, P., Heemskerk, M.H., Hagedoorn, R.S., Gostick, E., Price, D.A., Falkenburg, J.H., et al.

2011. Allogeneic HLA-A*02-Restricted WT1-Specific T Cells from Mismatched Donors Are Highly Reactive but Show Off-Target Promiscuity. *J Immunol* 187:2824-2833.
45. Amir, A.L., D'Orsogna, L.J., Roelen, D.L., van Loenen, M.M., Hagedoorn, R.S., de Boer, R., van der Hoorn, M.A., Kester, M.G., Doxiadis, II, Falkenburg, J.H., et al. Allo-HLA reactivity of virus-specific memory T-cells is common. *Blood*.
46. Nadazdin, O., Boskovic, S., Murakami, T., O'Connor, D.H., Wiseman, R.W., Karl, J.A., Tuscher, J.J., Sachs, D.H., Madsen, J.C., Tocco, G., et al. 2010. Phenotype, distribution and alloreactive properties of memory T cells from cynomolgus monkeys. *Am J Transplant* 10:1375-1384.
47. Felix, N.J., and Allen, P.M. 2007. Specificity of T-cell alloreactivity. *Nat Rev Immunol* 7:942-953.
48. Sallusto, F., and Lanzavecchia, A. 2009. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. *Eur J Immunol* 39:2076-2082.
49. Pepper, M., and Jenkins, M.K. 2011. Origins of CD4(+) effector and central memory T cells. *Nat Immunol* 12:467-471.
50. Zielinski, C.E., Corti, D., Mele, F., Pinto, D., Lanzavecchia, A., and Sallusto, F. 2011. Dissecting the human immunologic memory for pathogens. *Immunol Rev* 240:40-51.
51. Coffman, R.L., and Carty, J. 1986. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma. *J Immunol* 136:949-954.
52. Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-2357.
53. Harrington, L.E., Janowski, K.M., Oliver, J.R., Zajac, A.J., and Weaver, C.T. 2008. Memory CD4 T cells emerge from effector T-cell progenitors. *Nature* 452:356-360.
54. Lohning, M., Hegazy, A.N., Pinschewer, D.D., Busse, D., Lang, K.S., Hofer, T., Radbruch, A., Zinkernagel, R.M., and Hengartner, H. 2008. Long-lived virus-reactive

- memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *J Exp Med* 205:53-61.
55. Pepper, M., Linehan, J.L., Pagan, A.J., Zell, T., Dileepan, T., Cleary, P.P., and Jenkins, M.K. 2010. Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. *Nat Immunol* 11:83-89.
56. Yang, Y., An, J., and Weng, N.P. 2008. Telomerase is involved in IL-7-mediated differential survival of naive and memory CD4+ T cells. *J Immunol* 180:3775-3781.
57. Chevalier, N., Jarrossay, D., Ho, E., Avery, D.T., Ma, C.S., Yu, D., Sallusto, F., Tangye, S.G., and Mackay, C.R. 2011. CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol* 186:5556-5568.
58. Pepper, M., Pagan, A.J., Igyarto, B.Z., Taylor, J.J., and Jenkins, M.K. 2011. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* 35:583-595.
59. Gran, B., Chu, N., Zhang, G.X., Yu, S., Li, Y., Chen, X.H., Kamoun, M., and Rostami, A. 2004. Early administration of IL-12 suppresses EAE through induction of interferon-gamma. *J Neuroimmunol* 156:123-131.
60. Voorthuis, J.A., Uitdehaag, B.M., De Groot, C.J., Goede, P.H., van der Meide, P.H., and Dijkstra, C.D. 1990. Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon-gamma in Lewis rats. *Clin Exp Immunol* 81:183-188.
61. Billiau, A., Heremans, H., Vandekerckhove, F., Dijkmans, R., Sobis, H., Meulepas, E., and Carton, H. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol* 140:1506-1510.
62. Willenborg, D.O., Fordham, S.A., Staykova, M.A., Ramshaw, I.A., and Cowden, W.B. 1999. IFN-gamma is critical to the control of murine autoimmune encephalomyelitis and

- regulates both in the periphery and in the target tissue: a possible role for nitric oxide. *J Immunol* 163:5278-5286.
63. Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., and Cua, D.J. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233-240.
64. Murphy, C.A., Langrish, C.L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R.A., Sedgwick, J.D., and Cua, D.J. 2003. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 198:1951-1957.
65. Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., and Weaver, C.T. 2005. Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132.
66. Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., et al. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.
67. Peters, A., Lee, Y., and Kuchroo, V.K. 2011. The many faces of Th17 cells. *Curr Opin Immunol* 23:702-706.
68. Ivanov, II, McKenzie, B.S., Zhou, L., Tadore, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. 2006. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell* 126:1121-1133.
69. McGeachy, M.J., Bak-Jensen, K.S., Chen, Y., Tato, C.M., Blumenschein, W., McClanahan, T., and Cua, D.J. 2007. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* 8:1390-1397.
70. Chen, Z., Laurence, A., Kanno, Y., Pacher-Zavisin, M., Zhu, B.M., Tato, C., Yoshimura, A., Hennighausen, L., and O'Shea, J.J. 2006. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci U S A* 103:8137-8142.

71. Hue, S., Ahern, P., Buonocore, S., Kullberg, M.C., Cua, D.J., McKenzie, B.S., Powrie, F., and Maloy, K.J. 2006. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med* 203:2473-2483.
72. Kullberg, M.C., Jankovic, D., Feng, C.G., Hue, S., Gorelick, P.L., McKenzie, B.S., Cua, D.J., Powrie, F., Cheever, A.W., Maloy, K.J., et al. 2006. IL-23 plays a key role in *Helicobacter hepaticus*-induced T cell-dependent colitis. *J Exp Med* 203:2485-2494.
73. Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Fili, L., Ferri, S., Frosali, F., et al. 2007. Phenotypic and functional features of human Th17 cells. *J Exp Med* 204:1849-1861.
74. Suryani, S., and Sutton, I. 2007. An interferon-gamma-producing Th1 subset is the major source of IL-17 in experimental autoimmune encephalitis. *J Neuroimmunol* 183:96-103.
75. Lee, Y.K., Turner, H., Maynard, C.L., Oliver, J.R., Chen, D., Elson, C.O., and Weaver, C.T. 2009. Late developmental plasticity in the T helper 17 lineage. *Immunity* 30:92-107.
76. Das, J., Ren, G., Zhang, L., Roberts, A.I., Zhao, X., Bothwell, A.L., Van Kaer, L., Shi, Y., and Das, G. 2009. Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation. *J Exp Med* 206:2407-2416.
77. Ahern, P.P., Schiering, C., Buonocore, S., McGeachy, M.J., Cua, D.J., Maloy, K.J., and Powrie, F. 2010. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity* 33:279-288.
78. Hirota, K., Duarte, J.H., Veldhoen, M., Hornsby, E., Li, Y., Cua, D.J., Ahlfors, H., Wilhelm, C., Tolaini, M., Menzel, U., et al. 2011. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol* 12:255-263.
79. Kamanaka, M., Huber, S., Zenewicz, L.A., Gagliani, N., Rathinam, C., O'Connor, W., Jr., Wan, Y.Y., Nakae, S., Iwakura, Y., Hao, L., et al. 2011. Memory/effector (CD45RB(lo)) CD4 T cells are controlled directly by IL-10 and cause IL-22-dependent intestinal pathology. *J Exp Med* 208:1027-1040.

