THE UNIVERSITY OF CHICAGO

IMMUNE MECHANISMS MAINTAINING TRANSPLANTATION TOLERANCE

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES

AND THE PRITZKER SCHOOL OF MEDICINE

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

COMMITTEE ON IMMUNOLOGY

BY

CHRISTINE MARIA MCINTOSH

CHICAGO, ILLINOIS

AUGUST 2020

This dissertation is dedicated to my parents for their unwavering support and encouragement.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AICD: activation-induced cell death

AP-1: activator protein 1

APC: antigen presenting cell

Areg: amphiregulin

ASV: amplicon sequence variant

ATP: adenosine triphosphate

BCR: B cell receptor

Bcl-2: B cell lymphoma 2

Bim: Bcl-2 interacting mediator of cell death

CD: cluster of differentiation

CNI: calcineurin inhibitor

CNS: conserved non-coding DNA sequence

CTL: cytotoxic T cell

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

DAMP: damage-associated molecular pattern

DC: dendritic cell

DNA: deoxyribonucleic acid

DST: donor splenocyte transfusion

DT: diphtheria toxin

DTR: DT receptor

EAE: experimental autoimmune encephalomyelitis

ELISA: enzyme-linked immunosorbent assay

ELISpot: enzyme-linked immunosorbent spot assay

FasL: Fas ligand

FoxP3: forkhead box protein 3

Gata3: GATA binding protein 3

GVHD: graft-versus-host-disease

ICOS: inducible T cell co-stimulator

IDO: indoleamine 2,3-dioxygenase

Ig: immunoglobulin

IL: interleukin

IRI: ischemia reperfusion injury

iT_{REG}: T_{REG} induced in the periphery

Jax: Jackson Laboratory

LAG-3: lymphocyte activating 3

LCMV: lymphocytic choriomeningitis virus

Lm: *Listeria monocytogenes*

MED: Minimum Entropy Decomposition

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

mTEC: medullary thymic epithelial cell

MyD88: Deficiency in myeloid differentiation primary response 88

NFAT: nuclear factor of activated T cells

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

PAMP: pathogen-associated molecular pattern

PD-1: programmed cell death 1

PD-L1: programmed cell death 1 ligand 1

pMHC: peptide-MHC complex

PRR: pattern recognition receptor

RNA: ribonucleic acid

RNAseq: RNA sequencing

Rorγt: retanoic acid receptor-related orphan receptor gamma (t isoform)

scRNAseq: single cell RNAseq

T-bet: t-box expressed in T cells

Tac: Taconic Farms

TCR: T cell receptor

T_{CONV}: conventional FoxP3$^-$ CD4$^+$ T cell

TF: transcription factor

T_{FH}: follicular helper T cell

Tg: transgenic

T_H: helper CD4$^+$ T cell

TIGIT: T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif domains

TIM-3: T cell immunoglobulin and mucin domain containing 3

FMT: fecal microbiota transplantation

TLR: toll-like receptor

TNF: tumor necrosis factor

TOX: thymocyte-associated high mobility group box protein

TRAIL: TNF-related apoptosis-inducing ligand

T_{REG}: FoxP3$^+$ regulatory T cell

tT_{REG}: thymus-derived T_{REG}

VAT: visceral adipose tissue

WT: wild type

Acknowledgements

First and foremost, I would like to thank my thesis advisor, Marisa Alegre. I am immensely grateful to have spent the last five years learning from a scientist as brilliant, knowledgeable and kind as she. I have thoroughly enjoyed working through complex problems and developing experiments together. She has provided me with much-needed doses of optimism and perspective when experiments yielded disappointing results. I am also grateful to Marisa for keeping her door open to answer questions or solve problems at a moment's notice, a policy that has allowed me to salvage many an experiment gone awry. I have come away from my time in the Alegre Lab confident in my knowledge of immunology and ability to ask scientific questions because Marisa has spent so much time and effort helping me hone those skills. She has consistently treated my development as a scientist as a top priority. For those reasons, I could not imagine a better mentor.

I would like to thank the members of the Alegre Lab, who have made the lab an exciting and fulfilling place to work. I would especially like to thank Michelle Miller, whose work formed much of the foundation for my research. I am so fortunate to have had the opportunity to learn so much immunology and so many complex experimental techniques from someone as talented, patient and hard-working as Michelle. I am also incredibly grateful to Ying Wang, who performed countless heart transplants for my experiments. The majority of this work would have been impossible without her expertise. I would also like to thank Luqiu Chen for performing skin grafts for my experiments and for helping the lab with everything from performing experiments to ordering reagents. To Kevin Lei, thank you for helping me with multiple overnight sorts and for being a great friend. To Jen Allocco and Martin Sepulveda, thank you for being kind,

supportive friends. Jen, I have especially enjoyed getting to work with you on the avidity and T cell dysfunction projects. On top of helping me with experiments, you've also provided me with encouragement and a good laugh on many occasions. To all of the other members of the Alegre Lab, thank you for all of your advice and for being kind, helpful colleagues.

To the talented high school, undergraduate and rotation students who have worked directly with me, thank you for coming to lab striving to learn and contribute, especially Peter Wang, Victoria Rael and Maile Hollinger who made critical contributions to my experiments. To Peter, thank you for always offering to help me with experiments, for performing many Listeria infections for me and for your consistently positive attitude. To Victoria, thank you for your help with experiments and for keeping my mouse colony in order when my experiments piled up. To Maile, thank you for being the extra set of hands I needed during an incredibly busy time.

I would like to thank our collaborators in the Chong and Gajewski Labs for their advice on my project during lab meetings, and for generously sharing reagents. To Jay Williams, thank you for your technical advice and for helping me with an important overnight sort. I would also like to thank the Savage Lab for helping us generate TCR retrogenic mice, especially Victoria Lee who patiently taught me every technique involved. Thank you to Murat Eren and Alon Shaiber for performing bioinformatics analysis that was crucial for our microbiome study.

I would like to thank the members of my thesis committee, Pete Savage, Anita Chong and Bana Jabri for sharing their expertise to guide my project and help me determine which questions and experiments to prioritize.

Thank you to Jenny Zhang and Jiao-Jing Wang at Northwestern University for helping us perform heart transplants for an especially large experiment. I would also like to thank our

collaborators at Hôpital Erasme, Concetta Catalano and Alain Le Moine, for contributing clinical data on allospecific antibody production after transplantectomy.

To the members of the Gounari, Clark, Sperling and Kee labs, thank you for making the BSLC 3rd floor a friendly, collaborative place to work.

To members of the COI, thank you for many stimulating discussions during weekly journal clubs and for providing constructive feedback on my project.

To the Animal Resources Center, thank you for ensuring that our animal models are treated humanely. Thank you to David Leclerc, Bert Ladd, Mike Olson and Mandel Davis in the Flow Cytometry Core Facility for your technical expertise and for many laughs after overnight experiments. Thank you to the Environmental Sample Preparation and Sequencing Facility at Argonne National Lab for performing 16S sequencing for our microbiome study.

To the BSLC support staff, Angela Hayes, Gloria Davis and Luis Burgos, thank you for working hard to keep our research running smoothly. To Greg Washington, thank you for taking care of our facility. I have always enjoyed running into you during late nights in lab.

To Nancy Schwartz and Laurie Risner, thank you for ensuring that Pritzker students have the opportunity to pursue a PhD through the GDDTP. Thank you for cultivating the GDDTP community and promoting our career development through program dinners and seminars.

To my childhood teachers, George Hrab, Martin Fossett and Sophie Kan, thank you for showing me the value of science and giving me the confidence to challenge myself intellectually.

To Tim Huffaker and Owen O'Connor, thank you for welcoming me into your labs as an undergraduate and inspiring me to pursue a career in research. Thank you also to Beth Lalonde

and Enrica Marchi for being wonderful role models inside the lab and for training me in lab techniques with patience and kindness.

To my friends outside of the lab, especially Lea Hoefer, Rebecca Hirschfeld, Jess Reif and Beverly Yang, thank you for being a constant source of support and joy throughout graduate school.

To my parents, thank you for always providing me with the very best opportunities to learn. Thank you for giving me the courage to pursue big dreams, for pushing me to do my best and for loving me regardless of whether I have succeeded. I learned hard work, perseverance and an appreciation for the pursuit of knowledge from your example. To the rest of my family, especially my grandparents, thank you for showing me endless love and pride in my accomplishments. To Nobi and Misako Kambe, thank you for welcoming me into your family and showing me pure love and support.

To Yu, there are no words to describe how supportive you've been throughout my PhD and if there were, there would still be no words sufficient to thank you. Thank you for encouraging me through countless stressful and exhausting experiments. Thank you for celebrating every success with me and for seeing the value of my work. Your unwavering confidence in the scientific process has reassured me when I have felt discouraged. Your innate curiosity is inspiring. Thank you for making me a better scientist and a happier person. Thank you for consistently showing me unconditional love. I love you.

Abstract

While the development of powerful immunosuppressive medications enabled the use of organ transplantation as a treatment for end-stage organ failure, current immunosuppression protocols drive many co-morbidities; thus, they now present a critical limitation to long-term health and quality of life for transplant recipients. Replacing conventional immunosuppression with treatments to induce donor-specific immune tolerance would eliminate life-long exposure to drug toxicity and high risk of severe infections and malignancies for transplant recipients. Therefore, a large amount of research has been devoted to developing therapies to induce donor-specific immune tolerance. While there are treatments that induce permanent allograft acceptance in animal models and there are limited reports of immunosuppression-free graft survival in patients, tolerance observed in these circumstances is vulnerable to inflammatory challenges. To become a standard of care for transplant recipients, donor-specific tolerance must induce stable graft survival that resists bouts of inflammation. In this dissertation, we study the mechanisms maintaining tolerance long-term in murine models of transplantation. We find that that T_{REGs} play a more central role in the maintenance of tolerance than previously appreciated. T_{REGs} were required to constrain allospecific T cell expansion and avidity maturation during the maintenance phase of tolerance and depletion of T_{REGs} in tolerant allograft recipients swiftly led to rejection. We also investigated the factors driving development of cell-intrinsic dysfunction in T cells persisting in tolerant transplant recipients, finding that cell-extrinsic signals from lymphocytes in the tolerant host are required to program and maintain the dysfunctional state. We also found that there is heterogeneity in the functional profile of allospecific T cells persisting during tolerance. These findings may inform strategies to improve the resilience of tolerance and develop biomarkers to track the stability of tolerance in transplant recipients.

Chapter 1. Introduction

Introduction

Solid organ transplantation can cure end-stage organ failure. As the global population ages and rates of chronic diseases associated with organ failure increase, demand for organ transplantation is high. However, while organ transplantation is the best treatment option for many patients, transplant recipients experience significantly increased morbidity and mortality due to complications associated with their required lifelong immunosuppression regimens. With the exception of identical twins, all pairs of organ donor and recipient are genetically dissimilar. Due to this genetic disparity, the recipient's immune system will recognize the donor organ as foreign and mount a destructive immune response against it, a response known as transplant rejection. To prevent rejection, all transplant recipients must undergo immunosuppression. Indeed, development of new surgical techniques such as vascular anastomosis made solid organ transplantation technically possible in the early 20th century but its practice was limited to identical twins and autologous skin grafting until the addition of immunosuppressive medications fifty years later. Early immunosuppression regimens including total body irradiation or cytotoxic chemotherapeutic agents extended graft survival only modestly and were

accompanied by severe complications. It was not until the addition of steroids and cyclosporine in the 1950's that long-term graft survival improved sufficiently for transplantation to become a common practice. Indeed, steroids are still commonly used to control transplant rejection and nearly all patients receiving transplants today take medications with the same mechanism of action as cyclosporine. More recent developments in immunosuppression have included the discovery of new classes of immunosuppressants and combination of multiple immunosuppressants into treatment cocktails with the intention of reducing the dose, and thus side effects, of each component drug (Barker and Markmann 2013). While the development of immunosuppressive medications drove the widespread practice of solid organ transplantation, these same medications now represent one of the largest barriers to long-term health and quality of life in transplant recipients.

Current immunosuppression protocols for transplant recipients are associated with several critical shortcomings. First, immunosuppressive drugs that compose the standard of care for transplant recipients do not discriminate between harmful immune responses against the donor organ and desirable responses against pathogens and tumors. As a result, transplant recipients experience increased rates of cancer and life-threatening infections (Chapman, Webster, and Wong 2013, Fishman 2007, Engels et al. 2011, Bhat et al. 2018). Second, transplant recipients must take these immunosuppressive drugs indefinitely, exposing them to lifelong drug side effects and toxicity. Several common immunosuppressants are associated with increased risk of cardiovascular disease, which is one of the main causes of morbidity and mortality for transplant recipients (Kasiske 2001, Miller 2002). Renal toxicity associated with calcineurin inhibitors (CNIs) like cyclosporine can also lead to kidney failure necessitating dialysis or transplantation. Due to these disadvantages, as well as the prohibitive cost and

inconvenience of perpetual immunosuppression, many transplant recipients eventually become partially or fully non-adherent with their treatment, placing their donor organs at risk of rejection (Scheel et al. 2018, Cossart et al. 2019, Al-Sheyyab et al. 2019). Even in patients who adhere closely to their immunosuppression regimens, chronic rejection associated with vascular damage and fibrosis can lead to failure of the donor organ (Moreau et al. 2013, Demetris et al. 1997). Further, due to the shortage of donor organs and immune sensitization of the recipient during rejection of the first transplant, re-transplantation is frequently not possible. Thus, improvements upon current immunosuppression protocols are needed and would vastly improve clinical outcomes and quality of life for transplant recipients.

An attractive alternative to lifelong non-specific immunosuppression is the establishment of permanent donor-specific immune tolerance in the recipient prior to or at the time of transplantation. The concept of immunological tolerance was first proposed by Burnet, who hypothesized that to avoid autoimmunity, the immune system learns to discriminate in utero between "self" molecules expressed by the host and exogenous "non-self" molecules, resulting in specific non-responsiveness to self, or self-tolerance (Burnet 1941). Shortly thereafter, Owen published the first observations supporting this theory in nature. He found that "freemartin" fraternal twin cattle that share a placenta, and thus are exposed to each other's blood in utero, were almost always of the same blood type, despite random assortment of other genes. He later determined that freemartin cattle did not, in fact, express the same blood group antigens. Instead, their immune systems were non-reactive not only against their own blood type but also the blood type of their twin. He hypothesized that this was the result of exposure to the twin's blood in utero (Owen 1945). Billingham, Brent and Medawar later tested this hypothesis experimentally by inoculating mice with cells from other mouse strains in utero and later testing their reactivity

against the donor strain. Indeed, they found that as adults, the inoculated mice accepted skin grafts from the strain to which they had been exposed in utero, but rejected skin grafts from third party strains (Billingham, Brent, and Medawar 1956, 1953). As the molecular and cellular mechanisms inducing and maintaining self-tolerance were discovered in the ensuing decades, it became evident that the generation of self-tolerance was not limited to fetal development. Together, these findings supported the hypothesis that tolerance is an adaptive process that can be induced by exposure to antigen under tolerogenic circumstances and formed the basis for the therapeutic induction of tolerance.

Whereas self-tolerance refers to a lack of immune reactivity specifically towards self-antigens, donor-specific tolerance is a state where the recipient's immune system is trained not to respond to antigens within the donor organ, even after immunosuppression is stopped, while maintaining the ability to respond to third party antigens. Thus, by definition and in contrast to lifelong conventional immunosuppression, only a short period of treatment would be required to establish a tolerant state. This ensures that tolerant patients would not endure the complications or inconvenience associated with long-term immunosuppressant use. Critically, antigen-specificity of therapeutic tolerance would allow transplant recipients to respond normally to infections and tumors eliminating some of the largest causes of morbidity and mortality in transplant recipients. Finally, establishing a robust and stable state of donor-specific tolerance would eliminate acute rejection episodes due to periods of inadequate immunosuppression, which are thought to contribute to chronic rejection (Almond et al. 1993). Because generating donor-specific tolerance would vastly improve upon the disadvantages associated with conventional immunosuppression, a great deal of research has been devoted to the therapeutic induction of tolerance. However, as will be discussed in later sections, treatments to induce

donor-specific tolerance have thus far been more successful in animal models than in humans. A better understanding of the mechanisms inducing, maintaining and disrupting immune tolerance is needed to translate these findings to clinical transplantation,

While donor-specific tolerance has been difficult to achieve therapeutically in the clinic, some patients maintain stable graft function for years after stopping immunosuppression for medical indications or non-compliance, representing a proof of concept that clinical transplant acceptance in the absence of immunosuppression is possible in humans. Because these patients are defined clinically by the maintenance of graft function, rather than direct measures of immune reactivity to donor antigens, they are referred to as operationally tolerant. Indeed, operational tolerance may represent active immunological tolerance using mechanisms similar to those maintaining self-tolerance, but operational tolerance can also be explained by ignorance of the graft by immune cells, in the absence of inflammation. The latter state is expected to be less stable, as infection or injury in the graft would elicit a fully competent immune response to the new antigenic threat, which may awake a dormant immune response to the transplanted organ. It is also possible that operational tolerance is a heterogenous phenomenon with different mechanisms promoting graft survival. This would explain the wide variation in long-term graft survival across operationally tolerant patients (Massart et al. 2016, Orlando et al. 2010). In experimental models, donor-specific tolerance is observed as spontaneous acceptance of a graft from the strain to which the recipient was tolerized and rejection of third-party grafts. While it is not possible to perform such assays to identify true donor-specific tolerance in human transplant recipients, research is underway to identify biomarkers predicting operational tolerance in transplant hosts. Importantly, many of these biomarkers are direct measures of donor-specific immune responsiveness (Heidt and Wood 2012).

Operational tolerance is most frequently observed in liver transplant recipients, likely due to tolerogenic properties of the liver. Indeed, an estimated 20-30% of liver transplant recipients are expected to maintain stable graft function after cessation of immunosuppression (Sanchez-Fueyo 2013, Levitsky and Feng 2018). While it is a rare phenomenon, kidney transplant recipients may also experience operational tolerance (Massart et al. 2016, Orlando et al. 2010). Importantly, *ex vivo* assays have indicated that operationally tolerant kidney transplant recipients have reduced immune reactivity to donor antigens relative to third party antigens (Haynes et al. 2012, Sagoo et al. 2010). Spontaneous operational tolerance has never been reported in heart or lung transplant recipients in the clinic, organs which are more immunogenic than the liver and kidney in mouse models of transplantation (Zhang et al. 1996, Madariaga, Kreisel, and Madsen 2015).

Therapeutic induction of donor-specific immune tolerance would eliminate major complications associated with conventional immunosuppressants. The existence of operationally tolerant patients implies that long-term graft acceptance can be achieved without immunosuppression. While the exact cellular mechanisms preventing rejection in these patients are not well understood, there are correlations between tolerance and hypo-responsiveness specifically to donor antigens, suggesting that operational tolerance is associated with donor-specific tolerance. Biomarkers to identify tolerant patients who can be prospectively weaned from immunosuppression should vastly improve quality of life for those patients. However spontaneous tolerance is rare; thus, therapies to induce donor-specific tolerance are needed. Importantly, many treatments under investigation for the therapeutic induction of tolerance act by co-opting existing mechanisms that have evolved for self-tolerance. The following section

will describe mechanisms known to contribute to self-tolerance to establish a basis for the discussion of tolerance induction therapies under investigation.

Mechanisms that have evolved to maintain self-tolerance

Random generation of lymphocyte surface receptors necessitates mechanisms of self-tolerance

To generate specific adaptive immune responses against diverse pathogen- and tumor-derived antigens, T and B lymphocytes in vertebrates rearrange germline-encoded surface receptor components. Engagement of a surface receptor by an antigen results in activation of the lymphocyte and, when combined with proinflammatory signals from the cell environment and innate immune system, initiates an immune response directed towards that antigen. That generation of TCR and BCR sequences is stochastic rather than directed towards defined antigens allows specificity for new antigens that could arise through encounter with a pathogen or mutation in a tumor cell. Conversely, to instruct TCR or BCR rearrangement based on a set of known target antigens would place selective pressure on tumors and pathogens to mutate or down-regulate expression of these antigens to evade detection. The immense size and diversity of TCR and BCR repertoires make this mechanism of immune evasion much less likely. However, uninstructed generation of TCR and BCR specificities risks generating receptors specific for irrelevant targets or molecules endogenous to the healthy individual, known as self. Generating tolerance to self within this diverse immune system is thus an essential and formidable task. Self-tolerance is accomplished using several mechanisms divided into two main stages: central tolerance, which culls developing lymphocytes with self-reactive surface receptors before they have the potential to mount an immune response, and peripheral tolerance,

which acts on mature self-reactive lymphocytes that have evaded the mechanisms of central tolerance. The stochastic generation of TCR and BCR sequences also results in the production of allospecific lymphocytes specific for non-self antigens expressed within a donor organ. Allospecific T cells do not cause harm in non-transplanted individuals as they do not encounter their cognate antigen, thus there is no selective pressure driving the evolution of mechanisms to control or remove them. Indeed, studies have estimated that 1-30% of T cells in a naïve B6 mouse are reactive to directly or indirectly presented BALB/c-derived antigens (Suchin et al. 2001). Allospecific lymphocytes are required to reject a donor organ (Moreau et al. 2013), thus immunosuppression for transplant recipients typically targets lymphocytes, though in a non-antigen specific manner. Strategies for inducing donor-specific tolerance are meant to only eliminate responses by allospecific lymphocytes, leaving all other lymphocytes unaffected. Due to their shared requirements for suppressing immune responses against only some antigens, most treatments for the induction of donor-specific tolerance utilize mechanisms that evolved for central and peripheral self-tolerance. Thus, it is worth beginning with a discussion of how self-tolerance mechanisms operate under physiological conditions before elaborating on specific treatments for the establishment of donor-specific tolerance.

Central tolerance of T and B cells

Developing T cells are subjected to central tolerance in the thymic medulla. Once a thymocyte rearranges its TCR gene locus, the translated TCR is expressed on the cell surface and tested for reactivity against self-antigen-MHC complexes presented on thymic epithelial cells and dendritic cells (DCs). Developing thymocytes must first experience a minimal quantity of TCR signaling in the thymic cortex to survive, a process known as positive selection, which is thought to select against cells expressing TCRs that are structurally unstable or unable to recognize peptide:MHC

complexes (pMHC). While weak signaling through the TCR allows developing thymocytes to survive positive selection, cells with a strong response to self-pMHC must be removed from the T cell repertoire to prevent autoimmunity. Central tolerance, or the control and removal of self-reactive T cells, occurs in the thymic medulla. Positively selected thymocytes travel to the thymic medulla where they encounter antigen presenting medullary thymic epithelial cells (mTECs) and DCs. Most cells that experience strong TCR signaling in response to self-pMHC undergo apoptosis (Vrisekoop et al. 2014). An alternative fate is possible for CD4 single positive thymocytes with intermediate self-reactivity, by which they may up-regulate the transcription factor (TF) FoxP3 and develop into regulatory T cells (T_{REGs}) with immune suppressive function. The development of thymic T_{REGs} is thought both to promote a dominant mechanism of self-tolerance in the periphery, and to divert potentially self-reactive T cell clones from maturing and later developing into conventional $CD4^+$ T cells (T_{CONVs}) with potentially destructive effector functions (Xing and Hogquist 2012). While there is evidence that negative selection can occur in the thymic cortex (McCaughtry et al. 2008), the medulla has specific features that maximize the diversity of self-antigens presented to thymocytes and chemotaxis of thymocytes to the medulla appears necessary for preventing autoimmunity (Nitta et al. 2009, Kurobe et al. 2006). Unique to mTECs is expression of the transcriptional regulators Aire and Fezf2, which drive the expression of genes typically restricted to non-thymic tissues so that thymocytes reactive to tissue-restricted antigens can undergo central tolerance. Highlighting the importance of these molecules in generating self-tolerance, deficiency in either Aire or Fezf2 results in widespread autoimmunity (Anderson et al. 2002, Peterson et al. 1998, Takaba et al. 2015).

Developing B cells also undergo a process of central tolerance, which occurs in the bone marrow. B cells that have rearranged their BCR locus into a functional surface receptor are then

tested for autoreactivity. Random generation of BCR sequences frequently produces autoreactive B cell clones. Indeed, cloning of antibodies from developing B cells of healthy human donors revealed that greater than 75% of early B cell precursors expressed autoreactive BCRs (Wardemann et al. 2003). There are multiple potential outcomes for a developing B cell that signals strongly in the bone marrow. First, it can re-activate the BCR rearrangement machinery to potentially convert the autoreactive BCR sequence to an innocuous one, a process known as receptor editing. While there is controversial evidence that a similar process occurs in thymocytes (Mostoslavsky and Alt 2004, Kreslavsky et al. 2013), much more is known about the mechanism of receptor editing in B cells, where it clearly plays an important role in shaping the mature BCR repertoire. Alternatively, autoreactive B cells can be deleted (Nemazee 2017). B cell central tolerance does not come close to achieving perfect deletion of autoreactive B cells. Indeed, among 40% of BCRs sequenced from newly emigrated B cells were found to be autoreactive (Wardemann et al. 2003). These cells may remain ignorant in the periphery, or they may be tolerized through further deletion, receptor editing or anergy. T cell tolerance is also critically important for controlling autoreactive B cells in the periphery.

Generation of a germinal center response for B cell class-switching and somatic hypermutation requires help from activated $CD4^+$ T cells that recognize their cognate pMHC presented by the B cell. The peptides presented by B cells are typically generated by processing the antigen bound by the BCR, thereby linking the B cell specificity to the specificity of the helper T cell. In transplantation, acute and chronic graft injury tends to be associated with donor-specific IgG, whose production requires a germinal center response (Karahan, Claas, and Heidt 2017). Because effective tolerization of T cells should preclude such T cell-dependent B cell

responses, many strategies for inducing donor-specific tolerance focus on tolerizing allospecific T cells.

Thymic tolerance is not completely effective at eliminating self-reactive T cell clones, necessitating additional mechanisms to suppress autoimmunity. While TCR reactivity to self-antigen clearly contributes to T cell fate during thymic selection (Moran et al. 2011), not all self-reactive T cells are deleted or diverted into the T_{REG} population. Even among monoclonal thymocytes, individual T cells achieve varied fates (Bautista et al. 2009, Malchow et al. 2016). The overall proportion of thymocytes developing into T_{REGs} is dependent on TCR sequence as well as clonal frequency due to a limited T_{REG} developmental niche for each T cell clone (Bautista et al. 2009, Moran et al. 2011, Malchow et al. 2013). T cells with lower affinity for self-antigen may also present a challenge for central tolerance, as these cells signal only weakly in the thymus but may still mount an autoimmune response under some physiological conditions, for example, during an infection (Enouz, Carrié, et al. 2012).

Self-reactive T cells that escape central tolerance and enter the periphery as naïve cells are controlled by several peripheral tolerance mechanisms. Here, the discussion of peripheral tolerance will encompass all extrathymic mechanisms preventing a productive effector response by T cells. In addition to controlling self-reactive T cells, peripheral tolerance also plays a critical role in promoting maternal-fetal tolerance, colonization by commensal microbes, and lack of response to dietary antigens since fetal, commensal and diet-derived antigens are not endogenous to the host and thus cannot be expressed by thymic APCs.

Antigen presenting cells tolerize T cells in the periphery

In contrast to thymocytes, where T cell activation is generally undesirable, mature T cells require activation to perform their effector functions. Thus, differentiating between helpful and autoreactive T cell responses becomes more challenging in the periphery. This becomes a task for the DCs that initiate, or prime, a response by naïve T cells in the secondary lymphoid organs. DCs sense infection and tissue damage in their environment, which signal that an immune response is needed. The DC relays that information to the T cells that recognize pMHC on the DC surface by providing additional signaling through activating "co-stimulatory" molecules. An important consequence of MHC restriction of the TCR is the linkage between TCR signaling and these secondary co-signals during a T cell-DC interaction. Under some circumstances the DC and T cell may also express co-inhibitory surface molecules, whose interaction suppresses T cell priming. The role of co-inhibitory receptors during homeostasis is not completely understood, though they appear to play a role in limiting the size and duration of productive immune responses, likely limiting collateral damage to tissue during the response, and also preventing the priming of autoimmune responses. Overall, the balance of co-stimulatory and co-inhibitory signals delivered to the T cell determine whether it will mount a productive immune response or become tolerized. The regulation and molecular mechanisms of co-signaling pathways will be discussed below, followed by a discussion of the immunological consequences of T cell interaction with a stimulatory or tolerogenic DC.

A resting DC does not express sufficient co-stimulatory signals to activate a T cell response. To up-regulate surface expression of co-stimulatory molecules, the DC must be activated. DC activation occurs downstream of pattern recognition receptors (PRRs) that bind evolutionarily conserved molecules expressed by microbes and damaged tissue. PRR signaling

induces the DC to up-regulate antigen uptake, processing and presentation as well as expression of co-stimulatory molecules and pro-inflammatory cytokines. Microbe-derived PRR ligands called pathogen-associated molecular patterns (PAMPs) include, for example, bacterial cell membrane components and microbe-specific nucleic acid structures such as double stranded ribonucleic acid (RNA) (Amarante-Mendes et al. 2018). PRR ligands can also be endogenous molecules expressed by damaged tissue. Among these damage-associated molecular patterns (DAMPs) are stress-induced molecules such as heat shock proteins as well as molecules in abnormal cellular compartments, such as cytoplasmic deoxyribonucleic acid (DNA) and extracellular adenosine triphosphate (ATP) (Gong et al. 2020). Infections associated with tissue damage can result in a combination of PAMP and DAMP production (Cunha et al. 2012). Because organ transplantation typically involves sterile organs and is performed under aseptic conditions, PRR signaling after transplantation is mainly driven by DAMPs. Indeed, the ischemia-reperfusion injury (IRI) that occurs after transplantation generates many DAMPs. Purposely prolonged ischemia in mouse models of transplantation, as well as the enhanced ischemia associated with transplantation from a deceased versus living donor also result in greater production of DAMPs and are associated with an enhanced alloimmune response (Farrar, Kupiec-Weglinski, and Sacks 2013, Mori et al. 2014). Deficiency in myeloid differentiation primary response 88 (MyD88), an adaptor mediating signaling by some PRRs, can prolong the survival of minor-mismatched grafts significantly (Goldstein et al. 2003).

PRR signaling results in up-regulation of co-stimulatory molecules, allowing productive priming of T cells. The interaction between CD28 and its ligands CD80 and CD86 is one of the best studied co-stimulatory pathways. Briefly, CD28 expressed by the T cell can bind either CD80 or CD86, resulting in a signaling cascade within the T cell that both enhances TCR

signaling and also activates the TFs nuclear factor of activated T cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1), ultimately promoting T cell proliferation, differentiation, survival and memory formation (Esensten et al. 2016). An important outcome of CD28 signaling is production of IL-2 by the stimulated T cell, which acts in an autocrine manner to promote proliferation and differentiation (Fraser et al. 1991). Experiments using a CD28-Ig fusion protein have also indicated that signaling occurs within the DC downstream of CD80 and CD86 ligation, resulting in the production of inflammatory cytokines. However, the significance of this signaling mechanism *in vivo* is not yet understood (Orabona et al. 2004). Importantly, CD28 signaling results in the up-regulation of a second group of co-stimulatory molecules on the T cell, including inducible T cell co-stimulator (ICOS), CD154 and OX40. These co-stimulatory molecules enhance activation downstream of the TCR and also influence the differentiation of the primed T cell into different effector subsets (Chen and Flies 2013). For example, ICOS and OX40 promote the formation of follicular helper T cells (T_{FH}), which provide help to B cells for germinal center formation (Tahiliani et al. 2017, Walker et al. 1999). While some T cell responses can occur in the absence of CD28 signaling, CD28-deficient mice show significant impairments in antibody production and T cell proliferation, indicating that other co-stimulatory pathways cannot fully compensate for loss of CD28 in naïve T cells (Shahinian et al. 1993, Green et al. 1994). Because it has non-redundant functions and up-regulates expression of other co-stimulatory molecules, CD28 is thought to act upstream of other co-stimulatory pathways. For that reason, therapeutic manipulation of co-stimulation is often directed towards the CD28-CD80/CD86 axis.

Activated DCs can further up-regulate expression of co-stimulatory molecules following interactions with activated T_{CONVs} through a process known as DC licensing. DC licensing is

activated by interaction between CD40 expressed constitutively by DCs and CD154 expressed on activated T_{CONVs}. CD40-CD154 binding results in enhanced expression of co-stimulatory molecules such as CD80 and CD86, and production of cytokines such as IL-12 by the DC, ultimately promoting T cell priming and differentiation (Caux et al. 1994, Cella et al. 1996). DC licensing by T_{CONVs} is critical for generating optimal primary and memory $CD8^+$ T cell responses (Laidlaw, Craft, and Kaech 2016). Further, agonist anti-CD40 treatment can restore cytotoxicity by $CD8^+$ T cells primed by MHC II-deficient DCs, indicating that CD40 can compensate for a lack of help by T_{CONVs} (Ridge, Di Rosa, and Matzinger 1998).

Alternatively, co-inhibitory signaling can suppress T cell activation, often by interfering directly with TCR and co-stimulatory signaling. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) was one of the first co-inhibitory molecules discovered; thus, a relatively large amount is known about the molecular mechanisms of CTLA-4-mediated T cell inhibition. By binding the co-stimulatory molecules CD80 and CD86 on the APC surface with roughly 10-fold greater affinity than does CD28 (Sansom 2000), CTLA-4 acts as a competitive inhibitor of T cell priming. Further, CTLA-4-binding can induce the removal of CD80 and CD86 from a target APC followed by endocytosis and degradation of CD80 and CD86 within the CTLA-4-expressing T cell, a process known as trogocytosis (Qureshi et al. 2011, Chen and Flies 2013). Some co-inhibitory molecules, including CTLA-4 as well as programmed cell death 1 (PD-1) and T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory domains (TIGIT) recruit inhibitory phosphatases to the immunological synapse, where they deactivate signaling molecules downstream of the TCR and co-stimulatory receptors by dephosphorylation (Chen and Flies 2013). Lymphocyte activation gene 3 (LAG-3) is a co-inhibitory molecule with homology to CD4. While LAG-3 does bind MHC Class II, the

importance of MHC Class II as a ligand for LAG-3 is under debate (Maruhashi et al. 2018). Fibrinogen-like Protein 1 was recently identified as a LAG-3 ligand and likely plays a more significant role than MHC Class II in LAG-3-mediated suppression. Indeed, Fibrinogen-like Protein 1 was required for LAG-3-mediated suppression of anti-tumor immunity in mice (Wang et al. 2019). Several other co-inhibitory molecules have been identified, though the molecular mechanisms by which they suppress immunity are not well understood. Some of them likely utilize mechanisms similar to those described for CTLA-4, PD-1, TIGIT and LAG-3 (Chen and Flies 2013). It is important to note that expression of co-inhibitory receptors is not limited to T cells. PD-1 in particular has also been described in B cells and myeloid cells, where it suppresses an immune response by these cells (Zhu, Yao, and Chen 2011, Chen and Flies 2013).

Co-signaling is largely regulated by surface expression of co-signaling receptors and ligands. In some cases, both binding partners must be up-regulated prior to signaling. For other co-signaling pathways, one binding partner is expressed constitutively while the other is regulated. For example, CD28 is expressed constitutively by T cells whereas CD80 and CD86 expression is up-regulated by the DC after activation. Thus, up-regulation of CD80 and CD86 after DC activation is the limiting step for this co-signaling pathway. Further, CD86 is expressed before CD80, suggesting that it is more important for initiation of T cell priming. Other co-stimulatory molecules are up-regulated only after CD28 signaling. Finally, co-inhibitory molecules are often up-regulated by the T cell after activation. The asynchronous kinetics of co-signaling molecule expression has been referred to as tide-like, continuously rising towards a peak as more co-stimulatory molecules are expressed, and then regressing as co-inhibitory signaling increases and co-stimulatory molecules are deactivated (Zhu, Yao, and Chen 2011).

Up-regulation of surface co-signaling molecule expression is accomplished by increasing rates of transcription, translation and trafficking to the cell surface. Indeed, DC activation is accompanied by many changes to transcription, including at loci encoding co-stimulatory receptors (Brown et al. 2019). Termination of co-signaling can occur through a combination of reduced transcription as well as proteolytic degradation of the binding partners. Many co-signaling molecules, including CD28 and PD-1 are internalized and degraded by the proteasome following signaling (Linsley et al. 1993, Eck et al. 1997, Meng et al. 2018). Trogocytosis by CTLA-4 aids the removal of CD80 and CD86 from the cell surface, further contributing to the termination of CD28-CD80/CD86 signaling (Qureshi et al. 2011). Interestingly, LAG-3 signaling is terminated via cleavage of its external domain by metalloproteases (Andrews et al. 2017). Post-translational modifications such as glycosylation may also modulate co-signaling molecule activity, as appears to be true for LAG-3 (Chen and Flies 2013).

Integrating co-stimulatory and co-inhibitory signaling is a complex task, especially because different co-signaling molecules are expressed asynchronously and may bind multiple ligands. While it is not yet possible to predict the outcome of a given T cell-DC interaction based on the expression of co-stimulatory signals, it does appear that the decision between a productive or tolerogenic interaction generally relies on the balance of co-stimulatory and co-inhibitory signals. Successful priming of a T cell response requires the DC to have been activated by encountering DAMPs and/or PAMPs, signaling the need for a protective immune response. On the other hand, antigens presented in the absence of a pathogen or tissue damage are likely to be self-antigens, which should not elicit an immune response. Importantly, naïve T cells that signal without adequate co-stimulation do not remain naïve. As will be discussed in the following

section, these cells are tolerized through deletion or the development of cell-intrinsic dysfunction.

T cell-intrinsic mechanisms of peripheral tolerance: deletion and dysfunction

T cell apoptosis can be induced in the periphery under several circumstances. T cells interacting with DCs that are resting or supply greater co-inhibitory than co-stimulatory signals may undergo deletion. This is thought to occur due to a reduction in pro-survival signals downstream of TCR signaling. Co-stimulation may provide additional pro-survival signals to replace those that are lost, but in the absence of co-stimulation, the T cell will undergo apoptosis. Additionally, repeated T cell stimulation or particularly strong TCR signaling may also induce T cell death. These mechanisms add to the clonal deletion experienced by T cells in the thymus (Van Parijs, Ibraghimov, and Abbas 1996). Finally, T cells undergo widespread cell death during the contraction phase of a productive immune response, though this is not a tolerogenic process since a memory response will persist after the contraction of the effector phase and promote, rather than inhibit, a secondary immune response upon re-stimulation with the same antigen.

Two molecular pathways of apoptosis contribute to clonal deletion of T cells in the periphery: the "intrinsic" apoptotic pathway dependent on B cell lymphoma 2-interacting mediator of cell death (Bim) and a pathway dependent on death receptors. Briefly, Bim is a pro-apoptotic molecule that promotes the release of cytochrome-c from mitochondria, resulting in activation of caspases, the effector molecules that carry out the process of apoptosis. In contrast, the death receptor-dependent apoptotic pathway is initiated by interaction between a surface death receptor such as Fas with its ligand. As a result, the death receptor activates a signaling cascade that results in caspase activation. The Bim-dependent and death receptor-dependent

pathways do not converge until the activation of caspases, at which point apoptosis is inevitable, thus the two pathways are regulated independently (Galluzzi et al. 2018). Bim- and death receptor-dependent signals both appear to be involved in peripheral T cell death. Studies have suggested that T cell death due to inadequate co-stimulation or cytokine starvation is mediated specifically by Bim, whereas apoptosis due to repeated TCR signaling is more likely the result of death receptor activation (Green, Droin, and Pinkoski 2003). Regardless of the apoptotic pathway involved, clonal deletion of T cells in the periphery significantly shapes the T cell repertoire.

T cells that survive TCR stimulation without adequate co-stimulation become intrinsically dysfunctional, a state known as anergy. Anergy was first described in T cell clones stimulated *in vitro* by chemically fixed APCs, which cannot up-regulate co-stimulatory molecules. T cells primed under these conditions were unable to proliferate in response to re-stimulation by viable APCs, indicating that they had become intrinsically dysfunctional (Jenkins and Schwartz 1987). Stimulation with pMHC, anti-CD3 or the T cell mitogen concanavalin A alone yielded similar results (Schwartz et al. 1989). Later, it was determined that co-stimulation through CD28 could prevent the development of anergy, likely by enabling the T cell to produce IL-2 (Schwartz 2003, Harding et al. 1992). Stemming from these initial findings, a large amount of research over the last 20 years has investigated the molecular mechanisms and functional consequences of T cell dysfunction.

In vivo, self-reactive T cells also become dysfunctional, a state which is sometimes referred to as anergy, but which may be heterogenous and fairly distinct from the anergic state observed *in vitro* (Schwartz 2003). In common between the anergic states induced *in vitro* and *in vivo* is a lack of proliferation and effector response upon re-stimulation. These are associated

with multiple impairments in proximal TCR signaling, a reduction in effector gene expression and an up-regulation of genes associated with T cell inhibition. These changes are thought to result from unique gene targeting by TCR-activated NFAT when its binding partner AP-1 has not been up-regulated by co-stimulation (Wells 2009, Macián et al. 2002). Anergy is also associated with epigenetic modifications, which likely explain how self-reactive T cells that are made temporarily functional by transfer into lymphopenic hosts eventually revert to a dysfunctional state (Schietinger et al. 2012).

Dysfunction has also been described in T cells responding to tumors and infections. T cells responding to a chronic viral infection are thought to become "exhausted" as a result of persistent stimulation. Thus, despite the persistence of these virus-specific T cells, they fail to eliminate the infection. Exhaustion appears to worsen over time, with the gradual loss of effector cytokine production, potential to proliferate and increased expression of co-inhibitory molecules. Despite expression of CD44, indicating antigen experience, exhausted cells do not differentiate into long-lived memory cells and fail to express memory cell markers. Importantly, the initial phases of T cell exhaustion appear to be reversible whereas later stages are epigenetically enforced. Exhausted cells also require persistent TCR activation to survive since, unlike naïve or memory T cells, exhausted T cells do not respond to the survival cytokines IL-7 or IL-15 (Wherry and Kurachi 2015). A similar phenotype and functional profile is seen in T cells in the tumor microenvironment (Thommen and Schumacher 2018).

Recently, the TF thymocyte-associated high mobility group box protein (TOX) has been implicated as a driver of T cell exhaustion. TOX is up-regulated by NFAT signaling and is required for the up-regulation of co-inhibitory molecule expression in T cells responding to chronic viral infection (Khan et al. 2019). However, another study found that expression of TOX

was neither required nor sufficient for the development of an impaired effector response. This finding calls into question the importance of co-inhibitory molecules in driving the dysfunction of exhausted cells (Scott et al. 2019).

A third category of T cell-intrinsic dysfunction is senescence. As in other hematopoietic and non-hematopoietic cells, T cell senescence describes a state where cellular replication has shortened telomeres to the extent that further replication is not possible. T cell senescence is associated with low expression of CD28 and up-regulation of CD57 (Bellon and Nicot 2017, Frimpong et al. 2019). T cell senescence is associated with repeated T cell stimulation, chronic viral infection and old age. However, in contrast to T cell exhaustion or anergy, senescent T cells retain the ability to produce effector cytokines. Inability to proliferate upon re-challenge is the main defect apparent in senescent cells (Chou and Effros 2013).

Self-reactive T cells in the periphery appear to be controlled by a combination of clonal deletion and development of dysfunction. While the characterization of different forms of T cell dysfunction may suggest that there is a discrete number of distinct dysfunctional states, a single autoreactive T cell is likely to experience a combination of signals associated with multiple types of dysfunction. For example, autoreactive T cells are likely to signal chronically and without co-stimulation. Further research is needed to determine whether such combinations of dysfunction-inducing inputs result in a T cell state that is closer to exhaustion, anergy, senescence or another state altogether and which tolerogenic signals induce particular functional and phenotypic changes. Importantly, T cell deletion and dysfunction are both mechanisms that act in a cell-intrinsic fashion, thus they represent recessive forms of tolerance. The following section will discuss mechanisms exerting dominant tolerance upon T cells by suppressing of T cell priming and effector function in a cell-extrinsic manner.