80. Lee, Y., Awasthi, A., Yosef, N., Quintana, F.J., Xiao, S., Peters, A., Wu, C., Kleinewietfeld, M., Kunder, S., Hafler, D.A., et al. 2012. Induction and molecular signature of pathogenic TH17 cells. *Nat Immunol* 13:991-999.
81. Zielinski, C.E., Mele, F., Aschenbrenner, D., Jarrossay, D., Ronchi, F., Gattorno, M., Monticelli, S., Lanzavecchia, A., and Sallusto, F. 2012. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature* 484:514-518.
82. Salomon, B., and Bluestone, J.A. 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu Rev Immunol* 19:225-252.
83. Bouguermouh, S., Fortin, G., Baba, N., Rubio, M., and Sarfati, M. 2009. CD28 co-stimulation down regulates Th17 development. *PLoS One* 4:e5087.
84. de Wit, J., Souwer, Y., van Beelen, A.J., de Groot, R., Muller, F.J., Klaasse Bos, H., Jorritsma, T., Kapsenberg, M.L., de Jong, E.C., and van Ham, S.M. 2011. CD5 costimulation induces stable Th17 development by promoting IL-23R expression and sustained STAT3 activation. *Blood* 118:6107-6114.
85. Ying, H., Yang, L., Qiao, G., Li, Z., Zhang, L., Yin, F., Xie, D., and Zhang, J. 2010. Cutting edge: CTLA-4--B7 interaction suppresses Th17 cell differentiation. *Journal of immunology* 185:1375-1378.
86. Paulos, C.M., Carpenito, C., Plesa, G., Suhoski, M.M., Varela-Rohena, A., Golovina, T.N., Carroll, R.G., Riley, J.L., and June, C.H. 2010. The inducible costimulator (ICOS) is critical for the development of human T(H)17 cells. *Science translational medicine* 2:55ra78.
87. Santarlasci, V., Maggi, L., Capone, M., Querci, V., Beltrame, L., Cavalieri, D., D'Aiuto, E., Cimaz, R., Nebbioso, A., Liotta, F., et al. 2012. Rarity of human T helper 17 cells is due to retinoic acid orphan receptor-dependent mechanisms that limit their expansion. *Immunity* 36:201-214.

88. Nurieva, R.I., Treuting, P., Duong, J., Flavell, R.A., and Dong, C. 2003. Inducible costimulator is essential for collagen-induced arthritis. *J Clin Invest* 111:701-706.
89. Chatterjee, M., Rauen, T., Kis-Toth, K., Kyttaris, V.C., Hedrich, C.M., Terhorst, C., and Tsokos, G.C. 2012. Increased expression of SLAM receptors SLAMF3 and SLAMF6 in systemic lupus erythematosus T lymphocytes promotes Th17 differentiation. *J Immunol* 188:1206-1212.
90. Chatterjee, M., Hedrich, C.M., Rauen, T., Ioannidis, C., Terhorst, C., and Tsokos, G.C. 2012. CD3-T cell receptor co-stimulation through SLAMF3 and SLAMF6 receptors enhances ROR γ recruitment to the IL17A promoter in human T lymphocytes. *J Biol Chem* 287:38168-38177.
91. Fabrega, E., Lopez-Hoyos, M., San Segundo, D., Casafont, F., and Pons-Romero, F. 2009. Changes in the serum levels of interleukin-17/interleukin-23 during acute rejection in liver transplantation. *Liver Transpl* 15:629-633.
92. Loong, C.C., Hsieh, H.G., Lui, W.Y., Chen, A., and Lin, C.Y. 2002. Evidence for the early involvement of interleukin 17 in human and experimental renal allograft rejection. *J Pathol* 197:322-332.
93. Vanaudenaerde, B.M., Dupont, L.J., Wuyts, W.A., Verbeken, E.K., Meyts, I., Bullens, D.M., Dilissen, E., Luyts, L., Van Raemdonck, D.E., and Verleden, G.M. 2006. The role of interleukin-17 during acute rejection after lung transplantation. *Eur Respir J* 27:779-787.
94. Chung, B.H., Kim, K.W., Sun, I.O., Choi, S.R., Park, H.S., Jeon, E.J., Kim, B.M., Choi, B.S., Park, C.W., Kim, Y.S., et al. 2012. Increased interleukin-17 producing effector memory T cells in the end-stage renal disease patients. *Immunol Lett* 141:181-189.
95. Litjens, N.H., van de Wetering, J., van Besouw, N.M., and Betjes, M.G. 2009. The human alloreactive CD4⁺ T-cell repertoire is biased to a Th17 response and the

- frequency is inversely related to the number of HLA class II mismatches. *Blood* 114:3947-3955.
96. Burrell, B.E., Csencsits, K., Lu, G., Grabauskiene, S., and Bishop, D.K. 2008. CD8+ Th17 mediate costimulation blockade-resistant allograft rejection in T-bet-deficient mice. *J Immunol* 181:3906-3914.
97. Yuan, X., Ansari, M.J., D'Addio, F., Paez-Cortez, J., Schmitt, I., Donnarumma, M., Boenisch, O., Zhao, X., Popoola, J., Clarkson, M.R., et al. 2009. Targeting Tim-1 to overcome resistance to transplantation tolerance mediated by CD8 T17 cells. *Proc Natl Acad Sci U S A* 106:10734-10739.
98. Yuan, X., Paez-Cortez, J., Schmitt-Knosalla, I., D'Addio, F., Mfarrej, B., Donnarumma, M., Habicht, A., Clarkson, M.R., Iacomini, J., Glimcher, L.H., et al. 2008. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *J Exp Med* 205:3133-3144.
99. Benghiat, F.S., Craciun, L., De Wilde, V., Dernies, T., Kubjak, C., Lhomme, F., Goldman, M., and Le Moine, A. 2008. IL-17 production elicited by allo-major histocompatibility complex class II recognition depends on CD25posCD4pos T cells. *Transplantation* 85:943-949.
100. Aruffo, A., and Seed, B. 1987. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc Natl Acad Sci U S A* 84:8573-8577.
101. Gmunder, H., and Lesslauer, W. 1984. A 45-kDa human T-cell membrane glycoprotein functions in the regulation of cell proliferative responses. *Eur J Biochem* 142:153-160.
102. Linsley, P.S., and Nadler, S.G. 2009. The clinical utility of inhibiting CD28-mediated costimulation. *Immunol Rev* 229:307-321.
103. Bour-Jordan, H., Esensten, J.H., Martinez-Llordella, M., Penaranda, C., Stumpf, M., and Bluestone, J.A. 2011. Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/ B7 family. *Immunol Rev* 241:180-205.

104. Sanchez-Lockhart, M., Marin, E., Graf, B., Abe, R., Harada, Y., Sedwick, C.E., and Miller, J. 2004. Cutting edge: CD28-mediated transcriptional and posttranscriptional regulation of IL-2 expression are controlled through different signaling pathways. *J Immunol* 173:7120-7124.
105. Narayan, P., Holt, B., Tosti, R., and Kane, L.P. 2006. CARMA1 is required for Akt-mediated NF-kappaB activation in T cells. *Mol Cell Biol* 26:2327-2336.
106. Park, S.G., Schulze-Luehrman, J., Hayden, M.S., Hashimoto, N., Ogawa, W., Kasuga, M., and Ghosh, S. 2009. The kinase PDK1 integrates T cell antigen receptor and CD28 coreceptor signaling to induce NF-kappaB and activate T cells. *Nat Immunol* 10:158-166.
107. Jones, R.G., Parsons, M., Bonnard, M., Chan, V.S., Yeh, W.C., Woodgett, J.R., and Ohashi, P.S. 2000. Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo. *J Exp Med* 191:1721-1734.
108. Khoshnan, A., Tindell, C., Laux, I., Bae, D., Bennett, B., and Nel, A.E. 2000. The NF-kappa B cascade is important in Bcl-xL expression and for the anti-apoptotic effects of the CD28 receptor in primary human CD4+ lymphocytes. *J Immunol* 165:1743-1754.
109. Chen, C., Edelstein, L.C., and Gelinas, C. 2000. The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). *Mol Cell Biol* 20:2687-2695.
110. Burr, J.S., Savage, N.D., Messah, G.E., Kimzey, S.L., Shaw, A.S., Arch, R.H., and Green, J.M. 2001. Cutting edge: distinct motifs within CD28 regulate T cell proliferation and induction of Bcl-XL. *J Immunol* 166:5331-5335.
111. Yokosuka, T., Kobayashi, W., Sakata-Sogawa, K., Takamatsu, M., Hashimoto-Tane, A., Dustin, M.L., Tokunaga, M., and Saito, T. 2008. Spatiotemporal regulation of T cell costimulation by TCR-CD28 microclusters and protein kinase C theta translocation. *Immunity* 29:589-601.

112. Pagan, A.J., Pepper, M., Chu, H.H., Green, J.M., and Jenkins, M.K. 2012. CD28 Promotes CD4+ T Cell Clonal Expansion during Infection Independently of Its YMNM and PYAP Motifs. *J Immunol*.
113. Linsley, P.S., and Ledbetter, J.A. 1993. The role of the CD28 receptor during T cell responses to antigen. *Ann Rev Immunol* 11:191-212.
114. Bertram, E.M., Lau, P., and Watts, T.H. 2002. Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection. *J Immunol* 168:3777-3785.
115. Halstead, E.S., Mueller, Y.M., Altman, J.D., and Katsikis, P.D. 2002. In vivo stimulation of CD137 broadens primary antiviral CD8+ T cell responses. *Nat Immunol* 3:536-541.
116. McAdam, A.J., Farkash, E.A., Gewurz, B.E., and Sharpe, A.H. 2000. B7 costimulation is critical for antibody class switching and CD8(+) cytotoxic T-lymphocyte generation in the host response to vesicular stomatitis virus. *J Virol* 74:203-208.
117. Fuse, S., Obar, J.J., Bellfy, S., Leung, E.K., Zhang, W., and Usherwood, E.J. 2006. CD80 and CD86 control antiviral CD8+ T-cell function and immune surveillance of murine gammaherpesvirus 68. *J Virol* 80:9159-9170.
118. Boesteanu, A.C., and Katsikis, P.D. 2009. Memory T cells need CD28 costimulation to remember. *Semin Immunol* 21:69-77.
119. Viola, A., and Lanzavecchia, A. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science* 273:104-106.
120. Bachmann, M.F., Gallimore, A., Linkert, S., Cerundolo, V., Lanzavecchia, A., Kopf, M., and Viola, A. 1999. Developmental regulation of Lck targeting to the CD8 coreceptor controls signaling in naive and memory T cells. *J Exp Med* 189:1521-1530.
121. Flynn, K., and Mullbacher, A. 1996. Memory alloreactive cytotoxic T cells do not require costimulation for activation in vitro. *Immunol Cell Biol* 74:413-420.

122. Kim, S.K., Schluns, K.S., and Lefrancois, L. 1999. Induction and visualization of mucosal memory CD8 T cells following systemic virus infection. *J Immunol* 163:4125-4132.
123. Croft, M., Bradley, L.M., and Swain, S.L. 1994. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J. Immunol.* 152:2675-2685.
124. Shahinian, A., Pfeffer, K., Lee, K.P., Kundig, T.M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P.S., Thompson, C.B., and Mak, T.W. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261:609-612.
125. Borowski, A.B., Boesteanu, A.C., Mueller, Y.M., Carafides, C., Topham, D.J., Altman, J.D., Jennings, S.R., and Katsikis, P.D. 2007. Memory CD8+ T cells require CD28 costimulation. *J Immunol* 179:6494-6503.
126. Belz, G.T., Wilson, N.S., Smith, C.M., Mount, A.M., Carbone, F.R., and Heath, W.R. 2006. Bone marrow-derived cells expand memory CD8+ T cells in response to viral infections of the lung and skin. *Eur J Immunol* 36:327-335.
127. Zammit, D.J., Cauley, L.S., Pham, Q.M., and Lefrancois, L. 2005. Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity* 22:561-570.
128. Fuse, S., Zhang, W., and Usherwood, E.J. 2008. Control of memory CD8+ T cell differentiation by CD80/CD86-CD28 costimulation and restoration by IL-2 during the recall response. *J Immunol* 180:1148-1157.
129. Ndejemi, M.P., Teijaro, J.R., Patke, D.S., Bingaman, A.W., Chandok, M.R., Azimzadeh, A., Nadler, S.G., and Farber, D.L. 2006. Control of memory CD4 T cell recall by the CD28/B7 costimulatory pathway. *J Immunol* 177:7698-7706.

130. Linsley, P.S. 1995. Distinct roles for CD28 and cytotoxic T lymphocyte-associated molecule-4 receptors during T cell activation? [comment]. [Review]. *J. Exp. Med.* 182:289-292.
131. Linsley, P.S., Greene, J.L., Tan, P., Bradshaw, J., Ledbetter, J.A., Anasetti, C., and Damle, N.K. 1992. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J. Exp. Med.* 176:1595-1604.
132. Wu, Y., Guo, Y., Huang, A., Zheng, P., and Liu, Y. 1997. CTLA-4-B7 interaction is sufficient to costimulate T cell clonal expansion. *Journal of Experimental Medicine* 185:1327-1335.
133. Damle, N.K., Klussman, K., Leytze, G., Myrdal, S., Aruffo, A., Ledbetter, J.A., and Linsley, P.S. 1994. Costimulation of T lymphocytes with integrin ligands intercellular adhesion molecule-1 or vascular cell adhesion molecule-1 induces functional expression of CTLA-4, a second receptor for B7. *J. Immunol.* 152:2686-2697.
134. Walunas, T.L., Lenschow, D.J., Bakker, C.Y., Linsley, P.S., Freeman, G.J., Green, J.M., Thompson, C.B., and Bluestone, J.A. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1:405-413.
135. Krummel, M.F., and Allison, J.P. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation [see comments]. *J. Exp. Med.* 182:459-465.
136. Waterhouse, P., Penninger, J.M., Timms, E., Wakeham, A., Shahinian, A., Lee, K.P., Thompson, C.B., Griesser, H., and Mak, T.W. 1995. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science* 270:985-988.
137. Walunas, T.L., Bakker, C.Y., and Bluestone, J.A. 1996. CTLA-4 ligation blocks CD28-dependent T cell activation. *J Exp Med* 183:2541-2550.
138. Krummey, S.M., Cheeseman, J.A., Conger, J.A., Jang, P.S., Mehta, A.K., Kirk, A.D., Larsen, C.P., and Ford, M.L. 2014. High CTLA-4 expression on Th17 cells results in