T cell-extrinsic mechanisms of peripheral tolerance: suppression

Inhibition of immunity by suppressive T cells is a key component of peripheral tolerance and plays an important role in many strategies for induction of donor-specific tolerance. Suppressive T cells, though long hypothesized to exist, were first observed in 1995 when Sakaguchi and colleagues identified CD25 as a marker for a $CD4^+$ T cell population highly enriched for suppressive T cells (Sakaguchi et al. 1995). Later, FoxP3 was identified as a more specific marker for suppressive T cells, as CD25 is also expressed on recently activated T_{CONVs} in mice, and studies of FoxP3 function revealed that it is responsible for programming many of the suppressive functions found in T_{REGs} (Fontenot, Gavin, and Rudensky 2003, Marson et al. 2007). The importance of regulatory T cells can be seen in FoxP3-deficient "scurfy" mice or in mice expressing the diphtheria toxin receptor (DTR) under control of the FoxP3 promoter ($FoxP3^{DTR}$), where injection of diphtheria toxin (DT) selectively depletes $FoxP3^+$ cells. In these mouse models, the constitutive absence or induced depletion of $FoxP3^+$ T_{REGs} results in severe systemic autoimmunity (Kim, Rasmussen, and Rudensky 2007, Brunkow et al. 2001). More recent studies have identified other suppressive T cell populations, such as $FoxP3^-$ $CD4^+$ and $CD8^+$ T cells displaying suppressive activity as well as $CD8^+$ $FoxP3^+$ T cells (Flippe et al. 2019, Guillonneau, Picarda, and Anegon 2010, Gregori, Goudy, and Roncarolo 2012). This section will focus primarily on $CD4^+$ $FoxP3^+$ T_{REGs}, which are the most abundant and have been most thoroughly studied among the suppressive T cell subsets.

T_{REGs} are generated in the thymus (tT_{REGs}) as a result of intermediate self-reactivity, or they can be induced from naïve $CD4^+$ T cells in the secondary lymphoid organs (iT_{REGs}) upon stimulation in the presence of T_{REG} differentiation promoting factors such as TGF-β, IL-10 and retinoic acid. Both T_{REG} subsets can be expanded in the periphery upon antigen encounter (Plitas

and Rudensky 2016). Helios and neuropilin-1 have been used as markers to identify tT_{REGs} found in the periphery and differentiate them from iT_{REGs}, though studies have shown that these markers are not exclusive to tT_{REGs} (Szurek et al. 2015, Singh et al. 2015). While they differentiate in distinct organs, there are many similarities in the mechanisms of iT_{REG} and tT_{REG} differentiation and immune suppression. Optimal generation of both iT_{REGs} and tT_{REGs} requires the transcription factors forkhead box O1 and forkhead box O3 (Kerdiles et al. 2010) as well as dynamic expression of the chromatin organizer special AT-rich sequence binding protein 1 (Beyer et al. 2011, Kitagawa et al. 2017). However, there are also key distinctions between iT_{REGs} and tT_{REGs}. Activity in conserved non-coding sequence (CNS) 1 within the FoxP3 locus is necessary for iT_{REG} induction but dispensable for tT_{REG} differentiation. That CNS1 contains a SMAD binding site highlights the importance of TGF-β signaling in iT_{REG} induction (Zheng et al. 2010). Importantly, the TCR repertoires in iT_{REGs} and tT_{REGs} are largely distinct (Pohar, Simon, and Fillatreau 2018). tT_{REGs} are mainly thought to be specific for self-antigens encountered in the thymus and therefore, they primarily suppress autoimmune responses. Conversely, iT_{REGs} are derived from naïve T cells that left the thymus without exhibiting autoreactivity. iT_{REGs} are therefore thought to be mainly specific for environmental antigens whose presentation is limited to the periphery. This explains why CNS1-deficient mice specifically lacking iT_{REGs} experience inflammation localized at barrier sites (Josefowicz et al. 2012).

T_{REGs} have traditionally been thought to suppress immunity using 3 general mechanisms: secreting inhibitory cytokines, expressing inhibitory surface molecules and competing for survival and stimulation factors. The following sections will begin with a description of these three classical T_{REG} suppressive mechanisms, then expand to more recently discovered and less

well appreciated mechanisms followed by discussions of the importance of multiple suppressive mechanisms and T_{REG} subtypes in providing optimal suppression of deleterious immune responses. In the following survey of the functions of T_{REGs}, a broader role will be evident in the suppression of other classes of innate and adaptive immune cells and in the maintenance of tissue homeostasis in colonized and non-colonized tissues. Because T cell activation is required to trigger auto- or alloimmunity in the absence of T_{REGs}, T cell priming will remain the primary focus of this section, but it is important to acknowledge the potential that other cells having an independent interplay with T_{REGs} may also participate in the immune response or return to homeostasis. While there is evidence that T_{REGs} can suppress responses by previously activated cells *in vitro*, they are much more effective at suppressing the priming of naïve cells in mouse models of transplantation (Yang et al. 2007).

The main inhibitory cytokines known to be secreted by T_{REGs} are TGF-β, IL-10 and IL-35. TGF-β signals through the TGF-β receptor, a dimeric serine/threonine kinase receptor expressed on a wide range of target cells, resulting in activation of the TFs SMAD3 and SMAD4. TGF-β is a pleiotropic cytokine with critical roles in the development and homeostasis of many tissue types. Which genes are regulated by TGF-β depends on the target cell type and context due to differential expression of signal transduction components, inhibitors and co-factors as well as differences in chromatin availability. TGF-β suppresses immunity through several mechanisms including promoting differentiation and function of iT_{REGs} while inhibiting the differentiation of T_{CONV} subsets, suppressing antigen presentation by DCs and inhibiting IFN-γ expression in NK cells (Sanjabi, Oh, and Li 2017, Li, Wan, and Flavell 2007). A non-canonical SMAD-independent TGF-β signaling pathway also exists downstream of the TGF-β receptor, which exerts effects complementary to the canonical signaling pathway, including

changes in target cell metabolism, cytoskeleton organization and generation of extracellular matrix components (Massagué 2012, Hata and Chen 2016). In contrast to TGF-β, the functions of IL-10 and IL-35 described thus far have largely been restricted to immune cells. IL-10 signals through the heterodimeric IL-10 receptor on target cells and activates the TF STAT3. IL-10 signaling in myeloid cells down-regulates production of pro-inflammatory cytokines and reduces antigen presentation. IL-10 also binds T cells directly to promote T_{REG} survival and activity and suppress T_{CONV} responses (Coomes et al. 2017). Finally, IL-35 is a heterodimer consisting of EBI3 and IL-12a, which are also subunits of IL-27 and IL-12, respectively. IL-35 signals via a homodimer or heterodimer of IL-12Rβ2 and gp130, subunits also present in the IL-12 and IL-27 receptors, respectively. Binding of IL-35 to its receptor results in activation of the TFs STAT1 and STAT4, which suppress T cell proliferation and effector function and reduce antibody production (Olson, Sullivan, and Burlingham 2013, Collison et al. 2007).

T_{REGs} also express co-inhibitory ligands such as CTLA-4 and PD-L1, whose functions were described in depth in a previous section. CTLA-4 is constitutively expressed in T_{REGs} though stable surface expression only occurs after T_{REG} activation (Schneider and Rudd 2014). In addition to the mechanisms of CTLA-4-dependent suppression described previously, studies in T_{REGs} have also revealed that CTLA-4 induces expression and augments activity of indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades tryptophan, in DCs. The resulting tryptophan starvation and generation of active metabolites through the kynurenine degradation pathway suppress T cell proliferation, induce cell death and promote differentiation of iT_{REGs} (Fallarino et al. 2003, Grohmann et al. 2002, Fallarino et al. 2006). Importantly, engagement of co-inhibitory receptors such as PD-1 and LAG-3 on the T_{REG} surface can inhibit T_{REG} suppressive function, promoting an immune response (Kamada et al. 2019, Zhang et al. 2017).

Finally, T_{REGs} inhibit effector T cell responses by competing for TCR signaling and survival factors. T_{REGs} were originally identified by their expression of the high affinity IL-2 receptor component CD25, allowing them to compete with T_{CONVs} for IL-2. Indeed, IL-2 deprivation is thought to explain the Bim-dependent apoptosis observed in T_{CONVs} activated in the presence of T_{REGs} (Pandiyan et al. 2007). T_{REGs} may also compete with T_{CONVs} for other critical activating ligands on the APC surface. This was previously exemplified by competitive binding of CD80/86 by CTLA-4. T_{REGs} can also compete for binding of pMHC on the APC surface, preventing recognition by T_{CONVs}. Similar to trogocytosis of CD80/86, T_{REGs} can also remove MHC from the APC surface (Akkaya et al. 2019).

There are several more recently appreciated functions of T_{REGs} as well as interesting hypothesized suppressive mechanisms yet to be studied in depth. A subset of T_{REGs} secretes perforin and granzyme to kill other immune cells including T cells and DCs, resulting in suppression of an immune response (Cao et al. 2007, Grossman et al. 2004, Boissonnas et al. 2010). There is also some evidence for Fas-dependent killing of APCs and $CD8^+$ T cells and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-dependent killing of activated $CD4^+$ T cells by T_{REGs} *in vitro* (Gorbachev and Fairchild 2010, Strauss, Bergmann, and Whiteside 2009, Ren et al. 2007) though it is not yet clear how significant a role these mechanisms play in T_{REG} function *in vivo*. Depletion of T_{REGs} at the time of antigen challenge has long been known to result in an enhanced T cell-dependent antibody response (Sakaguchi et al. 1995), though only recently have T_{REGs} been shown to directly suppress activation of T_{FH} cells and B cells (Sage and Sharpe 2016). T_{REGs} also express CD39 and CD73 on their surface. Together, these enzymes degrade extracellular ATP, which promotes inflammation, into adenosine, which has immune suppressive functions. Indeed, blockade of CD39 or CD73

appears to reduce T_{REG}-mediated suppression (Deaglio et al. 2007, Stagg et al. 2011). While the study of exosome signaling in T cells is in its infancy, secretion of microvesicles, such as exosomes, by T_{REGs} may also provide a mechanism for delivery of multiple extracellular and intracellular suppressive signals as a packaged unit over relatively long distances. Indeed, exosomes derived from T_{REGs} *in vitro* whose contents include CD39, CD73 and microRNAs have been shown to suppress T cell activation *in vitro* and *in vivo* (Okoye et al. 2014, Akkaya et al. 2019). It is hypothesized that similar exosomes are produced by T_{REGs} *in vivo*, though *in vivo* models eliminating exosome production by T_{REGs} are not yet available.

Why is it necessary for T_{REGs} to have many unique mechanisms of suppression? It seems that multiple mechanisms of suppression by T_{REGs} may play somewhat compensatory roles. For example, CTLA-4 deficient T_{REGs} are capable of suppressing colitis induced by adoptive transfer of naïve T cells, but suppression of colitis by CTLA-4 deficient T_{REGs} relies more heavily on IL-10 than suppression by wild type (WT) T_{REGs} (Read et al. 2006). In other contexts, however, there is not adequate compensation for CTLA-4, which is evident in the widespread autoimmunity seen in mice born with global or T_{REG}-specific CTLA-4 deficiency (Tivol et al. 1995, Wing et al. 2008). Thus, different suppressive mechanisms may also play a dominant role in different tissues or physiological contexts. Indeed, some T_{REG}-derived cytokines can play pro- or anti-inflammatory roles depending on the presence of other modulating factors in the environment. Despite its many immune suppressive functions, when in the presence of IL-6, TGF-β drives the development of pro-inflammatory IL-17-producing T_{CONVs} (T_{H17} cells) rather than T_{REGs}. This may explain why TGF-β exacerbates experimental autoimmune encephalomyelitis (EAE) (Gutcher et al. 2011, Bettelli et al. 2006). Similarly, organism-wide and T_{REG}-specific IL-10 or IL-10R deficiency leads to severe colitis, indicating the importance of

its immune suppressive activity (Engelhardt and Grimbacher 2014, Kühn et al. 1993, Rubtsov et al. 2008, Chaudhry et al. 2011) but type 2 cytokines such as IL-4 potentiate pro-inflammatory effects of IL-10 such as promoting the proliferation and activity of mast cells and enhancing B cell activity (Polukort et al. 2016, Thompson-Snipes et al. 1991, Rousset et al. 1992). Further, IL-10 enhances NK cell proliferation, cytotoxicity and IFN-γ production in the presence of type I cytokines (Shibata et al. 1998, Cai, Kastelein, and Hunter 1999). These pro-inflammatory functions may explain why treatment with recombinant IL-10 generally seems more effective at preventing inflammation than reversing inflammatory disorders after their onset and may in fact exacerbate ongoing inflammatory conditions (Couper, Blount, and Riley 2008). Thus, certain suppressive functions may be best suited for particular tissues or undermined by specific cytokines. T_{REGs} may employ multiple suppressive mechanisms both for protective redundancy and to best suit the varied environments across different tissues and inflammatory conditions.

T_{REGs} also contribute to homeostasis in non-lymphoid tissues (Panduro, Benoist, and Mathis 2016). The gastrointestinal tract, skin and lungs are rich in T_{REGs}, which promote colonization by commensal microbes and prevent damage to host tissues during immune responses to microbial and other foreign antigens abundant at these barrier tissues. Ablation of IL-10 results in spontaneous colitis in colonized but not germ-free mice, indicating that IL-10 plays an important role in suppressing inflammation in response to microbes in the intestine. While IL-10 is produced by other cell types in the intestine, ablation of IL-10 specifically in T_{REGs} still results in colitis (Rubtsov et al. 2008, Sellon et al. 1998). In neonatal mice, T_{REGs} infiltrate the skin to establish immune tolerance to commensal microbes. Blocking skin infiltration by T_{REGs} results in expansion of commensal-specific effector T cells and inflammation when the barrier is disrupted by abrasion (Scharschmidt et al. 2015). Systemic

T_{REG} depletion also resulted in increased type 2 immunity and skin fibrosis (Kalekar et al. 2019). Thus, T_{REGs} are critical for maintaining homeostasis in barrier tissues. While colonized and sterile tissues host resident T_{REG} populations under homeostatic conditions, T_{REGs} may also travel to sites of inflammation to mitigate or repair tissue damage during an immune response. For example, after a viral respiratory infection, T_{REGs} infiltrate the lungs and prevent tissue damage (Arpaia et al. 2015, Antunes and Kassiotis 2010).

Sterile tissues such as visceral adipose tissue (VAT) and skeletal muscle also host resident T_{REG} populations, which have distinct transcriptional profiles and are likely to play tissue-specific functional roles. Though mouse models ablating only tissue T_{REGs} or impairing effector functions in tissue T_{REGs} specifically are lacking, several findings already suggest a meaningful role for these cells. Despite their residence in non-lymphoid tissue, VAT T_{REGs} are as effective as splenic T_{REGs} at suppressing T cell responses *in vitro* (Feuerer et al. 2009). VAT and skeletal muscle T_{REGs} express particularly high levels of the suppressive cytokine IL-10, suggesting that these cells suppress inflammation in the tissue. Interestingly, the IL-10 receptor is expressed not only on immune cells in the tissue but also on adipocytes (Lumeng, Bodzin, and Saltiel 2007, Rajbhandari et al. 2018), suggesting a direct functional impact of T_{REGs} on these stromal cells. Indeed, *in vitro* studies suggest that IL-10 signaling may reduce inflammatory cytokine production and promote glucose uptake by adipocytes (Feuerer et al. 2009, Lumeng, Bodzin, and Saltiel 2007) and *in vivo*, IL-10 deficient mice resist age-related weight gain (Rajbhandari et al. 2018). VAT, skeletal muscle and intestinal T_{REGs} also express high levels of the growth factor amphiregulin (Areg), which does not appear to play a role in T_{REG}-mediated immune suppression but does promote tissue healing, for example after an infection or tissue damage (Arpaia et al. 2015, Burzyn et al. 2013). Further supporting a role for T_{REGs} in promoting

homeostasis in the tissue, *in vivo* studies in mice show increased insulin resistance in the VAT and impaired healing of injured skeletal muscle after systemic T_{REG} depletion with anti-CD25 or DT administration in FoxP3^{DTR} mice (Burzyn et al. 2013, Feuerer et al. 2009), though it is difficult to interpret these results as T_{REG} deficiency results in widespread inflammation, which may play a confounding role. More specific depletion models are needed to determine the true impact of tissue-resident T_{REGs} and to study their effect in the absence of the severe autoimmunity and inflammation caused by systemic T_{REG} depletion.

While one study has shown that T_{REGs} can suppress T cell responses *in vitro* in the absence of ongoing stimulation (Szymczak-Workman, Workman, and Vignali 2009), it is well documented that T_{REGs} require stimulation from their cognate pMHC to suppress immune responses *in vivo* (Schmidt, Oberle, and Krammer 2012). Because several mechanisms of T_{REG}-mediated suppression require that the suppressed T cell be activated by the same APC as the activated T_{REG}, T_{REGs} are thought to mainly suppress T cell responses in an antigen-specific manner. For example, suppression due to competition for and trogocytosis of pMHC molecules requires matched pMHC specificity of the T_{REG} and T_{CONV}. On the other hand, other mechanisms of T_{REG} suppression seem only to require that the T_{REG} and T_{CONV} are activated by the same APC, even if they are specific for distinct pMHC complexes, a phenomenon known as linked suppression (Qin et al. 1993). For example, BALB/c mice tolerized to B6 heart allografts rapidly reject secondary C3H allografts but can accept F1 (C3H x B6) allografts long-term. In this case, spontaneous tolerance to C3H antigens is linked to the previously induced tolerance to B6 antigens (Li et al. 2008). Finally, T_{REGs} mediate "infectious tolerance" by secreting TGF-β and other factors that induce naive CD4$^+$ T cells to differentiate into iT_{REGs} during priming (Belladonna et al. 2009). Infectious tolerance between T_{REGs} and T cells with APC-linked but not

identical pMHC specificities also provides a means to expand upon the epitopes recognized within the T_{REG}, similar to epitope spreading in effector T cells (Yang et al. 1999).

Importantly, there is functional heterogeneity in the T_{REG} population. For example, recent work determined that the suppressive cytokines IL-10 and IL-35 are made by largely distinct subsets of T_{REGs} and that these cytokines make non-redundant contributions to the suppression of anti-tumor immunity (Sawant et al. 2019). In the lymph nodes and spleen, only a subset of T_{REGs} expresses CXCR5, which is necessary for them to traffic to the B cell follicle and suppress germinal center responses (Chung et al. 2011, Linterman et al. 2011). Similarly, a subpopulation of T_{REGs} expresses the T_{H1} master TF T-bet, which drives the expression of CXCR3, allowing them to colocalize with T_{H1} cells. Expression of T-bet in these T_{REGs} is necessary for optimal suppression of T_{H1} responses (Koch et al. 2009). The variety in T_{REG} subtypes is perhaps best appreciated in tissue T_{REGs}, which experience diverse stromal environments. A recent study analyzed the transcriptional profiles of skin, lung and lymph node T_{REGs} at the single cell level. Analysis of skin and lung T_{REGs} revealed multiple unique subsets of T_{REGs} in these tissues, with each tissue primarily colonized by distinct subtypes (Kalekar et al. 2019). The majority of intestinal T_{REGs} in mice express either Rorγt, the master TF of T_{H17} cells, or Gata3, which drives differentiation of T_{H2} cells, though interestingly expression of these TFs does not confer upon T_{REGs} the full effector functionality of the other T cell subtypes (Zhou et al. 2008, Wohlfert et al. 2011). Transcription of Gata3 and of Rorγt appear to be mutually exclusive, defining two unique subsets in addition to Gata3$^-$ Rorγt$^-$ T_{REGs}. Further, these subsets have unique functional roles and appear to be derived mainly from different pools of T_{REGs}, Gata3 being expressed mainly in tT_{REGs} and Rorγt expressed mainly in iT_{REGs}. Deletion of the Rorc gene in T_{REGs} results in exacerbation of disease in mouse models of colitis and enhanced anti-helminth immunity (Sefik

et al. 2015, Ohnmacht et al. 2015) whereas Gata3^{+} T_{REGs} accumulate preferentially in response to IL-33 and produce Areg (Schiering et al. 2014), suggesting that they may be particularly important for tissue repair. Tissue-resident T_{REGs} can even express TFs associated with the stromal cells in their surrounding tissue, such as PPAR-γ in visceral adipose tissue T_{REGs}. Indeed PPAR-γ expression drives transcription of some factors associated with the tissue repair functions of VAT T_{REGs}, including Gata3 (Cipolletta et al. 2012). In lymphoid and non-lymphoid tissue, distinct T_{REG} subtypes are optimized for context and tissue-specific functions.

There is increasing acknowledgment of plasticity between T cell subpopulations, including between T_{REG} subtypes. IL-10 and IL-35 producing T_{REGs} appear to interconvert *in vitro* though it is not yet known whether this occurs *in vivo* (Sawant et al. 2019). Fate mapping of FoxP3-expressing cells revealed a population of "ex- T_{REGs}" that had lost expression of FoxP3, lacked expression of other T_{REG}-associated molecules such as CD25 and CTLA-4 and performed functions associated with T_{CONVs} (Zhou et al. 2009). However, further investigation revealed that ex-T_{REGs} are very rarely generated in adult mice and may mainly represent a population of T_{CONVs} that undergo transient FoxP3 expression (Miyao et al. 2012, Sawant and Vignali 2014, Rubtsov et al. 2010).

Other populations of immune and stromal cells may also suppress immunity, often utilizing molecular mechanisms in common with T_{REGs}. Subsets of FoxP3^{-} T cells, for example, produce many of the same suppressive molecules as T_{REGs}, such as IL-10, TGF-β and CTLA-4. Priming in the presence of IL-27 has been shown to promote the differentiation of FoxP3^{-} CD4^{+} T cells with these properties. Such cells tend to have high surface expression of co-inhibitory molecules, including LAG-3 and PD-1, but these markers are not exclusive to suppressive T cells as they are also expressed on the surface of recently activated effector cells. Better markers for

FoxP3$^-$ suppressor T cells are needed to understand their development and function. Regulatory B cells represent another source of adaptive immunosuppression. These B cells were first identified by their ability to produce IL-10 after *in vitro* stimulation (Fillatreau et al. 2002). B cell-specific IL-10 deficiency has since been found to enhance autoimmunity (Fillatreau et al. 2002, Carter et al. 2011). Finally, expression of co-inhibitory ligands on stromal cells may also suppress immune responses, particularly within the graft where inflammation from ischemia-reperfusion injury is likely to enhance expression of these molecules (Castro et al. 2018).

Many mechanisms have evolved to prevent autoimmunity in the presence of T and B cell repertoires with highly diverse, randomly-generated TCRs and BCRs. Having multiple mechanisms allows for compensation if one mechanism were to fail and allows for modulation of suppression to suit a particular tissue or context. Importantly, most of these suppressive mechanisms act in an antigen-dependent manner. The next section will focus on the ways in which these mechanisms for self-tolerance are co-opted to therapeutically induce tolerance to foreign antigens present within an allograft.