- increased sensitivity to CTLA-4 coinhibition and resistance to belatacept. *Am J Transplant* 14:607-614.
139. Teft, W.A., Kirchof, M.G., and Madrenas, J. 2006. A molecular perspective of CTLA-4 function. *Annu Rev Immunol* 24:65-97.
140. Finn, P.W., He, H., Wang, Y., Wang, Z., Guan, G., Listman, J., and Perkins, D.L. 1997. Synergistic induction of CTLA-4 expression by costimulation with TCR plus CD28 signals mediated by increased transcription and messenger ribonucleic acid stability. *J Immunol* 158:4074-4081.
141. Ouyang, W., Beckett, O., Ma, Q., Paik, J.H., DePinho, R.A., and Li, M.O. 2010. Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. *Nat Immunol* 11:618-627.
142. Kerdiles, Y.M., Beisner, D.R., Tinoco, R., Dejean, A.S., Castrillon, D.H., DePinho, R.A., and Hedrick, S.M. 2009. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nat Immunol* 10:176-184.
143. Walunas, T.L., and Bluestone, J.A. 1998. CTLA-4 regulates tolerance induction and T cell differentiation in vivo. *J Immunol* 160:3855-3860.
144. Perez, V.L., Van Parijs, L., Biuckians, A., Zheng, X.X., Strom, T.B., and Abbas, A.K. 1997. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* 6:411-417.
145. Chambers, C.A., Kuhns, M.S., Egen, J.G., and Allison, J.P. 2001. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol* 19:565-594.
146. Judge, T.A., Wu, Z., Zheng, X.G., Sharpe, A.H., Sayegh, M.H., and Turka, L.A. 1999. The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival. *J Immunol* 162:1947-1951.

147. Lin, H., Rathmell, J.C., Gray, G.S., Thompson, C.B., Leiden, J.M., and Alegre, M.L. 1998. Cytotoxic T lymphocyte antigen 4 (CTLA4) blockade accelerates the acute rejection of cardiac allografts in CD28-deficient mice: CTLA4 can function independently of CD28. *J Exp Med* 188:199-204.
148. Ariyan, C., Salvalaggio, P., Fecteau, S., Deng, S., Rogozinski, L., Mandelbrot, D., Sharpe, A., Sayegh, M.H., Basadonna, G.P., and Rothstein, D.M. 2003. Cutting edge: transplantation tolerance through enhanced CTLA-4 expression. *J Immunol* 171:5673-5677.
149. Fecteau, S., Basadonna, G.P., Freitas, A., Ariyan, C., Sayegh, M.H., and Rothstein, D.M. 2001. CTLA-4 up-regulation plays a role in tolerance mediated by CD45. *Nat Immunol* 2:58-63.
150. Liu, D., Krummey, S.M., Badell, I.R., Wagener, M., Schneeweis, L.A., Stetsko, D.K., Suchard, S.J., Nadler, S.G., and Ford, M.L. 2014. 2B4 (CD244) induced by selective CD28 blockade functionally regulates allograft-specific CD8+ T cell responses. *J Exp Med* 211:297-311.
151. Zhang, T., Fresnay, S., Welty, E., Sangrampurkar, N., Rybak, E., Zhou, H., Cheng, X.F., Feng, Q., Avon, C., Laaris, A., et al. 2011. Selective CD28 blockade attenuates acute and chronic rejection of murine cardiac allografts in a CTLA-4-dependent manner. *Am J Transplant* 11:1599-1609.
152. Bachmann, M.F., Waterhouse, P., Speiser, D.E., McKall-Faienza, K., Mak, T.W., and Ohashi, P.S. 1998. Normal responsiveness of CTLA-4-deficient anti-viral cytotoxic T cells. *J Immunol* 160:95-100.
153. Sevilla, N., Homann, D., von Herrath, M., Rodriguez, F., Harkins, S., Whitton, J.L., and Oldstone, M.B. 2000. Virus-induced diabetes in a transgenic model: role of cross-reacting viruses and quantitation of effector T cells needed to cause disease. *J Virol* 74:3284-3292.

154. Tai, X., Van Laethem, F., Pobeziński, L., Guintier, T., Sharrow, S.O., Adams, A., Granger, L., Kruhlak, M., Lindsten, T., Thompson, C.B., et al. 2012. Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells. *Blood* 119:5155-5163.
155. Corse, E., and Allison, J.P. 2012. Cutting edge: CTLA-4 on effector T cells inhibits in trans. *J Immunol* 189:1123-1127.
156. Wang, C.J., Kenefeck, R., Wardzinski, L., Attridge, K., Manzotti, C., Schmidt, E.M., Qureshi, O.S., Sansom, D.M., and Walker, L.S. 2012. Cutting edge: cell-extrinsic immune regulation by CTLA-4 expressed on conventional T cells. *J Immunol* 189:1118-1122.
157. Qureshi, O.S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E.M., Baker, J., Jeffery, L.E., Kaur, S., Briggs, Z., et al. 2011. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 332:600-603.
158. Hainz, U., Jurgens, B., and Heitger, A. 2007. The role of indoleamine 2,3-dioxygenase in transplantation. *Transpl Int* 20:118-127.
159. Schneider, H., Downey, J., Smith, A., Zinselmeyer, B.H., Rush, C., Brewer, J.M., Wei, B., Hogg, N., Garside, P., and Rudd, C.E. 2006. Reversal of the TCR stop signal by CTLA-4. *Science* 313:1972-1975.
160. Zhang, N., Schroppel, B., Lal, G., Jakubzick, C., Mao, X., Chen, D., Yin, N., Jessberger, R., Ochando, J.C., Ding, Y., et al. 2009. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity* 30:458-469.
161. Salomon, B., Lenschow, D.J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J.A. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431-440.

162. Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T.W., and Sakaguchi, S. 2000. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192:303-310.
163. Read, S., Malmstrom, V., and Powrie, F. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 192:295-302.
164. Walker, L.S., and Sansom, D.M. 2011. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nat Rev Immunol* 11:852-863.
165. Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322:271-275.
166. Zhang, R., Huynh, A., Whitcher, G., Chang, J., Maltzman, J.S., and Turka, L.A. 2013. An obligate cell-intrinsic function for CD28 in Tregs. *J Clin Invest* 123:580-593.
167. Zheng, Y., Manzotti, C.N., Burke, F., Dussably, L., Qureshi, O., Walker, L.S., and Sansom, D.M. 2008. Acquisition of suppressive function by activated human CD4+ CD25- T cells is associated with the expression of CTLA-4 not FoxP3. *J Immunol* 181:1683-1691.
168. Lu, Y., Schneider, H., and Rudd, C.E. 2012. Murine regulatory T cells differ from conventional T cells in resisting the CTLA-4 reversal of TCR stop-signal. *Blood* 120:4560-4570.
169. Riella, L.V., and Sayegh, M.H. 2013. T-cell co-stimulatory blockade in transplantation: two steps forward one step back! *Expert Opin Biol Ther* 13:1557-1568.
170. Burrell, B.E., and Bishop, D.K. 2010. Th17 cells and transplant acceptance. *Transplantation* 90:945-948.