Strategies to induce donor-specific transplantation tolerance

Induced chimerism through stem cell and bone marrow transplantation

Following their discovery that adaptive tolerance was generated in utero in mice, and that tolerance could be manipulated to allow acceptance of genetically distinct skin grafts, Billingham, Brent and Medawar extended their findings to chickens. They observed that dizygotic “natural twin” chickens hatched from double-yolked eggs, as well as “artificial twin” chickens from single-yolked eggs whose embryonic blood supplies were surgically fused with

those from other eggs were tolerant to skin grafts from their natural or artificial twins as adults. Further, the authors found that the resulting chickens were chimeric, containing red blood cells of their twin's genotype sometimes over 5 months after hatching. Robust chimerism persisted in some chickens beyond the 120-day lifespan of red blood cells, indicating that these chickens contained a persisting source of new red blood cells of their twin's genotype, which we now would identify as hematopoietic stem cells. Supporting a hypothesis that persisting chimerism was necessary for maintaining tolerance, the authors found that some artificial twin chickens rejected their skin grafts late after transplantation, and this rejection was associated with a loss of chimerism (Billingham, Brent, and Medawar 1956). In more recent years, several tolerance induction strategies have focused on generating hematopoietic chimerism of donor cells in transplant recipients.

Bone marrow transplantation is a well-established method for generating long-term hematopoietic chimerism. Anecdotes of patients who develop tolerance to solid organ transplants after having previously received bone marrow transplants from the same donor provided proof of principle for this approach in clinical transplantation (Svendsen et al. 1995, Sayegh et al. 1991, Helg et al. 1994, Jacobsen et al. 1994). A group at Harvard subsequently pioneered clinical combined kidney and bone marrow transplantation. Patients undergoing concurrent hematopoietic cell and solid organ transplantation received both transplants on the same day, and were temporarily treated with conventional immunosuppression, typically for less than one year. Of the ten patients initially transplanted, seven were able to stop immunosuppression without rejecting their grafts for 4.5-11.4 years, though three patients did eventually experience rejection, requiring reinstatement of conventional immunosuppression (Kawai et al. 2014). Hematopoietic stem cells may also be harvested from donor peripheral blood, in a procedure that is less invasive

than harvesting bone marrow. More recently, two US medical centers have conducted clinical trials of concurrent hematopoietic stem cell transplantation with kidney transplantation, which have thus far reported similar levels of success in achieving immunosuppression-free graft survival (Scandling et al. 2015, Leventhal et al. 2013).

While the mechanisms preventing acute rejection in these patients are not fully understood, both clonal deletion (Morris et al. 2015) and induction of allospecific T_{REGs} (Sprangers et al. 2017) have been detected. These findings, and possibly cell-intrinsic dysfunction, contribute to donor-specific hypo-responsiveness observed in immune cells from recipients after combined hematopoietic cell and solid organ transplantation (Kawai et al. 2014). In mice, donor-specific tolerance induced by bone marrow transplantation has similarly been associated with clonal deletion of donor-reactive T cells, and anergy in donor-specific T cells that persist (Tomita, Khan, and Sykes 1994). Interestingly, the persistence of donor cell chimerism was not associated with graft outcomes in kidney recipients who received concurrent bone marrow transplants. In these patients, donor cells become undetectable 2-3 weeks after transplantation (Kawai et al. 2014). In patients receiving concurrent hematopoietic stem cell transplantation, the relation between stable chimerism and tolerance is less clear. Some patients with transient chimerism were more likely to undergo rejection, but in many cases these patients also received a lower dose of donor cells, had lower peak chimerism and different numbers of disparities in HLA mismatches compared to patients with stable chimerism, thus it is not clear whether the lack of chimerism is the cause of inadequate tolerance in these patients or whether impaired tolerance and transient chimerism are independent readouts of other variables in the treatment protocol (Leventhal et al. 2013).

Bone marrow and hematopoietic stem cell transplantation are associated with severe complications, making them infeasible for many solid organ transplant recipients. To prevent rejection of the donor cells, hematopoietic cell transplant recipients undergo conditioning regimens. Such regimens have historically included high dose total body irradiation or chemotherapy, which place the patient at risk of many complications, including life-threatening infections, since they eliminate a large portion of the immune system. More recently, novel conditioning agents and lower doses of radiation have been adopted to reduce these complications, though they do not eliminate risk (Gyurkocza and Sandmaier 2014, Chhabra et al. 2017). The need for pre-transplant conditioning also precludes the use of combined hematopoietic cell transplantation with deceased donor organs, since it is not possible to predict availability of a deceased donor to begin the conditioning treatment. GVHD is also a common complication in bone marrow transplant recipients that can be life-threatening. These risks are amplified in the context of the end-stage organ failure and major surgery experienced by solid organ transplant recipients.

Intra-thymic introduction of alloantigen

Some attempts have been made to establish donor-specific tolerance by inducing the presentation of alloantigen in the thymus, thereby applying the mechanisms of central tolerance to allospecific thymocytes. To that effect, protocols to induce thymic expression of alloantigen have included intra-thymic injection of donor tissue or cells, purified alloantigen, or alloantigen-expressing retrovirus directly into the thymus. These procedures were largely successful at promoting long-term kidney, islet and heart allograft acceptance in rodent models (Perico et al. 1991, Marodon et al. 2006, Jones et al. 1998, Saborio et al. 1999, Chen, Sayegh, and Khoury 1998, Posselt et al. 1990). As expected, intra-thymic alloantigen presentation resulted in clonal deletion of

allospecific T cells and increased differentiation of allospecific T_{REGs} (Jones et al. 1998, Saborio et al. 1999, Marodon et al. 2006). Allospecific T cells that escaped thymic selection and persisted in the periphery were found to be hypo-responsive upon re-challenge, which was likely the result of T_{REG}-mediated suppression but may also have involved T cell-intrinsic dysfunction (Chen, Sayegh, and Khoury 1998, Posselt et al. 1990). Despite success in the pre-clinical setting, intra-thymic alloantigen injection failed to induce tolerance in a small cohort of human heart transplant recipients (Remuzzi et al. 1995). It is not known whether intra-thymic injection failed to adequately induce central tolerance to alloantigen in these patients or whether additional peripheral tolerance mechanisms are needed to maintain tolerance specifically in humans. The efficacy of intra-thymic introduction of antigen for tolerance induction may also be limited in adult humans due to involution of the thymus and reduction in the rate of T cell development with age.

Oral tolerance

The immune system is exposed to a large number of innocuous non-self antigens from the environment, including those derived from the diet or commensal microbes. To prevent inflammation and food allergy, the host must be tolerized against these antigens. Central tolerance is not thought to play a significant role in tolerization against non-self antigens as they are not expressed in the thymus or bone marrow. Thus, tolerization against dietary and commensal antigens occurs mainly through peripheral tolerance mechanisms including T cell deletion and dysfunction and T_{REG} induction. The route of antigen exposure is a key determinant of whether the host will be immunized or tolerized against a particular environmental antigen. Mucosal and intravenous exposure to antigen generally promotes tolerance while subcutaneous or intramuscular injection of antigen is immunogenic. Metabolites from some commensal

microbes can further promote the induction of T_{REGs} (Atarashi et al. 2013, Stefka et al. 2014). Thus, some studies have attempted to induce donor-specific tolerance by orally exposing the recipient to alloantigens (Mayer and Shao 2004). In animal models, exposure to donor alloantigen in the form of purified protein, donor splenocytes or donor bone marrow significantly prolonged allograft survival, though graft acceptance was not permanent (Zavazava et al. 2000, Ishido et al. 1999, Gorczynski et al. 1998). A pilot study found that feeding synthesized peptides with sequences derived from donor MHC to kidney transplant recipients was associated with reduced T cell reactivity to those peptides (Womer et al. 2008). Oral tolerance would be an ideal therapeutic approach for patients as it is non-invasive, but thus far oral tolerance has not generated robust enough tolerance to induce permanent graft acceptance. Further, the need to generate allogeneic peptides for treatment beginning potentially months prior to transplantation makes this approach infeasible for the majority of transplant recipients.

Increasing the frequency of regulatory T cells in the allospecific T cell population

T_{REGs} are well-suited to promote donor-specific tolerance as they can potently and specifically suppress alloimmune responses. Indeed, in mouse models and clinical transplantation, tolerance is associated with a high T_{REG}:T_{CONV} ratio (Young et al. 2018, Savage et al. 2018, Heidt and Wood 2012). The endogenous T_{REG} population is also required for several experimental tolerance induction treatments, as T_{REG} depletion at the time of transplantation can prevent tolerance induction. The most straightforward approaches for inducing tolerance through T_{REG}-mediated suppression aim to increase the T_{REG}:T_{CONV} ratio *in vivo*. This can be accomplished by selectively increasing the number of allospecific T_{REGs} and/or decreasing the number of allospecific T_{CONVs}.

Many tolerance induction strategies such as concurrent hematopoietic cell transplantation, oral tolerance and co-stimulation blockade are associated with an increased T_{REG}:T_{CONV} ratio, though these treatments also induce other, independent mechanisms of tolerance such as clonal deletion of allospecific T cells. Some treatments, such as low dose IL-2, directly and specifically expand T_{REGs} with the hypothesis that an increased T_{REG}:T_{CONV} ratio is sufficient for tolerance induction. Due to their expression of CD25, T_{REGs} outcompete other cells for binding of limiting doses of IL-2. Combination with rapamycin, which inhibits IL-2 signaling, has had a synergistic effect in promoting graft survival in mice (Pilon et al. 2014). However, as the IL-2 dose increases, a larger proportion of effector cells is activated, potentially resulting in severe systemic inflammation. It is difficult to identify and maintain an optimal dose of IL-2 to specifically promote suppression, making this a risky strategy for treatment. Recently, a study found that complexing of IL-2 with a specific anti-IL-2 clone (JES6-1) specifically inhibited binding to lower affinity IL-2 receptors expressed by mouse effector T cells, further restricting its activity to T_{REGs} (Spangler et al. 2015, Boyman et al. 2006). This complex was more effective than IL-2 alone at inducing acceptance of fully MHC mismatched islet allografts in mice. Interestingly, the expansion of T_{REGs} after IL-2/JES6-1 treatment was shown to be transient and T cells from mice that had accepted their grafts were not found to be hypo-responsive *in vitro* when compared to IL-2/JES6-1-treated mice that failed to accept their grafts. These findings suggest that allospecific T cells are not well controlled by suppression, dysfunction or depletion in the periphery. It is possible that the functional cells are simply ignorant after initial acceptance of the graft, or perhaps changes within the graft, such as an enrichment of T_{REGs}, protect it from rejection (Webster et al. 2009). The efficacy of IL-2/anti-IL-2 treatment in inducing tolerance in patients remains to be determined.

Some recent studies have utilized a novel drug delivery system to localize T_{REG} -promoting treatments to the graft. In one study, microparticles were injected into a rat hindlimb graft to locally and continuously release CCL22, a chemokine that preferentially attracts T_{REGs}. CCL22 microparticle treatment significantly increased hindlimb graft survival, though the efficacy was highly dose-dependent, with less than ten-fold increase or decrease in dosage nearly eliminating the effects of treatment. Still, the majority of rats treated with an optimal dose accepted their hindlimb allografts for at least 200 days, a significant feat for transplantation of highly immunogenic vascular composite allografts (Fisher et al. 2020). Similarly, this group injected a combination of microparticles containing rapamycin and the T_{REG} -promoting cytokines IL-2 and TGF-β into hindlimb allografts to locally promote T_{REG} induction and expansion. This treatment induced acceptance of the graft for at least 300 days in all but one recipient (Fisher et al. 2019). Both T_{REG} recruiting and T_{REG} inducing microparticle treatments were found to enhance suppression of donor-specific T cell responses *in vitro* (Fisher et al. 2019, Fisher et al. 2020).

The converse strategy of selectively inhibiting T_{CONVs} can be accomplished with immunosuppressants and depleting antibodies that are known to preserve T_{REGs}. For example, immunosuppression protocols may minimize the use of calcineurin inhibitors, which impair T_{REG} function, in favor of T_{REG}-sparing immunosuppressants such as Rapamycin. Monoclonal anti-CD3 antibodies and polyclonal T cell-binding antibodies such as anti-thymocyte globulin have been used to deplete T cells after transplantation. These antibodies appear to deplete T_{REGs} less well than other T cell subsets, thereby increasing the proportion of T_{REGs} in the T cell repertoire (Tang and Bluestone 2013, Penaranda, Tang, and Bluestone 2011). Indeed, anti-CD3 has been

shown to induce long-term acceptance of major mismatched islet allografts in mice in a T_{REG}-dependent mechanism (Besançon et al. 2017).

Rather than expanding T_{REGs} *in vivo*, there is considerable interest in transfusing transplant recipients with T_{REGs} expanded *ex vivo*. The process of T_{REG} cell therapy typically starts with enrichment of T_{REGs} from a patient's blood using surface markers such as CD25, CD45RA and CD127. The isolated cells are then expanded *ex vivo* either by stimulation with anti-CD3- and anti-CD28-coated beads or donor APCs and then transfused into the recipient shortly after transplantation. Using donor-derived stimulators is advantageous as they specifically expand donor-specific T_{REGs}, resulting in a more potent suppressive population. However, T_{REGs} specific for alloantigens presented indirectly on host APCs will not be expanded by this process. This problem may be mitigated if the donor-recipient pair has partially or fully matched MHC Class II alleles. Additionally, the expanded T_{REGs} may promote infectious tolerance *in vivo*. A small number of clinical trials are underway to test the efficacy of T_{REGs} expanded by donor APCs in promoting tolerance to liver and kidney transplants. It is already clear that generating enough allospecific T_{REGs} for tolerance induction will be a significant barrier for T_{REG} cell therapy as one clinical trial has been terminated due to manufacturing challenges (NCT02188719, ClinicalTrials.gov) and others have reported frequent failed T_{REG} batches due to low cell yield or contamination (Safinia et al. 2018, Tang and Vincenti 2017).

It is also technically possible to induce T_{REGs} from naïve T cells *in vitro*, rather than expanding existing allospecific T_{REGs}. However, T_{REGs} induced by existing protocols involving stimulation in the presence of T_{REG}-promoting cytokines *in vitro* are epigenetically and functionally unstable, frequently converting to dangerous effector T cells (Koenecke et al. 2009). This strategy may become more practical after protocols are developed to stabilize *in vitro*

generated T_{REGs}. Preliminary studies have also shown that exosomes derived from T_{REGs} stimulated *in vitro* can modestly promote allograft survival, though it is unclear how long the exosomes last *in vivo* and whether they suppress responses in an antigen-specific manner (Yu et al. 2013, Aiello et al. 2017, Robbins and Morelli 2014). Some studies have also attempted to induce tolerance through transfusion of other suppressive immune cell subsets such as myeloid-derived suppressor cells, tolerogenic dendritic cells cultured from bone marrow or monocytes, mesenchymal stromal cells or regulatory B cells, though they have had limited success in inducing long-term graft acceptance. T_{REG} induction and/or T_{CONV} deletion are typically central to the mechanisms of action of these other suppressive cells (Ochando et al. 2019, Chesneau et al. 2013, Marín, Cuturi, and Moreau 2018, Podestà, Remuzzi, and Casiraghi 2019).

Transducing T_{REGs} with allospecific surface receptors is another way of generating large populations of allospecific T_{REGs} for adoptive transfer. Some studies in mice have accomplished this by transducing T_{REGs} with allospecific TCRs, which promoted long-term acceptance of partially MHC mismatched heart allografts. However, it is much harder to predict which TCRs are allospecific in more genetically diverse combinations of human transplant donors and recipients (Tsang et al. 2008). To reduce the size of this hurdle, some groups have engineered T cells to express chimeric antigen receptors (CARs) consisting of an external Ig-like region that binds alloantigen fused to intracellular TCR signaling components. CAR-T_{REGs} have been effective at preventing GVHD, skin graft rejection and autoimmunity in mouse models (MacDonald et al. 2016, Zhang, Lu, et al. 2018). However, CAR-T_{REGs} still carry several disadvantages similar to *ex vivo* expanded T_{REGs}. Manufacturing of cell therapies for clinical use is a costly and complex process. It is also unclear whether CAR-T_{REGs} can suppress priming of allospecific T cell responses or only suppress effector responses after priming, since they

recognize epitopes that may not be present in the secondary lymphoid organs. The significance of these disadvantages may become clear as clinical trials of CAR-T_{REGs} begin in the near future. The first trial of CAR-T_{REGs} in kidney transplant recipients is expected to start recruiting patients in 2020.

T_{REGs} are known to play a critical role in self-tolerance, tolerance to environmental antigens and therapeutically induced tolerance to allografts. Treatments to support T_{REG}-mediated suppression by increasing the T_{REG}:T_{CONV} ratio have generally promoted graft survival, though effective T_{REG}-centered treatments have thus far failed to induce permanent donor-specific tolerance. Cell-based therapies may be a promising strategy to quickly increase the allospecific T_{REG}:T_{CONV} ratio but face major manufacturing and regulatory challenges. Additionally, transplant recipients who have been sensitized to alloantigens by rejection of a previous allograft, blood transfusion or pregnancy present a significant challenge as memory T cells are more resistant to T_{REG}-mediated suppression than naïve T cells (Yang et al.). Thus, there is interest in de-sensitizing these patients so that they can become tolerized. A recent study has reported that a small molecule inhibitor of CDK8 and CDK19 may convert memory T_{CONVs} into T_{REGs}, removing a particularly dangerous subset of allospecific T cells and increasing the number of T_{REGs}. While this treatment did convert a small percentage of memory T cells to T_{REGs} *in vivo*, it did not result in T cell population-wide desensitization (Akamatsu et al. 2019). Promoting suppression by T_{REGs} is a logical strategy for inducing donor-specific tolerance but there are clearly large hurdles ahead for clinical implementation of existing T_{REG}-based therapies and it is not yet clear whether altering the T_{REG}:T_{CONV} ratio is sufficient for induction of robust tolerance induction.

Co-stimulation blockade

Several treatments under investigation for tolerance induction work by blocking co-stimulatory signaling between T cells and APCs. As discussed in a previous section, transplantation is associated with the release of DAMPs, which stimulate the APCs taking up alloantigen, allowing them to activate allospecific T cells and initiate graft rejection. By blocking co-stimulatory signals therapeutically, naïve allospecific T cells instead receive a tolerogenic signal similar to the signal provided by a resting APC. Important to this tolerogenic strategy is the timing of treatment. Theoretically, indefinite treatment with co-stimulation blockade would globally compromise host adaptive immunity since the immune system would be unable to optimally prime a response against any foreign antigen. Instead, limiting co-stimulation blockade to a short period of time concurrent with transplantation is expected to limit the induction of tolerance mainly to alloantigens, since APCs activate or tolerize T cells in an antigen-dependent manner and the host is unlikely to encounter many novel foreign antigens beyond alloantigens during a short window of treatment. Also important to this strategy is the generation of permanent tolerance, which would continue to prevent rejection after cessation of co-stimulation blocking treatment.

Some of the first treatments for blocking co-stimulation in transplant recipients interfered with interactions between CD28 and CD80/CD86. This pathway was a particularly attractive target because it was thought to regulate other co-stimulatory pathways, and it had been shown that CD28-CD80/CD86 interaction could prevent T cell anergy (Harding et al. 1992, Chen and Flies 2013). This pathway could be effectively blocked by the addition of a soluble extracellular domain of CTLA-4 stabilized by fusion to the Fc region of IgG1 (CTLA-4-Ig). CTLA-4-Ig acts as a decoy to bind CD80 and CD86 with high affinity and competitively inhibit their interaction

with CD28. Additionally, CTLA-4-Ig induces expression and activation of IDO in the bound DC (Fallarino et al. 2003). Indeed, immunization in the presence of CTLA-4-Ig has been shown to result in clonal deletion of T cells (Wells et al. 1999). However, while CTLA-4-Ig treatment at the time of antigen exposure was shown to effectively eliminate T cell priming *in vivo*, it did not induce tolerance, as secondary immunizations after cessation of CTLA-4-Ig treatment could still elicit an immune response (Linsley et al. 1992). Still, treatment with CTLA-4-Ig could induce donor-specific tolerance in mouse models of transplantation, as evidenced by long-term graft acceptance and spontaneous acceptance of secondary donor-matched grafts (Pearson et al. 1994). However, in a clinical trial, treatment of kidney transplant recipients with CTLA-4-Ig was associated with a 2-3 fold increase in acute rejection rate compared to the conventional immunosuppressant cyclosporine (Vincenti et al. 2010). More recent studies have revealed that CTLA-4-Ig may undermine the suppressive function of T_{REGs} by starving them of CD28 signaling that is needed for their survival (Tang et al. 2003, Riella et al. 2012, Charbonnier et al. 2012). Additionally, signaling downstream of CTLA-4 within host T_{REGs} may promote their suppressive function and CTLA-4-Ig treatment prevents this signaling, thereby inhibiting an important source of peripheral tolerance (Masteller et al. 2000).

Treatments that interfere with CD40-CD154 interaction impair co-stimulation without harming CTLA-4-CD28 signaling by T_{REGs}. Blocking this interaction prevents DC licensing to limit allospecific T cell priming, and also inhibits germinal center responses. While treatment with anti-CD154 alone prolongs allograft survival, combination with a transfusion of donor splenocytes (DST) is necessary to consistently induce long-term survival and donor-specific tolerance (Parker et al. 1995, Hancock et al. 1996). The mechanisms by which DST synergizes with anti-CD154 to induce tolerance are not well understood. Originally, DST was added to

conventional immunosuppression with the intention of inducing tolerance through hematopoietic chimerism. It was later shown that chimerism was not required for anti-CD154/DST-induced tolerance as irradiated DST could also induce donor-specific tolerance when combined with anti-CD154 (Bushell et al. 1995). Additional studies have investigated the requirement of various molecules and cells within the DST for inducing tolerance, but none have identified a particular population or pathway intrinsic to the DST that is critical for tolerance induction. Because cells in DST express donor MHC Class I and II, one could hypothesize that the mechanism of DST relies on direct alloantigen presentation on DST cells. However, a recent study using MHC Class I and/or II-deficient mice has shown direct presentation by DST is not necessary for tolerance induced by anti-CD154 and DST (Kishimoto et al. 2004). Work is underway in our group to better understand the mechanism of DST, with the hypothesis that through increasing the dose or the physical distribution of antigen in the host, DST allows tolerization of more allospecific T cells than graft-derived antigen alone.

Multiple long-lasting changes to the host immune system have been shown to occur after anti-CD154/DST-induced tolerance. These effects that last beyond cessation of anti-CD154 treatment are likely important for long-term maintenance of tolerance. Promoting these mechanisms may increase the robustness of tolerance during an inflammatory challenge, and detection of these mechanisms may identify biomarkers for stable tolerance in patients with spontaneous or therapeutically-induced transplantation tolerance. Like CTLA-4-Ig, anti-CD154/DST induces deletion of allospecific T cells through abortive proliferation (Quezada et al. 2005). However, clonal deletion is not complete, resulting in a significant population of allospecific T cells that persist long-term. Control of these persisting cells, and of new thymic emigrants, appears to involve several mechanisms. In contrast to CTLA-4-Ig, anti-CD154

treatment is accompanied by expansion of the allospecific T_{REG} population, which may suppress persisting allospecific T_{CONVs} (Ferrer et al. 2011). Indeed, T_{REG} depletion at the time of transplantation prevents tolerance induction (You and Chatenoud 2018). A recent study has indicated that similar expansion of allospecific T_{REGs} occurs during acute allograft rejection, but that the lack of T_{CONV} expansion after anti-CD154 treatment results in a high allospecific T_{REG}:T_{CONV} ratio that promotes tolerance. Thus, the allospecific T_{REG}:T_{CONV} ratio may be more important than the absolute number of allospecific T_{REGs} for predicting tolerance (Young et al. 2018).

The allospecific B cell repertoire of anti-CD154/DST-treated murine heart allograft recipients also experiences long-term changes. Allospecific B cells neither expand nor are deleted during tolerance induction, persisting at quantities similar to those of naïve mice. Like allospecific T cells, allospecific B cells become intrinsically dysfunctional during tolerance, failing to mount a productive germinal center response, even when exposed to functional activated cognate $CD4^+$ T cells. Interestingly tolerant B cells could suppress responses by naïve B cells in secondary hosts in an antigen-specific manner (Khiew et al. 2020).

Interestingly, transient T_{REG} depletion late after transplantation with anti-CD154/DST does not break established tolerance, suggesting that additional mechanisms maintain tolerance. One such mechanism appears to be the development of T cell-intrinsic dysfunction resulting from anergizing TCR stimulation without co-stimulation and/or chronic stimulation by persisting alloantigen expressed by the accepted graft (Miller et al. 2019, Quezada et al. 2003). Our group has shown that allospecific T cells persisting during tolerance up-regulate expression of CD44, indicating that they are antigen-experienced, but similar to exhausted T cells responding to a chronic infection, tolerant T cells fail to up-regulate the memory cell marker CD127 and express

high levels of PD-1, a co-inhibitory receptor that is up-regulated after recent antigen stimulation. Using monoclonal tracer populations of allospecific $CD4^+$ T cells, this study reported that persisting tolerant allospecific T cells expanded less than memory cells when re-stimulated, even when isolated from other immune cells, indicating that their dysfunction was cell-intrinsic rather than dependent on suppression by factors and cells within the tolerized mouse (Miller et al. 2019).

After productive immunization or rejection, the responding polyclonal population of T cells increases in average sensitivity to antigen stimulation, a process known as avidity maturation. In contrast to affinity maturation by B cells, which involves BCR rearrangement in the periphery, T cell avidity maturation results from the selective expansion of existing T cell clones with high sensitivity to stimulation due to high affinity for pMHC and other biochemical parameters (Nikolich-Žugich, Slifka, and Messaoudi 2004). While direct measurement of T cell sensitivity to stimulation, or functional avidity, requires a dose-titration of peptide stimulus, functional avidity can be estimated by the intensity of T cell binding to pMHC multimer complexes, a measure of structural avidity (Savage, Boniface, and Davis 1999a). Higher intensity of fluorophore-conjugated pMHC-binding as measured by flow cytometry correlates with increased functional avidity. Using this method of approximation, our group has determined that the endogenous, polyclonal population of allospecific T cells in tolerized heart recipient mice does not undergo avidity maturation, retaining the lower avidity of allospecific T cells in a naïve mouse instead (Miller et al. 2018). Limiting the quantity of high avidity T cell clones may support tolerance if high avidity T cells could mediate a particularly destructive rejection response if poorly controlled, or if they are resistant to mechanisms maintaining tolerance such

as suppression or development of dysfunction. More work is needed to test these hypotheses and to determine which mechanisms restrict avidity maturation during tolerance induction.

Due to the efficacy of both CTLA-4-Ig and anti-CD154 in preventing rejection in mouse models, some attempts have been made to combine these therapies in hopes that they will synergize in inducing tolerance. However, there are inconsistencies in reports of the effectiveness of combining CTLA-4-Ig with anti-CD154 and DST. Some studies have reported synergy between CTLA-4-Ig and anti-CD154/DST in inducing tolerance to allografts (Larsen et al. 1996), while others have found that CTLA-4-Ig impairs CD154/DST-mediated tolerance by blocking inhibitory signaling downstream of CTLA-4 on T cells (Zheng et al. 1999). Undermining of tolerance by CTLA-4-Ig may stem, as mentioned earlier in this dissertation, from the role of CD28 in promoting T_{REG} function and a potential role for inhibitory CTLA-4 signaling on T_{REGs} and T_{CONVs} (Walunas et al. 1994, Lin et al. 1998).

While promising in animal models of transplantation, humanized anti-CD154 monoclonal antibodies did not succeed in clinical trials because of thromboembolitic complications resulting from treatment (Kawai et al. 2000). Focus subsequently shifted back to development of CTLA-4-Ig as a less effective but safer option for co-stimulation blockade. Recently, evidence has indicated that the Fc region of anti-CD154 binds to FcγRIIa expressed on the platelet surface. Because humans express a soluble form of CD154, the antibodies can become cross-linked by this soluble antigen, forming large complexes of platelets. One study has shown that the combination of anti-CD154 and soluble CD154 causes clotting in mice transgenically expressing human FcγRIIa on their platelets, supporting this model of anti-CD154-induced clotting (Robles-Carrillo et al. 2010). Success of anti-CD154 treatment in inducing long-term survival of islet (Kenyon, Chatzipetrou, et al. 1999, Kenyon, Fernandez, et al. 1999) and renal (Kirk et al. 1999)

allografts in non-human primates indicates that blockade of this pathway may induce donor-specific tolerance in patients. Monoclonal antibodies specific for CD40 are in clinical trials (Lowe et al. 2012, Pearson et al. 2002) as a strategy to block CD40-CD154 co-stimulation with less potential for clotting than anti-CD154. Indeed, early results from a phase 2 clinical trial revealed that replacing calcineurin inhibitors with anti-CD40 in a standard immunosuppression regimen did not increase risk of thromboembolitic complications, resulted in similar 1-year renal graft outcomes, and reduced chronic allograft damage (Farkash et al. 2019). Additionally, anti-CD154 clones with modified Fc regions that do not bind FcγRIIa are in development. The first clinical trial using such a modified anti-CD154 is underway to test its efficacy in treating multiple sclerosis (https://www.sanofigenzyme.com).

The presence or absence of co-stimulation determines whether a T cell activated in the periphery will be primed or tolerized. Thus, eliminating co-stimulation to mimic an interaction with a tolerogenic DC is a clever strategy for inducing donor-specific tolerance. Despite early challenges with clinical translation, co-stimulation blockade remains one of the more clinically feasible tolerance induction strategies, especially when compared to the complexity of other leading treatments such as concurrent hematopoietic cell transplantation or T_{REG} cell therapy. Considering the success of anti-CD154 in animal models of transplantation, newer engineered clones of anti-CD154 or anti-CD40 antibodies hold great promise for robust tolerance induction.

Robustness of transplantation tolerance induced with anti-CD154 and DST

Multiple compensatory immune suppressive mechanisms maintain tolerance induced by anti-CD154 and DST

For any tolerance induction treatment to become a standard for transplant recipients, it must induce a state of tolerance that is resistant to inflammatory challenges such as infections. The state of tolerance induced by treatment with anti-CD154 and DST is particularly robust when compared to other tolerance induction methods. Thus, it is important to understand the immune mechanisms responsible for maintaining this robust tolerance so that they can be replicated and tracked in tolerant patients. It is also important to identify insults that can break robust tolerance to understand and overcome the potential vulnerabilities of patients made tolerant to their transplants with co-stimulation blockade.

Our group has found that limitation of alloreactive T cell number, and suppression of alloreactive T cells by T_{REG} cells and programmed cell death 1 ligand 1 (PD-L1) are compensatory mechanisms simultaneously keeping tolerance intact in tolerant heart recipient mice. Only after disruption of all three mechanisms did tolerized heart recipients reject their grafts (Miller et al. 2016a). Additional work from our group indicates that the allospecific T cell population persisting in tolerant graft recipients is intrinsically dysfunctional and these cells have low structural avidity for alloantigen (Miller et al. 2019, Miller et al. 2018). These cell-intrinsic features may prevent graft rejection even if cell-extrinsic suppression fails. The robustness of anti-CD154/DST-induced tolerance in mouse models makes it an ideal tolerant state to study and emulate with newly developed tolerizing therapies.

Breaking tolerance induced by anti-CD154 and DST

Our group and others have described the robustness of anti-CD154/DST-induced tolerance during infection. While infections with *lymphocytic choriomeningitis virus* (LCMV) (Welsh et al. 2000) or *Staphylococcus aureus* (Ahmed et al. 2011) prevent the induction of tolerance with anti-CD154 and DST, these pathogens do not break tolerance after it is established. Only infection with *Listeria monocytogenes* (Lm) has been reported to break established tolerance induced by anti-CD154 and DST. The precise immune mechanisms by which Lm is able to break tolerance are not well understood, though they seem to result from the release of type I interferon (IFN) and IL-6 during the host response to Lm infection. Indeed, IL-6 and type I IFN were both required for rejection precipitated by Lm infection. Further, the combination of IL-6 and IFN-β was sufficient to break tolerance under sterile conditions (Wang et al. 2010b), suggesting that other viruses and bacteria capable of eliciting these cytokines may also break established tolerance (McNab et al. 2015, Kovarik et al. 2016, Rose-John, Winthrop, and Calabrese 2017). There may also be other cytokines sufficient to break tolerance, and pathogens or sterile causes of inflammation that break tolerance via mechanisms distinct from those required for Lm-induced rejection. Thus, while anti-CD154/DST induces a relatively robust tolerant state, it is likely still vulnerable to multiple different infections.

Understanding the mechanisms that contribute to the maintenance of tolerance, and how these mechanisms are disrupted during rejection after a period of stable tolerance is necessary to improve the stability of therapeutically-induced tolerance, and to monitor patients with spontaneous or therapeutically-induced transplantation tolerance for signs of rejection or unstable tolerance. While cohorts of tolerant patients have been too small to establish a statistical relationship between infection and rejection, several observations in transplant recipients support

a model that infection leads to rejection in patients who previously were tolerant to their grafts. Episodes of rejection in transplant recipients with spontaneous operational tolerance are frequently preceded by infection (Brouard et al. 2012). Rejection following infection has also been reported in patients tolerized to kidney allografts with concurrent hematopoietic stem cell or bone marrow transplantation (Kawai et al. 2008, Markmann and Kawai 2012, Leventhal et al. 2012). Understanding the mechanisms of rejection after various insults, including infection, in tolerized mice may shed light on the mechanisms of rejection in tolerant transplant patients.

Many studies investigating the immune mechanisms of tolerance induction have shown that long-term changes occur in the allospecific lymphocyte populations of tolerant graft recipients, including increased T_{REG}:T_{CONV} ratio, and T cell- and B cell-intrinsic dysfunction. Rejection after a period of stable tolerance may involve reversal of some of these mechanisms of tolerance, or may involve mechanisms distinct from rejection in the absence of immunosuppressive or tolerogenic treatment. Rejection after Lm required both $CD4^+$ and $CD8^+$ T cells, contrasting with acute rejection in the absence of tolerance induction where only $CD4^+$ T cells are required. Indeed, Lm infection was associated with restoration of the donor-specific T cell response as measured *in vitro* by IFN-γ enzyme-linked immunospot assay (ELISpot) (Wang et al. 2010b). Studies of Lm-induced fetal resorption have revealed that Lm infection impairs T_{REG} suppressive function while leaving T_{REG} quantity unchanged (Rowe et al. 2012). Additional studies have indicated that IL-6 and IFN-β can impair T_{REG} function (Srivastava et al. 2014, Nish et al. 2014). Thus, impaired T_{REG}-mediated suppression is likely a contributor to rejection of tolerized allografts after Lm infection, though both IL-6 and IFN-β are highly pleiotropic cytokines, thus additional effects by these cytokines may also contribute to Lm-induced rejection. Interestingly, tracking of allospecific T cells revealed that allospecific T cells do not

expand with one week of Lm infection in previously tolerant hosts. Further, allospecific T cells remain hypo-responsive and retain a surface phenotype associated with dysfunction following Lm-induced rejection (Miller et al. 2019). Thus, it is not clear which T cells are required for allograft rejection precipitated by Lm.

An additional study found that tolerance induced by anti-CD154 and DST could be broken by inducing mast cell degranulation. Similar to Lm-induced rejection, rejection after mast cell degranulation was T cell-dependent. Also similar to Lm infection, mast cell degranulation resulted in impaired T_{REG} function, which was associated with a reduction in T_{REG} expression of TGF-β, IL-10, EBI3 and granzyme B (De Vries et al. 2009). However, disruption of T_{REG}-mediated suppression is not thought to be sufficient to break tolerance as our findings indicate that tolerance is sustained even after depletion of T_{REGs} with anti-CD25 (Miller et al. 2016b).

Additional insults have been shown to break transplant tolerance induced by other protocols. For example, repeated injections of high dose IL-2 could break longstanding tolerance induced by concurrent bone marrow transplantation in non-human primates. Broken tolerance was associated with recovery of the allospecific T cell response. Unexpectedly, IL-2 increased both T_{REG} and T_{CONV} numbers such that the total T_{REG}:T_{CONV} ratio increased. It is possible that T_{REG} function was impaired or that the numbers of allospecific T_{REGs} and T_{CONVs} did not follow this trend, potentially explaining the restoration of donor-specific T cell response (Yamada et al. 2015). It is not known whether IL-2 injections can similarly break tolerance induced by co-stimulation blockade.

In this dissertation, we utilize tracer monoclonal allospecific T cells and pMHC multimers to study the phenotype and functional profile of allospecific T cells in a mouse model

of anti-CD154/DST-induced tolerance to fully MHC mismatched heart allografts. Using this strategy, we have identified restriction of T cell avidity maturation as a novel immune mechanism supporting donor-specific tolerance, as high avidity allospecific T cells are more potent mediators of graft rejection than their low avidity counterparts. We also investigate the vulnerabilities of tolerance, finding that continuous T_{REGs}-mediated suppression is required for maintaining tolerance. We additionally identify T cells specific for transiently expressed alloantigen as a population that retains functionality during tolerance, while persistently stimulated allospecific T cells become anergic. Finally, we investigate the response by allospecific T cells and B cells during the disruption of tolerance by Lm infection or T_{REG} depletion, finding a multitude of effects including restoration of T cell and B cell function and T cell avidity maturation, dependent on which insult was used to break tolerance. These findings highlight the importance of T_{REGs} in tolerance, indicate that there is diversity in fate of allospecific T cells dependent on their cognate alloantigen, and suggest that tolerance could be made more robust by subjecting a broader range of allospecific T cells to prolonged T_{REG}-mediated suppression.

Summary

Since Owen's and Billingham, Brent and Medawar's discoveries revealed that immune tolerance was an adaptive process, a great deal has been discovered about the mechanisms of tolerance. Importantly, it has become clear that tolerance occurs beyond the neonatal period and that mechanisms have evolved to generate tolerance to non-self antigens, such as dietary and commensal antigens, in the periphery. Many tolerance induction treatments apply these existing mechanisms to alloantigens. We have chosen to focus on tolerance induced by anti-CD154 and

DST as this strategy is both clinically feasible and generates a robust tolerant state that resists many inflammatory challenges. Disruption of established tolerance by Lm infection or mast cell degranulation indicates that there are vulnerabilities in the tolerant state that must be resolved in order to induce reliable tolerance in clinical transplant recipients. The following chapters describe findings that uncover immune mechanisms contributing to robust transplantation tolerance and the ways in which these mechanisms are disrupted to induce rejection in previously tolerant graft recipients. These findings will inform strategies to improve the robustness of tolerance and predict the disruption of tolerance in transplant recipients.

Chapter 2. T cells with high avidity for alloantigen are potent mediators of alloimmunity that are controlled by multiple immune mechanisms during transplantation tolerance.

Note: much of the data and text in the following section titled “T cells with high avidity for alloantigen are potent mediators of alloimmunity that are controlled by multiple immune mechanisms during transplantation tolerance” are reproduced from references (Miller et al. 2018) and (Gupta et al. 2019) with some rearrangement and addition of text and figures.

Abstract

Mechanisms implicated in robust transplantation tolerance at the cellular level can be broadly categorized into those that inhibit alloreactive T cells intrinsically (clonal deletion and dysfunction) or extrinsically through regulation. Recently, we have discovered that co-stimulation blockade prevents the expansion of T cells with high avidity for alloantigen after

transplantation. Whereas T cells with high avidity preferentially accumulated during acute rejection of allografts, the alloreactive T cells in tolerant recipients retained a low-avidity profile, comparable to naive mice despite evidence of activation. Here, we investigated whether controlling the expansion of high avidity cells supports transplantation tolerance. Indeed, we found that high avidity cells were more potent at rejecting allografts than low avidity cells with the same specificity. We also investigated which immune mechanisms constrain T cell avidity during tolerance, finding that exposure to alloantigen and co-stimulation were not sufficient to restore avidity maturation. T_{REG} depletion did restore avidity maturation of some T cell specificities, indicating that mechanisms known to support transplantation tolerance play a role in maintaining low avidity for alloantigen, but that there are additional mechanisms also responsible for preventing avidity maturation. Thus, peripheral transplantation tolerance involves control of alloreactive T cells at the population level, in addition to the individual cell level. Controlling expansion or eliminating high-affinity, donor-specific T cells long term may be desirable to achieve robust transplantation tolerance in the clinic. Identification of high avidity allospecific T cells may also serve as a biomarker for the robustness of tolerance.

Introduction

Given the vast size and sequence diversity of the T cell repertoire, multiple T cells in an individual are likely to respond to the same alloantigen. T cells with the same specificity may differ in their sensitivity to stimulation, a phenomenon that has been termed functional avidity (Nikolich-Žugich, Slifka, and Messaoudi 2004, Honjo, Xu, and Bucy 2000). In contrast to T cell affinity, which refers to the binding strength of a single TCR and pMHC interaction, T cell avidity integrates interactions between multiple TCRs and co-receptors with pMHC molecules

(Van Den Berg and Rand 2007). Differences in TCR avidity have been attributed to the affinity and geometry of TCR binding to pMHC (Zhong et al. 2013, Krogsgaard et al. 2003), level of TCR (Labrecque et al. 2001) and co-receptor (Cawthon, Lu, and Alexander-Miller 2001) expression, post-translational modifications to the TCR (Kuball et al. 2009) and spatial arrangement of TCR components (Minguet et al. 2007, Fahmy et al. 2001), whereas exposure to inflammatory cytokines can enhance T cell sensitivity to activation (Richer, Nolz, and Harty 2013). Self-reactivity during thymic development also tunes the sensitivity of a T cell to stimulation by non-self antigens in the periphery through negative regulation of TCR signaling (Persaud et al. 2014). While the TCR sequence largely contributes to affinity for pMHC and tuning of the TCR in the thymus, it is not the sole determinant of avidity because even monoclonal T cells have varied functional avidity (Ioannidou et al. 2017). Antigen experience also contributes to T cell avidity, as memory T cells are more sensitive to stimulation than naïve or recently stimulated T cells (von Essen, Kongsbak, and Geisler 2012, Hesse et al. 2001). Thus, the diversity of the T cell repertoire is not only represented by the range of individual TCR sequences or specificities but also by the combination of parameters that determine avidity for a particular pMHC.

Several animal models have demonstrated that the avidity profile of peripheral T cell populations increases following foreign antigen encounter (Busch and Pamer 1999, Savage, Boniface, and Davis 1999b) through the selective expansion of high-affinity T cells that may better compete for limited amounts of antigen and receive a stronger signal to proliferate and survive (Bos et al. 2012, Ozga et al. 2016). In addition, T_{REGs} may preferentially inhibit low-avidity T cells (Pace et al. 2012). This accumulation of high-affinity and/or avidity T cells, based on interactions with pMHC, has been termed “avidity maturation” at the T cell population level,

in contrast to “affinity maturation” of B cells. Using the intensity of staining with pMHC multimer as a correlate for functional avidity (Busch and Pamer 1999, Dutoit et al. 2001), our group showed that allospecific $CD4^+$ and $CD8^+$ T cells in mice rejecting major MHC mismatched heart allografts also experienced a population-wide increase in avidity (Miller et al. 2018). Increased avidity during allograft rejection was associated with skewing of the TCR sequence representation in the allospecific repertoire but not an increase in other potential contributors to avidity such as co-receptor or TCR expression levels. By contrast, alloreactive T_{CONVs} from mice that were made tolerant to heart allografts with anti-CD154 and DST did not undergo avidity maturation and likewise did not substantially shift their TCR repertoire despite up-regulation of the activation marker CD44. This low-avidity profile in tolerized mice was stable, even upon re-challenge with donor antigen in the absence of co-stimulation blockade, and was due, at least partly, to an inhibition of expansion of high-avidity clones. These data reveal that therapeutically induced peripheral tolerance can durably shape T cell repertoires and suggests that reducing the abundance of high-avidity, donor-specific effector T cell clones may be desirable to achieve long-term tolerance.

Here, we determined whether controlling the expansion of high avidity T cell clones was advantageous for preventing graft rejection during tolerance. By limiting the adaptive alloimmune response to a titrated dose of monoclonal $CD8^+$ T cells with varied avidities for the same alloantigen, we found that high avidity cells were more potent mediators of graft rejection than their lower avidity counterparts, though low avidity cells were still capable of rejection at high doses. Thus, high avidity allospecific T cells are particularly dangerous for allograft survival. We have also developed an approach for the generation of TCR-retrogenic $CD4^+$ T cells with varied avidities for 2W:I-A^b, which will allow us to determine whether our findings

translate to $CD4^+$ T cells, an important question as $CD4^+$ T cells are necessary for heart allograft rejection (Krieger, Yin, and Fathman 1996).

We also investigated which immune mechanisms prevent the expansion of high avidity T cell clones during tolerance. We found that T_{REGs} contributed to the restriction of avidity maturation of T cells during the maintenance of tolerance for some but not other allospecificities, suggesting that other active or passive suppressive mechanisms of T cell avidity maturation are likely in place. Interestingly, T cells of every allospecificity tested were found to expand upon re-challenge with T_{REG} depletion even if they did not avidity mature, indicating that expansion and avidity maturation may be regulated independently. Lm-induced rejection in previously tolerant heart allograft recipients was also accompanied by avidity maturation in T cells of some allospecificities. Understanding the mechanisms of Lm-induced rejection may, therefore, reveal additional factors that can control T cell avidity maturation.