171. Krummey, S.M., Floyd, T.L., Liu, D., Wagener, M.E., Song, M., and Ford, M.L. 2014. Candida-elicited murine Th17 cells express high Ctl4 compared with Th1 cells and are resistant to costimulation blockade. *J Immunol* 192:2495-2504.
172. Turka, L.A., Linsley, P.S., Lin, H., Brady, W., Leiden, J.M., Wei, R., Gibson, M.L., Zheng, X., Myrdal, S., Gordon, D., et al. 1992. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection *in vivo*. *Proc. Natl. Acad. Sci. USA* 89:11102-11105.
173. Lenschow, D., Zeng, Y., Thistlethwaite, J., Montag, A., Brady, W., Gibson, M., Linsley, P., and Bluestone, J. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. *Science* 257:789-792.
174. Larsen, C.P., Pearson, T.C., Adams, A.B., Tso, P., Shirasugi, N., Strobert, E., Anderson, D., Cowan, S., Price, K., Naemura, J., et al. 2005. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant* 5:443-453.
175. Racke, M.K., Scott, D.E., Quigley, L., Gray, G.S., Abe, R., June, C.H., and Perrin, P.J. 1995. Distinct roles for B7-1 (CD-80) and B7-2 (CD-86) in the initiation of experimental allergic encephalomyelitis. *J Clin Invest* 96:2195-2203.
176. Tang, Q., Henriksen, K.J., Boden, E.K., Tooley, A.J., Ye, J., Subudhi, S.K., Zheng, X.X., Strom, T.B., and Bluestone, J.A. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 171:3348-3352.
177. Ford, M.L., and Larsen, C.P. 2009. Translating costimulation blockade to the clinic: lessons learned from three pathways. *Immunol Rev* 229:294-306.
178. Lo, D.J., Anderson, D.J., Weaver, T.A., Leopardi, F., Song, M., Farris, A.B., Strobert, E.A., Jenkins, J., Turgeon, N.A., Mehta, A.K., et al. 2013. Belatacept and sirolimus prolong nonhuman primate renal allograft survival without a requirement for memory T cell depletion. *Am J Transplant* 13:320-328.

179. Lowe, M.C., Badell, I.R., Turner, A.P., Thompson, P.W., Leopardi, F.V., Strobert, E.A., Larsen, C.P., and Kirk, A.D. 2013. Belatacept and sirolimus prolong nonhuman primate islet allograft survival: adverse consequences of concomitant alefacept therapy. *Am J Transplant* 13:312-319.
180. Kitchens, W.H., Haridas, D., Wagener, M.E., Song, M., Kirk, A.D., Larsen, C.P., and Ford, M.L. 2012. Integrin Antagonists Prevent Costimulatory Blockade-Resistant Transplant Rejection by CD8(+) Memory T Cells. *Am J Transplant*.
181. Kitchens, W.H., Haridas, D., Wagener, M.E., Song, M., and Ford, M.L. 2012. Combined Costimulatory and Leukocyte Functional Antigen-1 Blockade Prevents Transplant Rejection Mediated by Heterologous Immune Memory Alloresponses. *Transplantation*.
182. Reisman, N.M., Floyd, T.L., Wagener, M.E., Kirk, A.D., Larsen, C.P., and Ford, M.L. 2011. LFA-1 blockade induces effector and regulatory T-cell enrichment in lymph nodes and synergizes with CTLA-4Ig to inhibit effector function. *Blood* 118:5851-5861.
183. Badell, I.R., Russell, M.C., Thompson, P.W., Turner, A.P., Weaver, T.A., Robertson, J.M., Avila, J.G., Cano, J.A., Johnson, B.E., Song, M., et al. 2010. LFA-1-specific therapy prolongs allograft survival in rhesus macaques. *J Clin Invest* 120:4520-4531.
184. Vincenti, F., Larsen, C.P., Alberu, J., Bresnahan, B., Garcia, V.D., Kothari, J., Lang, P., Urrea, E.M., Massari, P., Mondragon-Ramirez, G., et al. 2012. Three-year outcomes from BENEFIT, a randomized, active-controlled, parallel-group study in adult kidney transplant recipients. *Am J Transplant* 12:210-217.
185. Vincenti, F., Larsen, C., Durrbach, A., Wekerle, T., Nashan, B., Blancho, G., Lang, P., Grinyo, J., Halloran, P.F., Solez, K., et al. 2005. Costimulation blockade with belatacept in renal transplantation. *N Engl J Med* 353:770-781.
186. Vincenti, F., Blancho, G., Durrbach, A., Friend, P., Grinyo, J., Halloran, P.F., Klempnauer, J., Lang, P., Larsen, C.P., Muhlbacher, F., et al. 2010. Five-year safety and

- efficacy of belatacept in renal transplantation. *Journal of the American Society of Nephrology* : *JASN* 21:1587-1596.
187. Burrows, S.R., Khanna, R., Burrows, J.M., and Moss, D.J. 1994. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for graft-versus-host disease. *J Exp Med* 179:1155-1161.
188. Kjer-Nielsen, L., Clements, C.S., Purcell, A.W., Brooks, A.G., Whisstock, J.C., Burrows, S.R., McCluskey, J., and Rossjohn, J. 2003. A structural basis for the selection of dominant alphabeta T cell receptors in antiviral immunity. *Immunity* 18:53-64.
189. Corse, E., Gottschalk, R.A., and Allison, J.P. 2011. Strength of TCR-peptide/MHC interactions and in vivo T cell responses. *J Immunol* 186:5039-5045.
190. Zehn, D., King, C., Bevan, M.J., and Palmer, E. 2012. TCR signaling requirements for activating T cells and for generating memory. *Cell Mol Life Sci* 69:1565-1575.
191. Zehn, D., Lee, S.Y., and Bevan, M.J. 2009. Complete but curtailed T-cell response to very low-affinity antigen. *Nature* 458:211-214.
192. van Gisbergen, K.P., Klarenbeek, P.L., Kragten, N.A., Unger, P.P., Nieuwenhuis, M.B., Wensveen, F.M., ten Brinke, A., Tak, P.P., Eldering, E., Nolte, M.A., et al. 2011. The costimulatory molecule CD27 maintains clonally diverse CD8(+) T cell responses of low antigen affinity to protect against viral variants. *Immunity* 35:97-108.
193. Gottschalk, R.A., Corse, E., and Allison, J.P. 2012. Expression of Helios in peripherally induced Foxp3+ regulatory T cells. *J Immunol* 188:976-980.
194. Gronski, M.A., Boulter, J.M., Moskophidis, D., Nguyen, L.T., Holmberg, K., Elford, A.R., Deenick, E.K., Kim, H.O., Penninger, J.M., Odermatt, B., et al. 2004. TCR affinity and negative regulation limit autoimmunity. *Nat Med* 10:1234-1239.

195. King, C.G., Koehli, S., Hausmann, B., Schmalzer, M., Zehn, D., and Palmer, E. 2012. T cell affinity regulates asymmetric division, effector cell differentiation, and tissue pathology. *Immunity* 37:709-720.
196. Enouz, S., Carrie, L., Merkler, D., Bevan, M.J., and Zehn, D. 2012. Autoreactive T cells bypass negative selection and respond to self-antigen stimulation during infection. *J Exp Med* 209:1769-1779.
197. Miossec, P., Korn, T., and Kuchroo, V.K. 2009. Interleukin-17 and type 17 helper T cells. *N Engl J Med* 361:888-898.
198. Korn, T., Bettelli, E., Oukka, M., and Kuchroo, V.K. 2009. IL-17 and Th17 Cells. *Annu Rev Immunol* 27:485-517.
199. Wilke, C.M., Bishop, K., Fox, D., and Zou, W. 2011. Deciphering the role of Th17 cells in human disease. *Trends Immunol* 32:603-611.
200. Acosta-Rodriguez, E.V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M., Lanzavecchia, A., Sallusto, F., and Napolitani, G. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 8:639-646.
201. Merrill, J.T., Burgos-Vargas, R., Westhovens, R., Chalmers, A., D'Cruz, D., Wallace, D.J., Bae, S.C., Sigal, L., Becker, J.C., Kelly, S., et al. 2010. The efficacy and safety of abatacept in patients with non-life-threatening manifestations of systemic lupus erythematosus: results of a twelve-month, multicenter, exploratory, phase IIb, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* 62:3077-3087.
202. Sandborn, W.J., Colombel, J.F., Sands, B.E., Rutgeerts, P., Targan, S.R., Panaccione, R., Bressler, B., Geboes, K., Schreiber, S., Aranda, R., et al. 2012. Abatacept for Crohn's disease and ulcerative colitis. *Gastroenterology* 143:62-69 e64.