Determining whether high and low avidity cells pose unique threats to the allograft is important for understanding the mechanisms and potential shortcomings of tolerance. High avidity $CD8^+$ T cells are particularly strong mediators of rejection. Limiting the number of high avidity cells may therefore limit the potential damage if other mechanisms of tolerance were disrupted. We have also found that allospecific T cells vary widely in their potency for mediating rejection. Thus, it may be advantageous to distinguish between high and low avidity T cells in functional assays to monitor alloreactivity in patients. The presence of high avidity T cells may also serve as a useful biomarker to detect unstable tolerance in transplant recipients with spontaneous or therapeutic tolerance.

Materials and Methods

Mice

B6 and BALB/c mice were purchased from Envigo RMS, CD45.1^{+} mice and OT-I TCR-Tg Rag$^{-/-}$ (OT-I) mice were purchased from Jackson Laboratory, and P14 TCR-Tg Rag$^{-/-}$ (P14 Rag$^{-/-}$) mice were bred in house from mice purchased from Taconic Biosciences. OT-3 TCR-Tg TCRα$^{-/-}$ (OT-3) mice were obtained from Stephen Schoenberger (La Jolla Institute) and Vβ3-Tg FoxP3^{GFP}, Vβ8.3-Tg FoxP3^{GFP}, Vβ3-Tg TCRα$^{-/-}$ CD4^{cre} and Foxp3^{DTR} mice were obtained from Peter Savage (University of Chicago). Because DTR is incorporated into the endogenous FoxP3 locus on the X chromosome in these mice, only FoxP3$^{DTR/DTR}$ females and FoxP3$^{DTR/y}$ males were used in experiments. Mice expressing a membrane-bound fusion protein of 2W1S and ovalbumin (2W-mOva) on a B6 background, as previously described (Moon et al. 2011), were crossed to BALB/c mice to generate F1 progeny. Both male and female mice at least 6 weeks of age were used for the experiments. In some experiments, mice were treated with one-sixth to one-quarter spleen for DST (intravenous [i.v.]) on day 0 ± 0.6 mg αCD154 (i.v.). In some experiments, mice were re-challenged with a second DST injection i.v. 30 or 60 days after primary immunization in the presence or absence of a single 100 μg dose of agonistic anti-CD40 (FGK4.5) or 1 μg of DT (Sigma) on days 28, 29, and 31. Mice were housed under specific pathogen-free conditions.

Skin transplantation

Tail skin from F1 2W-mOva mice was transplanted onto the flank of P14 Rag$^{-/-}$ recipients as previously described (Kellersmann and Zhong 1998). Sorted OT-I or OT-3 T cells were

adoptively transferred on the day of transplantation. Bandages were removed 7 days post-transplantation. Rejection was reported when <20% of the transplanted skin was viable.

Heart transplantation

Cardiac transplantation was performed using a technique adapted from Corry et al (Corry, Winn, and Russell 1973). For induction of tolerance, mice were treated with 500-600 µg of anti-CD154 (MR1, BioXCell) on days 0, (i.v.) 7 and 14 (i.p.) post-transplantation and DST (i.v.) on day 0.

Magnetic enrichment

T cells were magnetically enriched by negative selection from spleens of recipient mice following binding with biotinylated anti-CD19 (Fitch Monoclonal Facility), anti-Ter119 (eBioscience), and anti-CD11b (eBioscience) and incubation with streptavidin magnetic beads (Pierce, Thermo Fisher Scientific). APCs were magnetically enriched by negative selection of splenocytes from CD45.1$^+$ B6 mice following staining with anti-Thy1.2-biotin (eBioscience) and incubation with streptavidin magnetic beads (Pierce).

Lm infection

An overnight culture of Lm engineered to express GFP (Lm-GFP) (Ertelt et al. 2009) was diluted 1:50 and grown until OD_{600} was within the log-phase of our Lm-GFP growth curve. OD_{600} was used to calculate colony forming units (CFU)/mL. Mice were infected i.p. with $1x10^6$ CFU Lm-GFP in 400 µL per mouse as this dose resulted in the highest rejection rate with minimal lethality. Lm-GFP was plated to confirm dose. Graft survival was monitored twice per week after infection.

Flow cytometry

$5x10^6$ T-enriched cells were stained with a fixable live/dead stain (Invitrogen) followed by phycoerytherin (PE)- or allophycocyanin (APC)-conjugated 2W:I-A^b or pK^d:I-A^b tetramer (NIH Tetramer Core Facility) for 1 hour in a room temperature (RT) water bath, washed, and then stained with PE- or APC-conjugated Ova:K^b pentamers (ProImmune) or H60:K^b tetramers (NIH Tetramer Core Facility) for 20 minutes in a room temperature (RT) water bath. Cells were then stained with fluorophore conjugated anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD44 (IM7), anti-TCRβ (H57-597), and anti-B220 (RA3-6B2). Surface-stained cells were then fixed with the FoxP3 fixation permeabilization buffer kit (eBioscience, San Diego, California) for 15 minutes at RT and washed with 1x permeabilization buffer. Some samples were intracellularly stained with anti-Ki67 (SolA15), anti-IRF4 (3E4), or anti-FoxP3 (FJK-16s) for 30 minutes at RT; washed with permeabilization buffer; and analyzed by flow cytometry. CFSE labeling was performed by labeling cells with CellTrace CFSE (Invitrogen) for 20 minutes at 37°C. All monoclonal antibodies (mAbs) were from BD Biosciences, eBioscience, or Invitrogen.

Adoptive cell transfer

Spleen and lymph node cells were isolated into single-cell suspensions and counted with an Accuri C6 flow cytometer (BD Biosciences, San Jose, California). For direct comparison of skin allograft rejection or homeostatic proliferation by OT-I and OT-3 cells, $CD8^+$ $CD44^{lo}$ TCR-Tg cells were sorted before adoptive transfer in 200 μL of PBS. In some experiments, APCs and B cells were magnetically depleted before sorting. For comparison of homeostatic proliferation of OT-I versus OT-3 cells, 1.5×10^5 cells were injected.

Tetramer sorting for *in vitro* stimulation

Spleens were isolated from B6 mice 7 days after immunization with F1 2W-mOVA splenocytes, pooled in groups of three spleens, homogenized, and magnetically enriched for T cells. Cells were stained with APC-conjugated 2W:I-A^b tetramers for 1 hour in a room temperature water bath, washed, and then stained with PE-conjugated Ova:K^b pentamers for 20 minutes in a room temperature water bath. Cells were then stained with anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD44 (IM7), and anti-B220 (RA3-6B2). Within the population of $CD4^+$ $CD44^+$ 2W:I-A^{b+} cells, 30% of cells with the brightest 2W:I-A^b staining were sorted as 2W:I-A^b Hi. The 20% next brightest were not sorted. The following 30% brightest were sorted as 2W:I-A^b Lo. The dimmest 20% of 2W:I-A^{b+} cells were not sorted. $CD4^+$ $CD44^+$ 2W:I-A^{b-} cells were sorted as 2W:I-A^b Neg. Gates were adjusted between samples before sorting so that the percentage of cells in each gate remained the same across samples.

Generation of TCR-Retrogenic mice

Spleens were isolated from B6 Vβ3-Tg $FoxP3^{GFP}$ mice 8 days after immunization with F1 2W-mOva splenocytes and then homogenized. Pilot experiments also investigated Vβ8.3-Tg $FoxP3^{GFP}$ mice as a source for $tetramer^+$ cells but Vβ3-Tg $FoxP3^{GFP}$ mice were selected as they yielded more $FoxP3^-$ $CD44^{hi}$ 2W:I-A^b $tetramer^+$ cells after immunization. Cells were stained with APC-coupled-2W:I-A^b tetramers for 1 hour in a room temperature water bath, washed, and then magnetically enriched for T cells as we had seen that enrichment after tetramer staining was associated with less non-specific tetramer staining than enrichment before tetramer staining. Enriched samples were then stained with anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD44 (IM7), and anti-B220 (RA3-6B2). All staining and wash buffers contained 50 nM of the protein kinase

inhibitor Dasatinib (AdooQ Bioscience) to prevent internalization of the TCR during multimer staining (Lissina et al. 2009). Within the population of $CD4^+$ $FoxP3^-$ $CD44^+$ 2W:I-A^{b+} cells, 30% of cells with the brightest 2W:I-A^b staining were sorted as 2W:I-A^b Hi. The 20% next brightest were not sorted. The following 30% brightest were sorted as 2W:I-A^b Lo. The dimmest 20% of 2W:I-A^{b+} cells were not sorted. Gates were set using a global average containing an equal number of cells from each sample. Cells were sorted directly into buffer RLT (QIAGEN) containing β-mercaptoethanol, then immediately homogenized, frozen in ethanol and dry ice and stored at -80˚C until RNA isolation.

Cell lysates of the same tetramer binding intensity were pooled into three samples from 3-4 mice each prior to RNA isolation with RNeasy micro kit (QIAGEN). Isolated RNA was sent to iRepertoire for TCRα sequencing. CDR3 sequences identified in at least 2 samples of the same tetramer binding intensity were considered candidates for Rg T cell generation. Seven CDR3 sequences were selected for cloning based on their relatively high abundance and diverse distribution in RNA from 2W:I-A^b Hi and Lo cells.

Full-length TCRα sequences were reconstructed from CDR3 sequences using the International Immunogenetics information system (IMGT) V-quest alignment tool. Gibson reactions were designed using NEBuilder for construction of full length TCRα from fragments encoding the variable (TRAV) and constant (TRAC) regions into a pMGflThy1.1 plasmid vector (McDonald et al. 2015). DNA encoding TRAVs was purchased from Genscript (and primers were purchased from Integrated DNA Technologies. DNA encoding TRAC and the pMGflThy1.1 vector were obtained from the Savage Lab. TRAV and TRAC fragments were amplified using Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich,

Massachusetts) and amplification products were purified using a QIAquick Gel Extraction Kit (QIAGEN). pMGflThy1.1 was linearized by simultaneous digestion with High Fidelity AgeI and High Fidelity NotI restriction enzymes in CutSmart Buffer (New England BioLabs). Gibson assembly of TRAV, TRAC and linearized pMGflThy1.1 was performed with Gibson Assembly Master Mix (New England BioLabs) under the conditions specified by NEBuilder. Assembly products were cloned into 5-alpha Competent E. coli (New England BioLabs). Colonies resistant to ampicillin were screened by sequencing the inserted TRAV and TRAC. Maxi-prep of plasmids was performed using an EndoFree Plasmid Maxi Kit (QIAGEN).

Platinum E (PlatE) cells obtained from the Savage Lab were transfected with pMGflThy1.1-TRAC-TRAV constructs using Lipofectamine 2000 (Invitrogen). Transfected cells were cultured in DMEM with 10% fetal bovine serum (FBS) for 24-48 hours. Transfection was confirmed by GFP expression. Retrovirus was collected by passing culture supernatant through a 0.45 μm filter. Filtrate was frozen in ethanol and dry ice and stored at -80˚C until use.

Vβ3-Tg TCR$\alpha^{-/-}$ CD4^{cre} mice were injected with 150 mg/kg 5-Fluorouracil (APP Pharmaceuticals) 3 days prior to bone marrow harvest. Bone marrow harvested from femura and tibiae was cultured for 2 days in X-Vivo 10 (Lonza) with 1% penicillin and streptomycin (pen/strep), 15% FBS, 100 ng/mL mouse stem cell factor (SCF), 10 ng/mL mouse IL-3 and 20 ng/mL human IL-6 (Biolegend). Cultured cells were transduced by centrifugation at 900 g for 90 minutes at 37˚C in the presence of virus, 4 μg/mL polybrene (EMD Millipore), mouse SCF, mouse IL-3 and human Il-6. Transduced cells were cultured for 24 hours before being harvested for BMT.

BMT recipient mice (B6 CD45.1^{+}) were lethally irradiated 24 hours before i.v. injection with 1x10^{6} transduced bone marrow cells and 5x10^{6} freshly isolated filler bone marrow cells from a Rag$^{-/-}$ donor. At least 6 weeks after BMT, peripheral blood of recipient mice was stained with antibodies to identify CD45.2/2 Thy1.1^{+} Rg T cells. Mice were used in experiments only after Rg T cells had been identified in their peripheral blood.

In vitro stimulation

5×10^{3} sorted 2W:I-A^{b} Hi, 2W:I-A^{b} Lo, or 2W:I-A^{b} Neg cells were co-cultured with 2×10^{4} APC-enriched CD45.1^{+} B6 splenocytes and 25 μg/mL 2W peptide for 6 hours at 37°C and 5% CO_2. For peptide stimulation of OT-I and OT-3 T cells, splenocytes were isolated from CD45.1^{+} OT-I and OT-3 mice 7 days after immunization with 2W-mOva DST and then magnetically enriched for T cells. 1×10^{4} T cell-enriched OT-I or OT-3 T cells were then co-cultured with 4×10^{4} CD45.1^{-} APC-enriched B6 splenocytes, and the indicated dose of SIINFEKL peptide for 6 hours at 37°C and 5% CO_2. Stimulated cells were then stained with a fixable live/dead stain (Aqua, Invitrogen), washed, and stained with anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD45.1 (A20), and anti-CD45.2 (104). Using the FoxP3 fixation buffer set as mentioned earlier, surface-stained cells were fixed and intracellularly stained with anti-IRF4 (3E4) for 30 minutes at room temperature and then were analyzed by flow cytometry.

To determine the specificity and avidity of Rg T cells, spleens and lymph nodes were harvested from Rg BMT recipients CD45.2/2 Thy1.1^{+} cells were detected in their peripheral blood. Cells were homogenized, labeled with CellTrace Violet (CTV) (Invitrogen) for 20 minutes, magnetically enriched for T cells and sorted for CD4^{+} CD8^{-} CD45.2^{+} Thy1.1^{+} CD44^{lo} cells. 2x10^{4} sorted T cells were co-cultured with 8x10^{4} APC-enriched B6 or F1 APCs with or

without 10 μg/mL OT-2 peptide (AAHAEINEA), a titration of 2W peptide (EAWGALANWAVDSA) (Genscript) or 1 μg/mL anti-CD3 (2C11). Unstimulated controls were cultured with 1 ng/mL human IL-7 (PeproTech). After 3 days of culture at 37˚C and 5% CO_2, dilution of CTV was observed in live Thy1.1$^+$ cells by flow cytometry.

Data analysis

Flow cytometry data were analyzed using FlowJo (Tree Star). Flow cytometry samples were gated on live single cells before analysis. Tetramer stained samples were also gated on B220- cells. Statistical analyses were performed where appropriate using GraphPad Prism (GraphPad). Each statistical test is listed in the figure legends.

Study Approval

The studies were performed in agreement with the University of Chicago's Institutional Animal Care and Use Committee, according to the NIH guidelines for animal use.

Results

T cell structural avidity is associated with functional avidity in vitro

To investigate whether differential pMHC binding avidity translated into distinct functional avidity, an injection of DST from 2W-mOva-transgenic F1 mice into B6 hosts was used to expand donor-reactive T cells. Seven days later, secondary lymphoid organ cells were stained with pMHC multimers and sorted by flow cytometry into high- and low-multimer binders (Fig. 2.1A) before re-stimulation with peptide for 6 hours. A larger percentage of the high than the low 2W:I-A^b-binding T cells up-regulated expression of interferon regulatory factor 4 (IRF4) (Fig.

2.1B), a quantifier of TCR signal strength (Krishnamoorthy et al. 2017), demonstrating more limited functional avidity of low 2W:I-A^b-binding T cells. A similar analysis with Ova:K^b-binding T cells could not be performed, because cell sorting using Ova:K^b pentamers resulted in maximal IRF4 expression even without peptide re-stimulation, an artifact of pMHC multimers inducing TCR signaling during the sorting process.

To circumvent these caveats, we obtained OT-I and OT-3 mice whose T cells can be sorted with congenic markers, thus leaving their TCRs untouched. As shown by their differential binding to Ova:K^b (Fig. 2.2A), OT-I and OT-3 T cells span the range of avidities that can represented by Ova:K^b tetramer binding. This difference in pMHC binding correlated with a difference in T cell signaling upon Ova peptide stimulation, as evidenced by IRF4 staining (Fig. 2.2B). Indeed, OT-3 cells required about 50-fold more peptide to elicit a similar magnitude of response as OT-I cells.

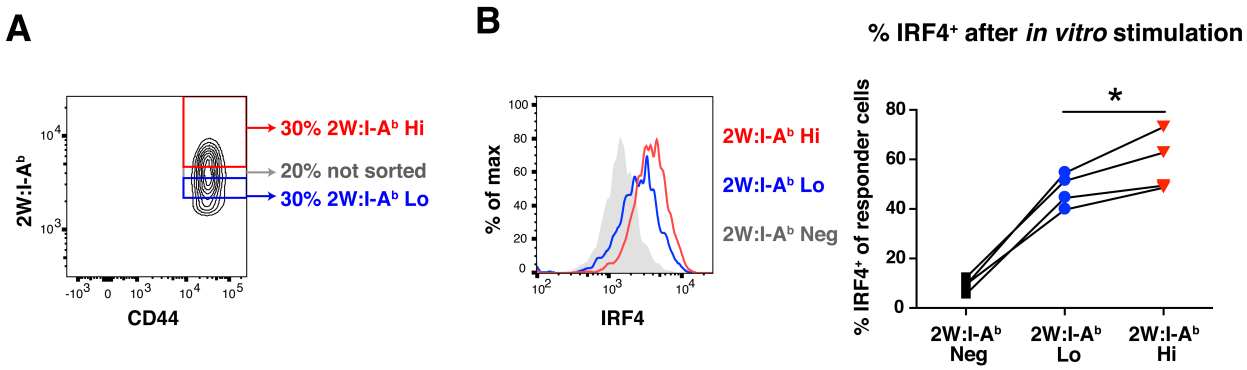

Figure 2.1 pMHC tetramer binding intensity correlates with functional avidity.

(A) Flow cytometry plot showing strategy for sorting 2W:I-A^b bright (Hi), dim (Lo), and negative (Neg) cells from B6 mice 7 days after immunization with 2W-mOva F1 splenocytes. (B) Left: representative histogram showing levels of IRF4 staining in tetramer-sorted, 2W:I-A^b-specific T cells after *in vitro* stimulation with 2W peptide. Right: percentage of IRF4$^+$ tetramer-sorted, 2W:I-A^b-specific T cells after *in vitro* stimulation with 2W peptide. Results are from one experiment. Data were analyzed by one-way paired ANOVA. *p<0.05.

High avidity CD8$^+$ T cells are more potent in rejecting skin allografts than low avidity CD8$^+$ T cells specific for the same alloantigen.

To determine the impact of T cell avidity for alloantigen on transplant rejection, we used an Ova-expressing skin allograft model. P14 Rag$^{-/-}$ mice we used as hosts of OT-I and OT-3 cells to prevent homeostatic proliferation of the transferred cells (Fig. 2.2D), while ensuring that only the transferred cells recognized the graft, as P14 T cells recognize an irrelevant antigen. Whereas 10^3 high-affinity OT-I T cells rejected Ova-expressing skin grafts readily, even 10^4 low-avidity OT-3 T cells failed to reject (Fig. 2.2C), demonstrating the superior capacity of high-avidity T cells to reject allografts over that of low-avidity T cells. Importantly, higher doses of OT-3 T cells were capable of rejecting Ova-expressing skin grafts.

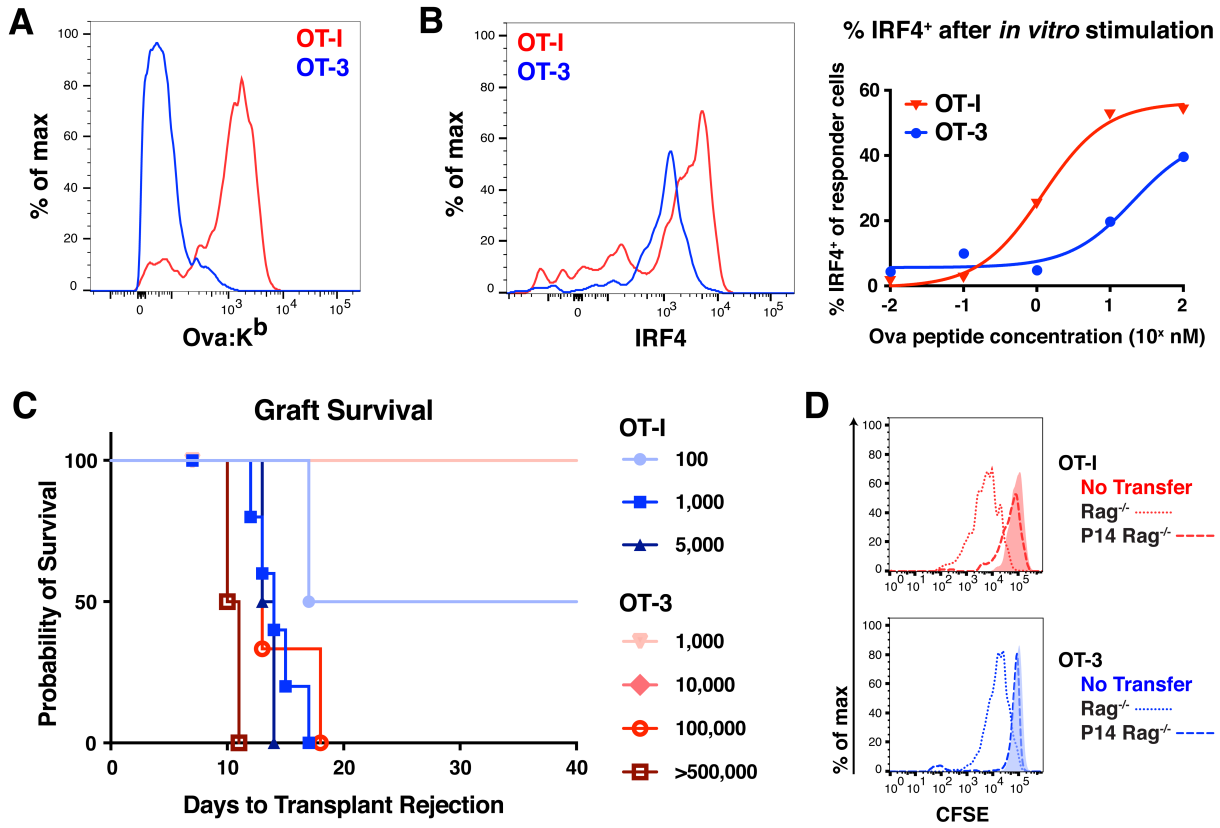

Figure 2.2 T cells with high avidity for alloantigen are more potent in rejecting skin grafts than T cells with lower avidity for the same antigen.

(A) Histogram showing intensity of Ova:K^b pentamer binding in naive OT-I and OT-3 T cells. (B) Left: representative histogram showing levels of IRF4 staining in OT-I and OT-3 T cells after *in vitro* stimulation with 10 nM SIINFEKL peptide. Right: percent $IRF4^+$ in OT-I or OT-3 responder T cells after *in vitro* stimulation with a dose titration of SIINFEKL peptide. Lines indicate nonlinear regression analysis. (C) Survival of 2W-mOva B6 skin allografts in P14 $Rag^{-/-}$ recipients adoptively transferred with the indicated number of $CD44^-$ OT-I or OT-3 T cells. (D) Histograms showing CFSE dilution in $CD44^-$ OT-I and OT-3 T cells 6 days after adoptive transfer into $Rag^{-/-}$ or P14 $Rag^{-/-}$ mice or after 6 days of *in vitro* culture without stimulation. Results in (C) are pooled from 2 independent experiments.

Co-stimulation and alloantigen exposure are not sufficient to increase T cell avidity for alloantigen after co-stimulation blockade.

Having determined that high avidity cells mediated a stronger alloimmune response on a per-cell basis, we next investigated the mechanisms preventing avidity maturation in co-stimulation blockade-treated mice. Similar to the increased population avidity reported upon secondary response to infections (Busch and Pamer, 1999), re-challenge of DST-immunized mice with

donor splenocytes resulted in a further increase in pMHC multimer binding, characteristic of a memory T cell response. In contrast, the low-avidity profile of allospecific T cells in mice primed with DST in the presence of co-stimulation blockade remained low after re-stimulation 30 days later (Miller et al. 2018). These results suggest the T cell-population avidity profile is altered long term upon antigen exposure in the presence of initial co-stimulation blockade, despite the potential input of new donor-reactive thymic emigrants.

We had found that adding co-stimulation in the form of agonistic anti-CD40 at the time of priming with anti-CD154 and DST could rescue avidity maturation. We hypothesized that providing agonistic anti-CD40 at the time of antigen re-challenge could also allow avidity maturation in mice that had been primed with anti-CD154 and DST. To test this, we treated mice with anti-CD154 and 2W-mOva F1 DST, then 30 days later re-challenged them with 2W-mOva F1 DST with or without anti-CD40 (Fig. 2.3A). 1 week after re-challenge, we measured the intensity of pMHC multimer binding among 2W-I-A^b, Ova:K^b and H60:K^b tetramer$^+$ cells. We did not see an increase in tetramer binding avidity in the group re-challenged with anti-CD40 compared to the group re-challenged without anti-CD40 (Fig. 2.3B). Thus, provision of additional co-stimulation through the CD40 pathway is not sufficient to rescue avidity maturation in mice treated with co-stimulation blockade at the time of priming. Interestingly, the quantity of 2W-I-A^b and H60:K^b tetramer$^+$ cells increased in mice after re-challenge (Fig. 2.3C), suggesting that avidity maturation and cell expansion are independently regulated.

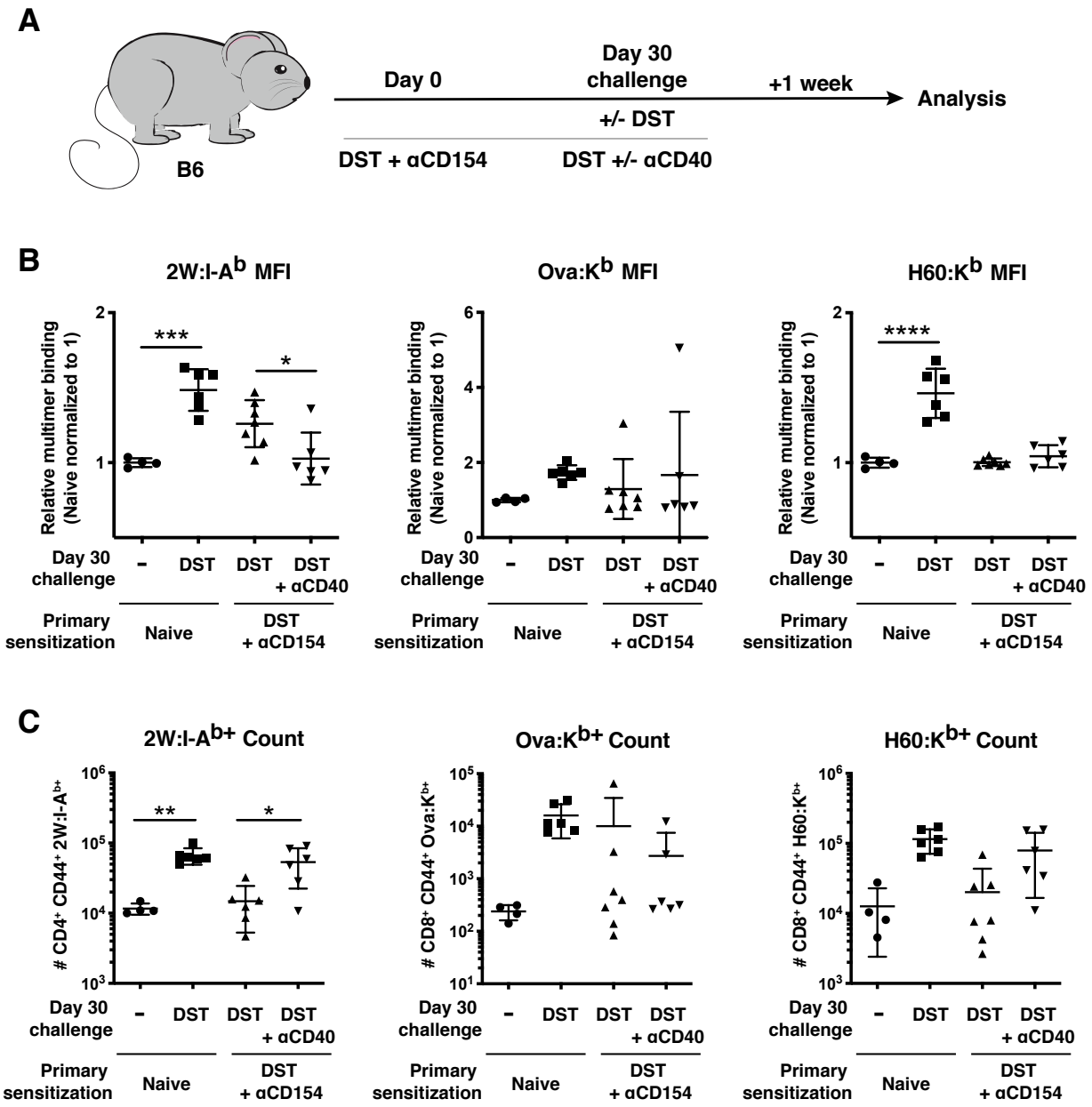

Figure 2.3 Co-stimulation and antigen re-challenge are not sufficient to drive avidity maturation in T cells primed in the presence of co-stimulation blockade.

(A) Experimental design. (B) Normalized MFI of 2W:I-A^b, Ova:K^b and H60:K^b multimer binding in tetramer$^+$ splenic T cells isolated from naïve mice, mice 30 days post-immunization with 2W-mOva DST or anti-CD154/DST-primed mice 1 week post-re-challenge. Cells from naïve mice were gated on CD44^{lo} and all other groups were gated on CD44^{hi} cells. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. (C) Number of 2W:I-A^b, Ova:K^b and H60:K^b multimer binding cells per spleen of mice treated as in (B). Data were pooled from 2 independent experiments. Each data point represents an individual mouse with lines indicating average +/- SD. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

After co-stimulation blockade T_{REGs} are necessary for maintaining low avidity for some alloantigens in the recipient T cell population.

Considering the importance of T_{REGs} in maintaining transplantation tolerance, we investigated whether T_{REGs} are necessary for constraining the avidity profile of allospecific T cells after anti-CD154/DST priming. To test this, we treated B6 WT or FoxP3^{DTR} mice with anti-CD154/2W-mOva F1 DST, then 30 days later re-challenged mice with 2W-mOva F1 DST while treating with DT to selectively deplete T_{REGs} in the FoxP3^{DTR} mice (Figs. 2.4A, B). We found that T_{REG} depletion upon re-challenge was associated with increased avidity for Ova:K^{b}, but not 2W:I-A^{b} or H60:K^{b}, indicating that constraint of avidity maturation does depend on T_{REGs} for some T cell specificities but not others. Similar to re-challenge with agonistic anti-CD40, re-challenge with T_{REG} depletion resulted in expansion of T cells of every specificity evaluated.

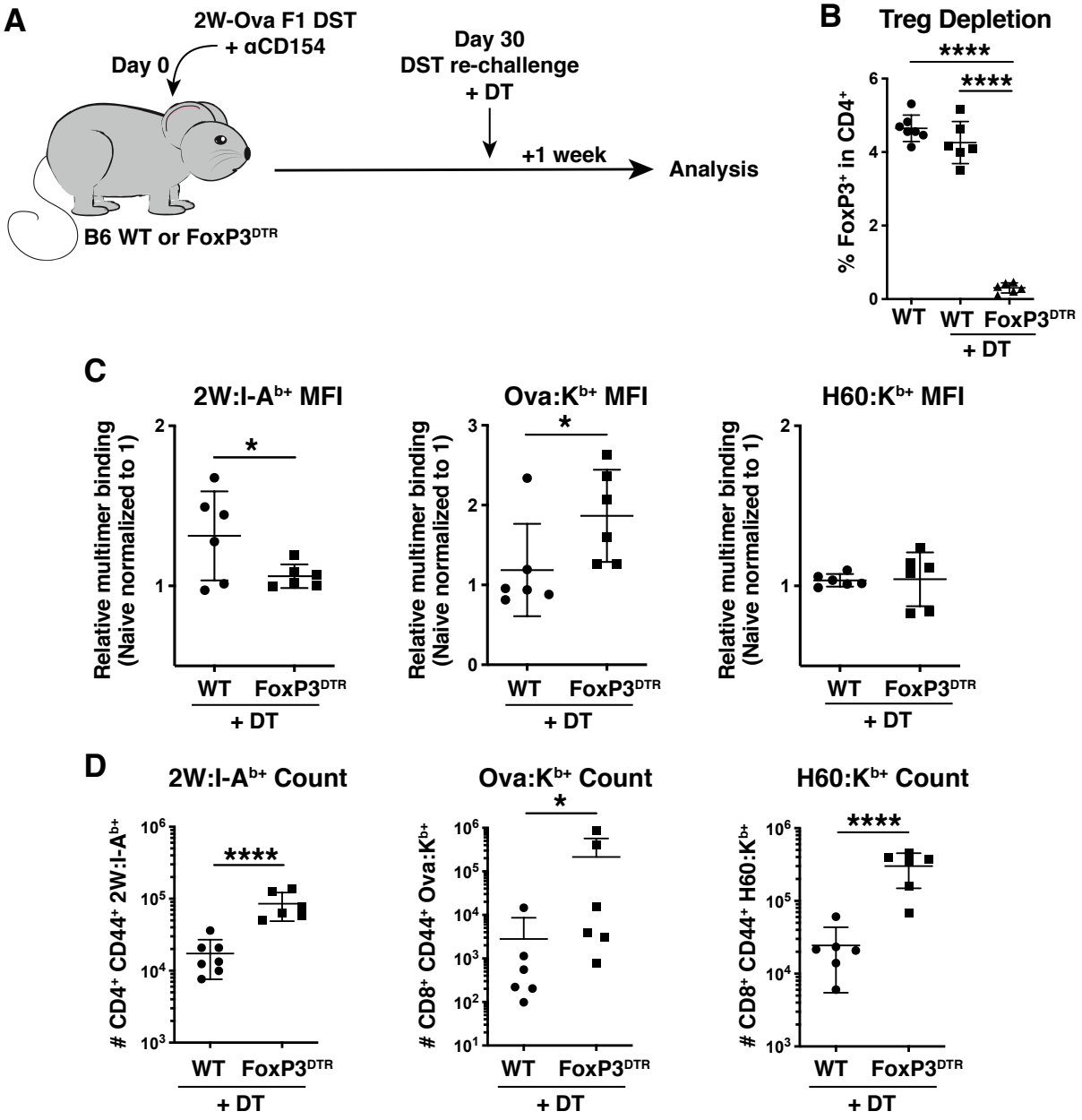

Figure 2.4 T_{REG} depletion at the time of antigen re-challenge allows avidity maturation of some T cells primed in the presence of co-stimulation blockade.

WT or FoxP3^{DTR} mice were primed with 2W-mOva F1 DST and treated with anti-CD154. 30 days after priming, mice were re-challenged with DST and treated with DT to deplete T_{REGs} from FoxP3^{DTR} mice. (A) Experimental design. (B) Percent FoxP3$^+$ in CD4$^+$ cells in peripheral blood of naïve WT mice or anti-CD154/DST-primed WT or FoxP3^{DTR} mice 1 day after the second injection of DT. (C) Normalized tetramer MFI and (D) number of 2W:I-A^b, Ova:K^b and H60:K^b tetramer-binding splenic T cells isolated from mice 1 week post-re-challenge. MFI values were normalized, with the average of the MFI for naïve mice in each experiment set to 1. Data were pooled from 2 independent experiments. Each data point represents an individual mouse with lines indicating average +/- SD. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

While allospecific T cells in un-transplanted anti-CD154/DST-treated mice experience TCR stimulation without adequate co-stimulation similar to transplant recipients tolerized with anti-CD154/DST, the persistence of antigen in tolerant graft recipients may apply additional mechanisms to constrain avidity maturation. Thus, we investigated whether T_{REGs} were required to prevent avidity maturation in allospecific T cells persisting in tolerant heart allograft recipients. B6 WT or FoxP3^{DTR} mice were tolerized to BALB/c heart allografts using anti-CD154/DST. At least 30 days post-heart transplantation, recipients were treated with DT, resulting in rejection in all FoxP3^{DTR} mice within 11 days and survival of all WT mice (Fig. 2.5). Tetramer binding intensity was measured in pK^{d}:I-A^{b} or H60:K^{b} tetramer-binding splenocytes 12 or 35 days after DT treatment. No difference was observed in pK^{d}:I-A^{b} tetramer MFI 12 days post-DT. On day 35 post-DT, however, we observed an increase in pK^{d}:I-A^{b} tetramer MFI in 2/3 mice compared to naïve and tolerant WT mice. The increase in avidity after DT exceeded that observed in B6 mice 35 days after DST immunization. Consistent with our findings in anti-CD154/DST-primed mice (Fig. 2.4), tetramer binding intensity in H60:K^{b} was unchanged after T_{REG} depletion.

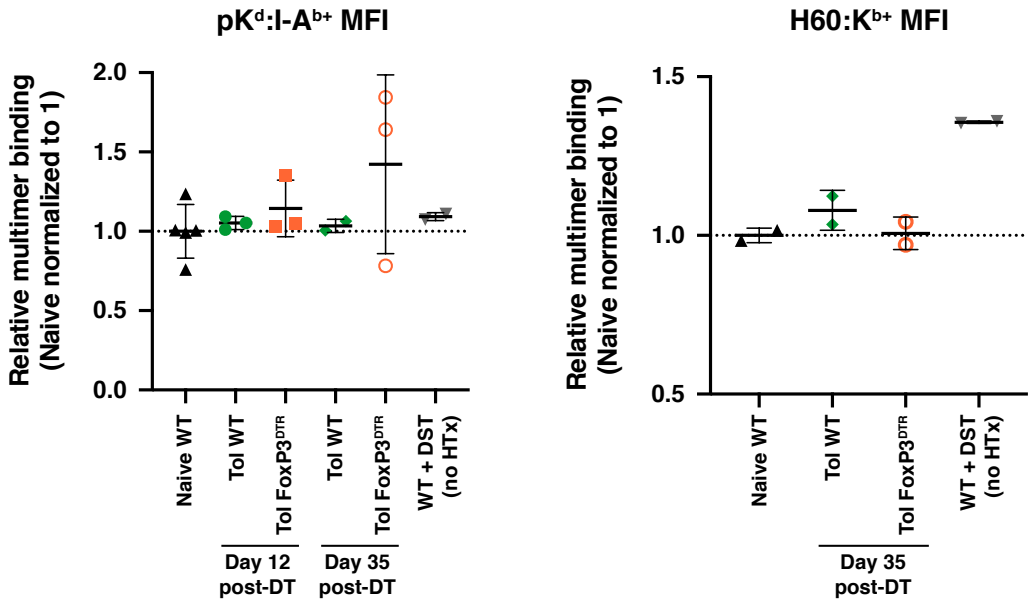

Figure 2.5 T_{REG} depletion in previously tolerant heart allograft recipients may result in avidity maturation of some allospecific T cells.

Normalized MFI of pK^d:$I\text{-}A^b$ and $H60:K^b$ multimer binding in endogenous alloreactive splenic T cells isolated from naïve WT mice, previously tolerant WT or $FoxP3^{DTR}$ BALB/c heart recipients 12 or 35 days after DT injection or WT mice 35 days after immunization with BALB/c DST. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Results were pooled from 1-2 independent experiments. Each data point represents an individual mouse with lines indicating average +/- SD.

Avidity maturation in allospecific T cells after Lm-induced rejection.

Lm infection results in T cell-dependent rejection in previously tolerant heart allograft recipients (Wang et al. 2010b). We sought to determine whether Lm-induced rejection was associated with loss of constraint of avidity maturation. B6 mice were tolerized to BALB/c heart allografts with anti-CD154/DST and 35 days later infected with Lm-GFP, which resulted in rejection in 2/5 mice and reduced allograft function without rejection in 2/5 mice. 40 days after Lm infection, the tetramer MFI was measured in pK^d:$I\text{-}A^b$ and $H60:K^b$ tetramer-binding splenocytes. While pK^d:$I\text{-}A^b$ MFI is not increased in stably tolerant mice compared to naïve mice (Fig. 2.5), we found a significant increase in pK^d:$I\text{-}A^b$ MFI after Lm-induced rejection compared

to naïve mice (Fig. 2.6). Consistent with previous findings (Figs. 2.4C, 2.5), H60:K^b MFI remained stable after tolerance was disrupted by Lm infection.

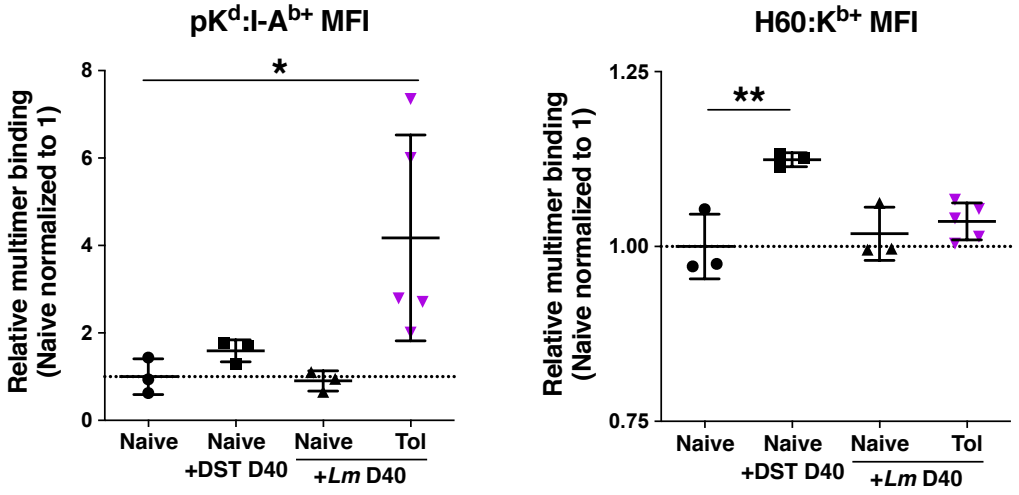

Figure 2.6 Lm-induced rejection in previously tolerant heart allograft recipients results in avidity maturation of T cells specific for some alloantigens.

Normalized MFI of pK^d:I-A^b and H60:K^b multimer binding in endogenous alloreactive splenic T cells isolated from naïve mice, mice 40 days after immunization with BALB/c DST or previously tolerant heart recipients 40 days after Lm-GFP infection. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Results are from one experiment. Each data point represents and individual mouse with lines indicating average +/- SD. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons. *$p<0.05$; **$p<0.01$.

Discussion

In animal models, co-stimulation blockade therapy has been shown to induce robust donor-specific tolerance through peripheral T cell mechanisms (Markees et al. 1998), including clonal deletion and anergy and/or exhaustion at a cell-intrinsic level and suppression by regulatory cells at a cell-extrinsic level (Pinelli et al. 2013, Quezada et al. 2005, Miller et al. 2019). Using allogeneic pMHC multimer binding intensity as a correlate for T cell sensitivity, or avidity, for alloantigen we recently observed that peripheral transplantation tolerance induced with anti-CD154/DST was also associated with the persistence of graft-reactive $CD4^+$ and $CD8^+$

T cells with low avidity for alloantigens. Unlike rejecting graft recipients, whose allospecific T cell populations increase in average avidity for alloantigen, a process known as avidity maturation, the allospecific $CD4^+$ and $CD8^+$ T cell populations in tolerant graft recipients maintained low avidity overall, similar to the avidity of alloreactive T cells observed in naïve mice. Constraining avidity depended on limiting the selective expansion of higher-avidity T cell clones (Miller et al. 2018). These findings highlight a previously unknown element of peripheral transplantation tolerance, in which allospecific T cells are shaped at the population-level. In this study, we have investigated the functional consequences of limiting T cell avidity for alloantigen and whether preventing the expansion of high avidity allospecific T cell clones supports graft survival during anti-CD154/DST-induced transplantation tolerance.

To compare the strength of alloimmune response mediated by T cells with high versus low avidity for alloantigen, we developed an experimental system to compare skin allograft rejection kinetics when the adaptive alloimmune response was limited to a defined number of adoptively transferred high or low avidity T cells. By comparing Ova^+ skin graft rejection in recipients adoptively transferred with matched numbers of high avidity $CD8^+$ OT-I T cells versus OT-3 T cells with 10-to-100-fold lower *in vitro* avidity for the same Ova:K^b alloantigen, we determined that OT-I T cells were far more potent at mediating allograft rejection on a per-cell basis. $>1x10^4$ OT-3 T cells were required to reject a graft whereas only $1x10^2$ OT-I T cells were able to consistently reject skin allografts. $1x10^5$ OT-3 T cells were required to reject skin grafts with similar kinetics to $1x10^3$ OT-1 T cells. In this model, a 100-fold greater dose of OT-3 T cells was generally required to mount an alloimmune response with similar strength as a response mediated by OT-I T cells. Thus, high avidity allospecific T cells present a particularly large risk to graft survival in the absence of immunosuppression or tolerance. Future studies,

which are discussed in a later chapter, will determine whether avidity for alloantigen impacts the strength of alloreactivity mediated by $CD4^+$ T cells as well.