203. Zhai, C., Zhang, J., Zhai, Y., Chen, J., Zuo, H., and Jiang, L. 2004. [Comparison of effect between multiplex and simplex nutritional intervention on hyperlipemia, hypertension or diabetes]. *Wei Sheng Yan Jiu* 33:719-721.
204. Ma, X., Qian, Y., Xie, M.X., Hou, Z., Meng, Y., Liu, J.Y., Zhai, Y.N., Zhu, Y.F., Zhou, Z.M., Sha, J.H., et al. 2004. [A two-dimensional reference map of mouse ovary proteins]. *Beijing Da Xue Xue Bao* 36:581-586.
205. Williams, M.A., Onami, T.M., Adams, A.B., Durham, M.M., Pearson, T.C., Ahmed, R., and Larsen, C.P. 2002. Cutting edge: persistent viral infection prevents tolerance induction and escapes immune control following CD28/CD40 blockade-based regimen. *J Immunol* 169:5387-5391.
206. Socie, G., and Blazar, B.R. 2009. Acute graft-versus-host disease: from the bench to the bedside. *Blood* 114:4327-4336.
207. Blazar, B.R., Murphy, W.J., and Abedi, M. 2012. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol* 12:443-458.
208. Paulos, C.M., Carpenito, C., Plesa, G., Suhoski, M.M., Varela-Rohena, A., Golovina, T.N., Carroll, R.G., Riley, J.L., and June, C.H. 2010. The inducible costimulator (ICOS) is critical for the development of human T(H)17 cells. *Sci Transl Med* 2:55ra78.
209. Amezcua-Guerra, L.M., Hernandez-Martinez, B., Pineda, C., and Bojalil, R. 2006. Ulcerative colitis during CTLA-4Ig therapy in a patient with rheumatoid arthritis. *Gut* 55:1059-1060.
210. Floyd, T.L., Wagener, M.E., Kitchens, W.H., Robertson, J.M., Cheeseman, J.A., Stempora, L., Larsen, C.P., and Ford, M.L. 2011. Limiting the Amount and Duration of Antigen Exposure During Priming Increases Memory T Cell Requirement for Costimulation During Recall. *J Immunol* 186:2033-2041.
211. Infante-Duarte, C., Horton, H.F., Byrne, M.C., and Kamradt, T. 2000. Microbial lipopeptides induce the production of IL-17 in Th cells. *J Immunol* 165:6107-6115.

212. McGeachy, M.J., Chen, Y., Tato, C.M., Laurence, A., Joyce-Shaikh, B., Blumenschein, W.M., McClanahan, T.K., O'Shea, J.J., and Cua, D.J. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol* 10:314-324.
213. Esplugues, E., Huber, S., Gagliani, N., Hauser, A.E., Town, T., Wan, Y.Y., O'Connor, W., Jr., Rongvaux, A., Van Rooijen, N., Haberman, A.M., et al. 2011. Control of TH17 cells occurs in the small intestine. *Nature* 475:514-518.
214. Alegre, M.L., Frauwirth, K.A., and Thompson, C.B. 2001. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* 1:220-228.
215. Roy, M., Waldschmidt, T., Aruffo, A., Ledbetter, J.A., and Noelle, R.J. 1993. The regulation of the expression of gp39, the CD40 ligand, on normal and cloned CD4+ T cells. *J. Immunol.* 151:2497-2510.
216. Larsen, C.P., and Pearson, T.C. 1997. The CD40 pathway in allograft rejection, acceptance, and tolerance. *Curr Opin Immunol* 9:641-647.
217. Awasthi, A., Riol-Blanco, L., Jager, A., Korn, T., Pot, C., Galileos, G., Bettelli, E., Kuchroo, V.K., and Oukka, M. 2009. Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. *J Immunol* 182:5904-5908.
218. Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., et al. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.
219. Jager, A., Dardalhon, V., Sobel, R.A., Bettelli, E., and Kuchroo, V.K. 2009. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol* 183:7169-7177.
220. Cosmi, L., De Palma, R., Santarlasci, V., Maggi, L., Capone, M., Frosali, F., Rodolico, G., Querci, V., Abbate, G., Angeli, R., et al. 2008. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J Exp Med* 205:1903-1916.

221. McGeachy, M.J., and Cua, D.J. 2008. Th17 cell differentiation: the long and winding road. *Immunity* 28:445-453.
222. Tsokos, G.C. 2011. Systemic lupus erythematosus. *N Engl J Med* 365:2110-2121.
223. Suchard, S.J., Davis, P.M., Kansal, S., Stetsko, D.K., Brosius, R., Tamura, J., Schneeweis, L., Bryson, J., Salcedo, T., Wang, H., et al. 2014. A monovalent anti-human CD28 domain antibody antagonist: preclinical efficacy and safety. *J Immunol* 191:4599-4610.
224. Ling, V., Wu, P.W., Finnerty, H.F., Sharpe, A.H., Gray, G.S., and Collins, M. 1999. Complete sequence determination of the mouse and human CTLA4 gene loci: cross-species DNA sequence similarity beyond exon borders. *Genomics* 60:341-355.
225. Hedrick, S.M., Hess Michelini, R., Doedens, A.L., Goldrath, A.W., and Stone, E.L. 2012. FOXO transcription factors throughout T cell biology. *Nat Rev Immunol* 12:649-661.
226. Powell, J.D., Pollizzi, K.N., Heikamp, E.B., and Horton, M.R. 2012. Regulation of immune responses by mTOR. *Annu Rev Immunol* 30:39-68.
227. Kerdiles, Y.M., Stone, E.L., Beisner, D.R., McGargill, M.A., Ch'en, I.L., Stockmann, C., Katayama, C.D., and Hedrick, S.M. 2010. Foxo transcription factors control regulatory T cell development and function. *Immunity* 33:890-904.
228. Kim, J.S., Sklarz, T., Banks, L.B., Gohil, M., Waickman, A.T., Skuli, N., Krock, B.L., Luo, C.T., Hu, W., Pollizzi, K.N., et al. 2013. Natural and inducible TH17 cells are regulated differently by Akt and mTOR pathways. *Nat Immunol* 14:611-618.
229. Wan, Q., Kozhaya, L., ElHed, A., Ramesh, R., Carlson, T.J., Djuretic, I.M., Sundrud, M.S., and Unutmaz, D. 2011. Cytokine signals through PI-3 kinase pathway modulate Th17 cytokine production by CCR6+ human memory T cells. *J Exp Med* 208:1875-1887.
230. Kavanagh, B., O'Brien, S., Lee, D., Hou, Y., Weinberg, V., Rini, B., Allison, J.P., Small, E.J., and Fong, L. 2008. CTLA4 blockade expands FoxP3+ regulatory and activated effector CD4+ T cells in a dose-dependent fashion. *Blood* 112:1175-1183.

231. Jameson, S.C., and Masopust, D. 2009. Diversity in T cell memory: an embarrassment of riches. *Immunity* 31:859-871.
232. Obar, J.J., and Lefrancois, L. 2010. Early signals during CD8 T cell priming regulate the generation of central memory cells. *J Immunol* 185:263-272.
233. van Faassen, H., Saldanha, M., Gilbertson, D., Dudani, R., Krishnan, L., and Sad, S. 2005. Reducing the stimulation of CD8⁺ T cells during infection with intracellular bacteria promotes differentiation primarily into a central (CD62L^{high}CD44^{high}) subset. *J Immunol* 174:5341-5350.
234. Floyd, T.L., Koehn, B.H., Kitchens, W.H., Robertson, J.M., Cheeseman, J.A., Stempora, L., Larsen, C.P., and Ford, M.L. 2011. Limiting the amount and duration of antigen exposure during priming increases memory T cell requirement for costimulation during recall. *J Immunol* 186:2033-2041.
235. Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J., and Ahmed, R. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439:682-687.
236. Nolz, J.C., and Harty, J.T. 2011. Protective capacity of memory CD8⁺ T cells is dictated by antigen exposure history and nature of the infection. *Immunity* 34:781-793.
237. Gottschalk, R.A., Hathorn, M.M., Beuneu, H., Corse, E., Dustin, M.L., Altan-Bonnet, G., and Allison, J.P. 2012. Distinct influences of peptide-MHC quality and quantity on in vivo T-cell responses. *Proc Natl Acad Sci U S A* 109:881-886.
238. Sabatino, J.J., Jr., Huang, J., Zhu, C., and Evavold, B.D. 2010. High prevalence of low affinity peptide-MHC II tetramer-negative effectors during polyclonal CD4⁺ T cell responses. *J Exp Med* 208:81-90.
239. Moon, J.J., Chu, H.H., Hataye, J., Pagan, A.J., Pepper, M., McLachlan, J.B., Zell, T., and Jenkins, M.K. 2009. Tracking epitope-specific T cells. *Nat Protoc* 4:565-581.

240. Smith-Garvin, J.E., Burns, J.C., Gohil, M., Zou, T., Kim, J.S., Maltzman, J.S., Wherry, E.J., Koretzky, G.A., and Jordan, M.S. 2010. T-cell receptor signals direct the composition and function of the memory CD8⁺ T-cell pool. *Blood* 116:5548-5559.
241. Hermiston, M.L., Xu, Z., and Weiss, A. 2003. CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol* 21:107-137.
242. Moran, A.E., Holzapfel, K.L., Xing, Y., Cunningham, N.R., Maltzman, J.S., Punt, J., and Hogquist, K.A. 2011. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med* 208:1279-1289.
243. Daniels, M.A., Teixeira, E., Gill, J., Hausmann, B., Roubaty, D., Holmberg, K., Werlen, G., Hollander, G.A., Gascoigne, N.R., and Palmer, E. 2006. Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. *Nature* 444:724-729.
244. Williams, M.A., Tyznik, A.J., and Bevan, M.J. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8⁺ memory T cells. *Nature* 441:890-893.
245. Rutishauser, R.L., Martins, G.A., Kalachikov, S., Chandele, A., Parish, I.A., Meffre, E., Jacob, J., Calame, K., and Kaech, S.M. 2009. Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31:296-308.
246. Pipkin, M.E., Sacks, J.A., Cruz-Guilloty, F., Lichtenheld, M.G., Bevan, M.J., and Rao, A. 2010. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 32:79-90.
247. Kalia, V., Sarkar, S., Subramaniam, S., Haining, W.N., Smith, K.A., and Ahmed, R. 2010. Prolonged interleukin-2Ralpha expression on virus-specific CD8⁺ T cells favors terminal-effector differentiation in vivo. *Immunity* 32:91-103.
248. Boyman, O., and Sprent, J. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol* 12:180-190.

249. Intlekofer, A.M., Takemoto, N., Wherry, E.J., Longworth, S.A., Northrup, J.T., Palanivel, V.R., Mullen, A.C., Gasink, C.R., Kaeck, S.M., Miller, J.D., et al. 2005. Effector and memory CD8⁺ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* 6:1236-1244.
250. Kitchens, W.H., Haridas, D., Wagener, M.E., Song, M., Kirk, A.D., Larsen, C.P., and Ford, M.L. 2012. Integrin antagonists prevent costimulatory blockade-resistant transplant rejection by CD8(+) memory T cells. *Am J Transplant* 12:69-80.
251. Ford, M.L., Koehn, B.H., Wagener, M.E., Jiang, W., Gangappa, S., Pearson, T.C., and Larsen, C.P. 2007. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J Exp Med* 204:299-309.
252. Xu, Z., and Weiss, A. 2002. Negative regulation of CD45 by differential homodimerization of the alternatively spliced isoforms. *Nat Immunol* 3:764-771.
253. Teixeira, E., Daniels, M.A., Hamilton, S.E., Schrum, A.G., Bragado, R., Jameson, S.C., and Palmer, E. 2009. Different T cell receptor signals determine CD8⁺ memory versus effector development. *Science* 323:502-505.
254. Wherry, E.J., Puorro, K.A., Porgador, A., and Eisenlohr, L.C. 1999. The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. *J Immunol* 163:3735-3745.
255. Wiehagen, K.R., Corbo, E., Schmidt, M., Shin, H., Wherry, E.J., and Maltzman, J.S. 2010. Loss of tonic T-cell receptor signals alters the generation but not the persistence of CD8⁺ memory T cells. *Blood* 116:5560-5570.
256. Wherry, E.J., McElhaugh, M.J., and Eisenlohr, L.C. 2002. Generation of CD8(+) T cell memory in response to low, high, and excessive levels of epitope. *J Immunol* 168:4455-4461.

257. Knudson, K.M., Goplen, N.P., Cunningham, C.A., Daniels, M.A., and Teixeira, E. 2013. Low-affinity T cells are programmed to maintain normal primary responses but are impaired in their recall to low-affinity ligands. *Cell Rep* 4:554-565.
258. Feau, S., Arens, R., Togher, S., and Schoenberger, S.P. 2011. Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells. *Nat Immunol* 12:908-913.
259. Bachmann, M.F., Wolint, P., Schwarz, K., Jager, P., and Oxenius, A. 2005. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol* 175:4686-4696.
260. Gagliani, N., Gregori, S., Jofra, T., Valle, A., Stabilini, A., Rothstein, D.M., Atkinson, M., Roncarolo, M.G., and Battaglia, M. 2011. Rapamycin combined with anti-CD45RB mAb and IL-10 or with G-CSF induces tolerance in a stringent mouse model of islet transplantation. *PLoS One* 6:e28434.
261. Gagliani, N., Jofra, T., Valle, A., Stabilini, A., Morsiani, C., Gregori, S., Deng, S., Rothstein, D.M., Atkinson, M., Kamanaka, M., et al. 2013. Transplant tolerance to pancreatic islets is initiated in the graft and sustained in the spleen. *Am J Transplant* 13:1963-1975.
262. Chen, G., Luke, P.P., Yang, H., Visser, L., Sun, H., Garcia, B., Qian, H., Xiang, Y., Huang, X., Liu, W., et al. 2007. Anti-CD45RB monoclonal antibody prolongs renal allograft survival in cynomolgus monkeys. *Am J Transplant* 7:27-37.
263. Yang, H., J. Arp, Y. Ma, I. Welch, A. Haig, W. Liu, K. Reimann, A. Jevnikar, D. Rothstein. 2014. Combination of Novel Anti-CD45RB and Anti-CD40 Chimeric Antibodies Promotes Operational Tolerance and Induction of T Regulatory Cells in Cynomolgus Monkey Renal Allograft Recipients. In *World Transplant Congress*. San Francisco, CA.
264. Luke, P.P., Deng, J.P., O'Brien, C.A., Everest, M., Hall, A.V., Chakrabarti, S., O'Connell, P.J., Zhong, R., and Jevnikar, A.M. 2003. Alteration in CD45RB^{hi}/CD45RB^{lo} T-cell

- ratio following CD45RB monoclonal-antibody therapy occurs by selective deletion of CD45RBhi effector cells. *Transplantation* 76:400-409.
265. Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76:17-27.
266. Ehst, B.D., Ingulli, E., and Jenkins, M.K. 2003. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am J Transplant* 3:1355-1362.
267. Trambley, J., Bingaman, A.W., Lin, A., Elwood, E.T., Waitze, S.Y., Ha, J., Durham, M.M., Corbascio, M., Cowan, S.R., Pearson, T.C., et al. 1999. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. *J Clin Invest* 104:1715-1722.
268. Nadazdin, O., Boskovic, S., Murakami, T., Tocco, G., Smith, R.N., Colvin, R.B., Sachs, D.H., Allan, J., Madsen, J.C., Kawai, T., et al. 2011. Host alloreactive memory T cells influence tolerance to kidney allografts in nonhuman primates. *Sci Transl Med* 3:86ra51.
269. Krummey, S.M., and Ford, M.L. 2012. Heterogeneity within T Cell Memory: Implications for Transplant Tolerance. *Front Immunol* 3:36.
270. Chen, L., and Flies, D.B. 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* 13:227-242.
271. Watts, T.H. 2005. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol* 23:23-68.
272. Kim, E.Y., and Teh, H.S. 2001. TNF type 2 receptor (p75) lowers the threshold of T cell activation. *J Immunol* 167:6812-6820.
273. Kim, E.Y., and Teh, H.S. 2004. Critical role of TNF receptor type-2 (p75) as a costimulator for IL-2 induction and T cell survival: a functional link to CD28. *J Immunol* 173:4500-4509.

274. Kim, E.Y., Priatel, J.J., Teh, S.J., and Teh, H.S. 2006. TNF receptor type 2 (p75) functions as a costimulator for antigen-driven T cell responses in vivo. *J Immunol* 176:1026-1035.
275. Mahmud, S.A., Manlove, L.S., Schmitz, H.M., Xing, Y., Wang, Y., Owen, D.L., Schenkel, J.M., Boomer, J.S., Green, J.M., Yagita, H., et al. 2014. Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nat Immunol* 15:473-481.
276. Evavold, B.D., and Allen, P.M. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science* 252:1308-1310.
277. Mehlhop-Williams, E.R., and Bevan, M.J. 2014. Memory CD8+ T cells exhibit increased antigen threshold requirements for recall proliferation. *J Exp Med* 211:345-356.
278. Aggarwal, B.B. 2003. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 3:745-756.
279. Sabbagh, L., Snell, L.M., and Watts, T.H. 2007. TNF family ligands define niches for T cell memory. *Trends Immunol* 28:333-339.
280. Croft, M. 2009. The role of TNF superfamily members in T-cell function and diseases. *Nat Rev Immunol* 9:271-285.
281. Slifka, M.K., and Whitton, J.L. 2000. Activated and memory CD8+ T cells can be distinguished by their cytokine profiles and phenotypic markers. *J Immunol* 164:208-216.
282. Shen, H., and Goldstein, D.R. 2009. IL-6 and TNF-alpha synergistically inhibit allograft acceptance. *J Am Soc Nephrol* 20:1032-1040.
283. Korngold, R., Marini, J.C., de Baca, M.E., Murphy, G.F., and Giles-Komar, J. 2003. Role of tumor necrosis factor-alpha in graft-versus-host disease and graft-versus-leukemia responses. *Biol Blood Marrow Transplant* 9:292-303.
284. Tsukada, N., Kobata, T., Aizawa, Y., Yagita, H., and Okumura, K. 1999. Graft-versus-leukemia effect and graft-versus-host disease can be differentiated by cytotoxic

- mechanisms in a murine model of allogeneic bone marrow transplantation. *Blood* 93:2738-2747.
285. Luz, J.G., Huang, M., Garcia, K.C., Rudolph, M.G., Apostolopoulos, V., Teyton, L., and Wilson, I.A. 2002. Structural comparison of allogeneic and syngeneic T cell receptor-peptide-major histocompatibility complex complexes: a buried alloreactive mutation subtly alters peptide presentation substantially increasing V(beta) Interactions. *J Exp Med* 195:1175-1186.
286. Bowerman, N.A., Colf, L.A., Garcia, K.C., and Kranz, D.M. 2009. Different strategies adopted by K(b) and L(d) to generate T cell specificity directed against their respective bound peptides. *J Biol Chem* 284:32551-32561.
287. Sykulev, Y., Brunmark, A., Tsomides, T.J., Kageyama, S., Jackson, M., Peterson, P.A., and Eisen, H.N. 1994. High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex class I proteins. *Proc Natl Acad Sci U S A* 91:11487-11491.
288. Sykulev, Y., Brunmark, A., Jackson, M., Cohen, R.J., Peterson, P.A., and Eisen, H.N. 1994. Kinetics and affinity of reactions between an antigen-specific T cell receptor and peptide-MHC complexes. *Immunity* 1:15-22.
289. Udaka, K., Tsomides, T.J., and Eisen, H.N. 1992. A naturally occurring peptide recognized by alloreactive CD8+ cytotoxic T lymphocytes in association with a class I MHC protein. *Cell* 69:989-998.
290. Cho, B.K., Lian, K.C., Lee, P., Brunmark, A., McKinley, C., Chen, J., Kranz, D.M., and Eisen, H.N. 2001. Differences in antigen recognition and cytolytic activity of CD8(+) and CD8(-) T cells that express the same antigen-specific receptor. *Proc Natl Acad Sci U S A* 98:1723-1727.
291. Chen, J., Eisen, H.N., and Kranz, D.M. 2003. A model T-cell receptor system for studying memory T-cell development. *Microbes Infect* 5:233-240.

292. Zehn, D., Turner, M.J., Lefrancois, L., and Bevan, M.J. 2010. Lack of original antigenic sin in recall CD8(+) T cell responses. *J Immunol* 184:6320-6326.
293. Intlekofer, A.M., Wherry, E.J., and Reiner, S.L. 2006. Not-so-great expectations: re-assessing the essence of T-cell memory. *Immunol Rev* 211:203-213.
294. Mele, D.A., Salmeron, A., Ghosh, S., Huang, H.R., Bryant, B.M., and Lora, J.M. 2013. BET bromodomain inhibition suppresses TH17-mediated pathology. *J Exp Med* 210:2181-2190.
295. Pandiyan, P., Conti, H.R., Zheng, L., Peterson, A.C., Mathern, D.R., Hernandez-Santos, N., Edgerton, M., Gaffen, S.L., and Lenardo, M.J. 2011. CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse *Candida albicans* Th17 cell infection model. *Immunity* 34:422-434.
296. Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., and Stockinger, B. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-189.
297. Xu, L., Kitani, A., Fuss, I., and Strober, W. 2007. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol* 178:6725-6729.
298. Kang, S.M., Tang, Q., and Bluestone, J.A. 2007. CD4+CD25+ regulatory T cells in transplantation: progress, challenges and prospects. *Am J Transplant* 7:1457-1463.
299. Ford, M.L., Adams, A.B., and Pearson, T.C. 2014. Targeting co-stimulatory pathways: transplantation and autoimmunity. *Nat Rev Nephrol* 10:14-24.
300. Vincenti, F., Grinyo, J., Charpentier, B., Medina-Pestana, J.D., Rostaing, L., Vanrenterghem, Y., Di Russo, G.B., Garg, P., Lin, C.S., and Larsen, C.P. 2009. Primary Outcomes from a Randomized, Phase III Study of Belatacept vs Cyclosporine in Kidney Transplant Receptients (BENEFIT study). *Am J Transplant* 9:191.