The functional enhancement of high versus low avidity T cells that contributes to their stronger potential for alloimmunity remains unknown, although functional differences between high and low avidity cells have been described, most frequently in $CD8^+$ T cells. For example, high avidity T cells were shown to produce more cytokines and effector molecules on a per-cell basis *in vivo* or when antigen was limiting *in vitro* (Zhong et al. 2013, Hesse et al. 2001, Enouz, Carrie, et al. 2012). Intravital imaging revealed that, compared with low avidity monoclonal $CD8^+$ T cells, high avidity T cells made longer contacts with dendritic cells (DCs) and were held in the lymph nodes longer, allowing greater overall expansion (Ozga et al. 2016). Polyclonal high avidity $CD8^+$ T cells were also better able to lyse target cells *in vitro* and *in vivo* (Alexander-Miller, Leggatt, and Berzofsky 1996, Zeh et al. 1999), and they were better able to receive help from $CD4^+$ T cells, further enhancing their function (Zhu et al. 2015). As a result of their enhanced response to antigen, high avidity T cells were more potent than low avidity T cells in mediating anti-tumor immunity as well as autoimmune reactions (Zhong et al. 2013, Zeh et al. 1999). We hypothesize that a lack of avidity maturation may support graft survival during tolerance by limiting the pool of cells that would be most harmful to the graft if the suppressive mechanisms maintaining tolerance were to be disrupted.

Low avidity T_{CONVs}, while less potent in mediating alloimmunity, may be more apt at resisting the suppressive mechanisms underlying induced donor-specific tolerance. During central tolerance to self, low avidity self-reactive T cells were more likely to escape thymic deletion and T_{REG} induction compared with high avidity T cells and persisted in the periphery, where they could be activated and mediate autoimmunity following infection (Enouz, Carrie, et

al. 2012, Zehn and Bevan 2006). Most treatments used to induce donor-specific tolerance rely on peripheral tolerance mechanisms, which also exert different effects on T cells depending on the avidity for self. There are conflicting reports on whether high or low avidity T cells are more likely to undergo activation-induced cell death in the periphery (Anderton and Fillatreau 2008, Black, Armstrong, and Jaffee 2014). Interestingly, a study found that both high and low avidity self-reactive $CD4^+$ T cells were susceptible to becoming anergic, but only high avidity anergic cells upregulated regulatory markers such as CTLA-4 and FoxP3 and suppressed the activation of naïve T cells (Mallone et al. 2005). Future studies using adoptively transferred TCR-Tg or TCR-Rg T cells will determine whether $CD4^+$ or $CD8^+$ T cells with high or low avidity for alloantigen are more likely to prevent tolerance induction or break established tolerance.

We also investigated the mechanisms by which avidity is constrained during tolerance. We had previously found that antigen re-challenge did not restore avidity maturation in mice primed in the presence of anti-CD154, indicating that a lasting change had occurred in the anti-CD154/DST-primed mice to permanently restrict avidity maturation, even in the presence of antigen. Avidity maturation is generally associated with expansion of high avidity clones. Indeed, pharmacologically inhibiting T cell proliferation prevents avidity maturation after priming. Because allospecific T cells in anti-CD154/DST-primed mice do not expand upon re-stimulation, impaired avidity maturation upon re-stimulation in these mice may be the consequence of poor expansion of allospecific T cells, as a consequence of various long-lived mechanisms of tolerance including suppression and/or cell-intrinsic hypo-responsiveness. Although deletion of high-avidity clones could also explain impaired avidity maturation upon re-stimulation, previous TCR spectratyping data from our group had found that there was no reduction in the total number of peaks detected in tolerant heart recipients versus naive mice

(Miller et al. 2018), strongly suggesting that clonal deletion had not occurred during anti-CD154/DST priming or tolerance induction. That allospecific T cells in tolerant graft recipients up-regulated expression of CD44 indicated that the lack of avidity maturation was not a consequence of ignorance by persisting allospecific T cells.

While our group had previously found that avidity maturation could be rescued by provision of additional co-stimulation by agonistic αCD40 at the time of anti-CD154/DST priming, αCD40 could not restore avidity maturation if given only at the time of re-stimulation. Impaired co-stimulation through the CD40 pathway therefore did not explain the permanent constraint of avidity in anti-CD154/DST-primed or tolerant mice. Interestingly, however, anti-CD40 treatment at the time of re-challenge did restore the expansion of allospecific T cell clones specific for 2W:I-A^b and H60:K^b. Other instances of polyclonal T cell expansion without avidity maturation have not been described. It is possible that T cell expansion would occur without avidity maturation under conditions where antigen presentation was abundant enough to exceed the dose required for a maximal response of both high and low avidity cells, and where interclonal competition did not hinder activation of low avidity cells. However, it is hard to imagine a single pMHC complex being this abundant *in vivo*. It is also possible that the initial priming in the presence of anti-CD154/DST tuned the avidity of allospecific T cells so that re-stimulation would elicit a similar response by high and low avidity T cells. The cellular mechanisms of avidity-neutral T cell expansion clearly require more investigation.

We found that resistance to population-level changes depended on the presence of T_{REGs} for Ova:K^b- and pK^d:I-A^b-binding populations, but not for H60:K^b- or 2W:I-A^b-binding populations. For these other specificities, resistance to avidity maturation may be the result of T cell-intrinsic hypo-responsiveness, which makes cells resistant to secondary encounter of antigen

(Philip et al., 2017, Schietinger et al., 2012), or may be constrained by regulatory cells distinct from classical T_{REGs}. Disruption of established tolerance by *L. monocytogenes* infection also induced avidity maturation in pK^d:I-A^b-binding, but not H60:K^b-binding T cells. While the mechanisms of Lm-induced rejection are not well understood, Lm infection has been found to impair T_{REG} function (Rowe et al. 2012). The similar patterns we have observed in avidity maturation after Lm infection and T_{REG} depletion in tolerized heart recipients provide further support for impaired T_{REG} function as a major contributor to Lm-induced rejection. It is not clear which factors determine whether avidity maturation will be constrained by T_{REGs} versus other factors. Work from our lab previously determined that multiple immune mechanisms simultaneously support transplantation tolerance. It is possible that different mechanisms act primarily on distinct allospecific T cells and these data suggest that specificity for a particular alloantigen influences which mechanisms act on a particular cell.

There are strong correlations between accumulation of high-avidity T cells and clearance of infections (Busch and Pamer, 1999), elimination of tumors (Black et al., 2014, Kuball et al., 2009, Soong et al., 2014), better memory responses (Turner et al., 2008, Zehn et al., 2009), and autoimmunity (Maeda et al., 2014). Low-avidity T cells, however, which are correlated with less effector functionality (Falta et al., 2005, Kuball et al., 2009, Tsang et al., 2011), would be beneficial for transplantation. This study demonstrates that high avidity T cells are particularly potent mediators of allograft rejection, and controlling the number and activation of these cells should be a priority in transplantation tolerance. Tracking the activation, suppression and number of high avidity T cells may also serve as an important biomarker for robust tolerance. We found that tolerance induction restricted the expansion of T cells with high avidity for alloantigen, partially dependent on T_{REGs}. We also observed that different T cell subsets are subject to

different immune mechanisms of tolerance, dependent on their specificity for a particular allogeneic pMHC complex. A strategy to apply additional mechanisms of tolerance to allospecific T cells whose control during tolerance depends on T_{REGs} may improve the robustness of tolerance during an inflammatory challenge such as Lm infection.

Chapter 3. Persistent suppression is necessary for maintaining allospecific T cell dysfunction during transplantation tolerance.

Abstract

T_{REGs} suppress alloimmune responses to prevent rejection during transplantation tolerance. During the maintenance phase of tolerance, additional features of the allospecific T cell repertoire, such as low population-wide avidity for allogeneic pMHC and development of cell-intrinsic dysfunction have also been observed. Redundancy in these mechanisms may support the robustness of tolerance, preventing rejection even when one or more mechanisms are disrupted. However, it is also possible that these mechanisms all result from one or more upstream tolerogenic mechanisms, with disruption of the upstream mechanism representing a critical vulnerability of tolerance. For example, T_{REGs} have been shown to suppress avidity maturation of allospecific T cells during the maintenance phase of tolerance. Here, we asked whether T_{REGs} are required for maintaining donor-specific transplantation tolerance. Indeed, we found that T_{REGs}

were required for the maintenance of anti-CD154/DST-induced transplantation tolerance, as T_{REG} depletion led to graft rejection in mice that had previously achieved stable tolerance to heart allografts. T_{REG} depletion-induced rejection was associated with allospecific T and B cell responses, which was surprising as prior studies had indicated that allospecific T and B cells persisting in tolerant hosts become intrinsically dysfunctional and thus should not respond, even after T_{REG} depletion. We thus investigated whether allospecific T and B cells could recover functionality, if starved of cell-extrinsic suppressive signals from the tolerant environment. Indeed, we found that signals in the tolerant mouse were required for maintaining dysfunction, as transfer to non-tolerant secondary hosts led to the return of function in previously tolerized T cells. These signals likely derive from suppressive lymphocytes such as T_{REGs} as we found that allospecific T cells remained functional in lymphopenic heart recipients treated with anti-CD154/DST. These findings suggest that bolstering T_{REG} function may make tolerance more resilient and tracking allospecific T_{REG} function and T_{CONV} dysfunction may be useful biomarkers for robust transplantation tolerance.

Introduction

Treatments to induce donor-specific transplantation tolerance have been under investigation for decades as an attractive alternative to the non-specific immunosuppressants that compose the standard of care for transplant recipients. Achieving donor-specific tolerance would eliminate the life-long exposure to drug toxicity, adverse effects and high risk of malignancy and infection resulting from conventional immunosuppression. Strategies to induce tolerance by blocking the CD40-CD154 co-stimulation pathway are being developed for use in clinical transplantation. One such treatment, a blocking antibody specific for CD154, has been shown to induce robust

donor-specific tolerance in mouse models of transplantation when combined with a transfusion of donor splenocytes (DST). However, even in mice that achieved stable graft acceptance with anti-CD154/DST, inflammatory challenges including infection can precipitate rejection (Wang et al. 2010a). Understanding which immune mechanisms are required to maintain transplantation tolerance may identify additional threats to stable tolerance and methods to make tolerance more robust.

Work from our group and others has identified multiple immune mechanisms that support anti-CD154/DST-induced tolerance in mice, including limiting the expansion of allospecific T cells, particularly those with high avidity for alloantigen, T_{REG}-mediated suppression, and development of cell-intrinsic dysfunction in allospecific T and B cells (Miller et al. 2018, Miller et al. 2016b, Khiew et al. 2020, Quezada et al. 2005, Quezada et al. 2003). It is possible that these are independent consequences of treatment with anti-CD154/DST, or they may all result and diverge from one upstream mechanism. Redundancy and independence in these mechanisms could allow tolerance to persist when one or more mechanisms are disrupted. On the other hand, if there is one keystone mechanism that drives tolerance, then disrupting that mechanism alone would precipitate rejection, representing a major vulnerability of the tolerant state.

Recent findings suggest that T_{REG}-mediated suppression may be required for maintaining other immune mechanisms supporting tolerance. Studies described in a previous chapter of this dissertation found that T_{REG}-mediated suppression was required to constrain expansion and avidity maturation in allospecific T cells during the maintenance phase of tolerance. There is also evidence that T_{REGs} are required to program and maintain dysfunction in T_{CONVs}. T cells that are tolerized to self or fetal antigens in the periphery may become anergic, expressing the surface markers CD73 and FR4 and failing to produce cytokines upon *in vitro* stimulation (Martinez et

al. 2012, Kalekar et al. 2016). However, self-reactive T cells failed to up-regulate these markers in the absence of T_{REGs} (Martinez et al. 2012). Anergic cells were also able to mount an autoimmune response after T_{REG} depletion (Kalekar et al. 2016). These data indicate that T_{REGs} are required for the development and maintenance of anergy in self-reactive T cells. It is not yet clear whether the dysfunction we and others have observed in allospecific T cells from anti-CD154/DST-tolerized graft recipients is functionally or epigenetically similar to anergy. Thus far, T cell dysfunction during transplantation tolerance has been defined by functional assays and a PD-1^{hi} $CD127^{lo}$ surface phenotype associated with chronic stimulation and exhaustion. One report showing increased FR4 and CD73 expression in graft-infiltrating T_{CONVs} from mice tolerized to islet transplants suggests that the anergic functional profile may be involved in transplantation tolerance (Besançon et al. 2017).

Further supporting a central role for T_{REGSs} in maintaining tolerance are findings that multiple challenges that can break established tolerance are also associated with disruption of T_{REG}-mediated suppression. Indeed, Lm infection or the combination of IL-6 and IFN-β can break anti-CD154/DST-induced tolerance to heart allografts (Wang et al. 2010b) and have each been shown to impair T_{REG} suppressive function (Srivastava et al. 2014, Nish et al. 2014, Rowe et al. 2012), though it is not yet clear whether T_{REG} impairment is required for these insults to break tolerance as they also disrupt tolerance through other mechanisms. We hypothesize that T_{REG}-mediated suppression is required for maintaining transplantation tolerance and may be required to maintain dysfunction in tolerized allospecific T cells as the central driver of other immune mechanisms known to support transplantation tolerance.

Previous work from our group suggested that other suppressive mechanisms such as co-inhibitory ligands, could compensate for T_{REGs}-mediated suppression during transplantation

tolerance. After induction of stable transplantation tolerance, grafts persisted after T_{REG} depletion with anti-CD25, blockade of PD-1/PD-L1 signaling, or adoptive transfer of naïve allospecific T cells. Only the combination of all three challenges could break established tolerance and induce rejection, suggesting that there is redundancy in these mechanisms during the maintenance phase of tolerance (Miller et al. 2016b). While these data do clearly indicate that multiple mechanisms are involved in the maintenance of tolerance, they do not eliminate the possibility that these three mechanisms are all derived from T_{REGs} or that T_{REGs} are not required for the maintenance of tolerance. Anti-CD25 does not deplete T_{REGs} well in tissues, including lymphoid organs where alloimmune responses are primed. Indeed, there are reported differences in the *in vivo* effects of partial T_{REG} depletion by anti-CD25 versus more complete T_{REG} depletion by DT treatment in mice expressing DTR under control of the FoxP3 promoter (Li et al. 2010). Further, T_{REGs} express PD-L1 and can be overwhelmed by high numbers of T cells requiring suppression, thus blocking PD-1/PD-L1 signaling and adoptively transferring allospecific T cells may break tolerance not by targeting three independent mechanisms but by further impairing suppression by a persisting T_{REG} population after anti-CD25 treatment.

In this study, we investigated whether T_{REGs} are necessary for maintaining transplantation tolerance induced with anti-CD154/DST. Indeed, we found that extensively depleting T_{REGs} in allograft recipients after establishing donor-specific tolerance swiftly led to rejection and increased alloimmune responses of T cells and B cells. As our prior findings showed that allospecific T_{CONVs} become dysfunctional during tolerance, we next investigated whether T_{CONVs} could recover functionality by isolating them from the tolerant environment and adoptively transferring them into non-tolerant secondary hosts. Indeed, tolerized T_{CONVs} fully recovered functionality in secondary hosts, and were able to reject skin allografts with similar kinetics as

memory T cells. Similar to anergic cells described in other contexts, tolerant allospecific T cells failed to develop dysfunction or acquire the $CD73^{hi}$ $FR4^{hi}$ anergy-associated phenotype in hosts lacking allospecific lymphocytes, which likely serve as a source of necessary dysfunction-promoting signals. While it is likely that the requisite anergy-inducing and -maintaining signals are derived from T_{REGs}, this will be determined definitively in future studies described in a later chapter. Our findings indicate that disrupting T_{REGs} alone is sufficient to induce rejection in previously tolerant allograft recipients. Thus, the tolerant state may be more vulnerable than previously appreciated as redundancy from other immunosuppressive mechanisms does not appear sufficient to maintain tolerance after the disruption of T_{REGs}. Further, the additional immune mechanisms that support transplantation tolerance may depend on persistent suppression by T_{REGs}. This work indicates that insults impairing T_{REG} function, even transiently, pose a significant risk to tolerant allograft recipients and strategies to improve and stabilize T_{REG} function may improve long-term outcomes in tolerant graft recipients.

Materials and Methods

Mice

B6 and BALB/c mice were purchased from Envigo RMS and P14 TCR-Tg $Rag^{-/-}$ (P14 $Rag^{-/-}$) mice were bred in house from mice purchased from Taconic Biosciences. TCR75 TCR-Tg mice were obtained from R. Pat Bucy (University of Alabama) and crossed with $Rag^{-/-}$ mice to generate TCR75 $Rag^{-/-}$ mice (TCR75). $Foxp3^{DTR}$ mice were obtained from Peter Savage (University of Chicago). Because DTR is incorporated into the endogenous FoxP3 locus on the X chromosome in these mice, only $FoxP3^{DTR/DTR}$ females and $FoxP3^{DTR/y}$ males were used in experiments. Mice were housed under specific pathogen-free conditions. DST for immunization

or tolerance induction was prepared by homogenizing and isolating a single cell suspension of splenocytes from BALB/c mice. Mice were injected i.v. with splenocytes from one-quarter spleen in 200 µL PBS.

Heart transplantation

Cardiac transplantation was performed using a technique adapted from Corry et al (Corry, Winn, and Russell 1973). For induction of tolerance, mice were treated with 500-600 µg of anti-CD154 (MR1, BioXCell) on days 0, (i.v.) 7 and 14 (i.p.) post-transplantation and DST (i.v.) on day 0.

Skin transplantation

Tail skin from BALB/c mice was transplanted onto the flank of P14 $Rag^{-/-}$ recipients as previously described (Kellersmann and Zhong 1998). Either $1x10^3$ or $3x10^3$ TCR75 cells sorted from primary hosts ≥30 days post-transplantation or DST were adoptively transferred into P14 $Rag^{-/-}$ secondary hosts on the day of transplantation. Bandages were removed 7 days post-transplantation. Rejection was reported when <20% of the transplanted skin was viable. TCR75 cells re-isolated from P14 $Rag^{-/-}$ skin allograft recipients were pooled based on the status of theirs skin graft (early, late or no rejection) prior to analysis.

Adoptive cell transfer

In experiments where TCR75 tracer cells were seeded before transplantation or immunization, spleen and lymph node cells (inguinal, axillary, brachial, cervical and mesenteric) from TCR75 mice were isolated into single-cell suspensions and counted with an Accuri C6 flow cytometer (BD Biosciences, San Jose, California). $5x10^4$ splenocytes from a naïve TCR75 mouse were injected i.v. in 200 µL of PBS 0-1 days before transplantation or immunization with DST.

ELISA to detect donor MHC-specific antibodies

Clear 96-well high binding microplates (Corning) were bound overnight at 4˚C with 5 μg/mL streptavidin (Jackson Immunoresearch) in pH 9.63 carbonate-bicarbonate coating buffer. Plates were washed then bound with biotinylated SYIPSAEKI:K^d or FIEWNKLRFRQGLEW:I-E^d monomers (NIH Tetramer Facility) diluted to 8.65 nM in wash buffer (PBS + 0.05% Tween-20) for 1 hour at RT. Some plates were not bound by pMHC monomers to evaluate non-specific binding of serum antibodies to the plate. After pMHC monomer binding, plates were washed, blocked with PBS + 1% bovine serum albumin (BSA), washed again and then incubated for 1 hour at RT with serum diluted 1:100 in wash buffer. Plates were washed then bound with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch) diluted 1:10,000 in wash buffer for 1.5 hours at RT. SigmaFAST para-nitrophenyl phosphate solution (Sigma) was added to detect alkaline phosphatase activity. OD_{405} was measured 15-30 minutes after addition of substrate. Background OD_{405} detection in wells without bound serum was subtracted from OD_{405} measurement in other wells before analysis.

Isolation of tracer TCR75 cells from primary and secondary hosts

Spleen and lymph nodes from primary or secondary hosts of tracer TCR75 cells were harvested and homogenized ≥30 days following transplantation or DST injection. Single cell isolates were stained with anti-CD45.1-biotin (eBioscience) and incubated with streptavidin magnetic beads (Miltenyi) for magnetic enrichment with LS columns (Miltenyi) or an AutoMACS machine (Miltenyi). In some experiments, cells form mice within the same experimental group were pooled after magnetic enrichment. Magnetically enriched cells were then stained with fluorescently coupled anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD4 (L3T4), anti-CD8 (Ly2),

anti-CD44 (IM7) and streptavidin and sorted for CD45.1^{+} CD4^{+} CD44^{hi} cells on a FACSAria cell sorter (BD Biosciences). Cells were sorted into FBS, then washed and re-suspended in PBS and subjected to further staining or functional analysis *in vitro* or *in vivo*.

Isolation of graft-infiltrating cells

Heart allografts were thoroughly rinsed with Hank's balanced salt solution (HBSS) with heparin, cut into small pieces and digested with 400 U/m: collagenase IV (Sigma), 0.01% DNase I (MP Biomedicals) and 10mM HEPES (Cellgro) in HBSS for 40 minutes at 37˚C. Cells were then filtered to isolate a single cell suspension, washed and then stained.

Flow cytometry

Sorted TCR75 cells or unenriched spleen and lymph node cells were stained with a fixable live/dead stain (Invitrogen). For tetramer staining 5x10^{6} cells followed by phycoerytherin (PE)- or allophycocyanin (APC)-coupled pK^{d}:I-A^{b} tetramer (NIH Tetramer Core Facility) for 1 hour in a room temperature (RT) water bath, washed, and then stained with PE- or APC-coupled H60:K^{b} tetramer (NIH Tetramer Core Facility) for 20 minutes in a room temperature (RT) water bath. In some experiments, tetramer-stained cells were then magnetically enriched for T cells as we had seen that enrichment after tetramer staining was associated with less non-specific tetramer staining than enrichment before tetramer staining. Cells were then stained with anti-CD4, anti-CD8, anti-CD44, anti-PD-1 (J43), anti-CD127 (A7R34), anti-FR4 (12A5), anti-CD73 (TY/11.8) and anti-B220 (RA3-6B2). Surface-stained cells were then fixed and permeablized with the FoxP3 fixation permeabilization buffer kit (eBioscience). Some samples were intracellularly stained with anti-Ki67 (SolA15) or anti-FoxP3 (FJK-16s) for 30 minutes at RT; washed with permeabilization buffer; and analyzed by flow cytometry. CFSE labeling was performed by

labeling cells with CellTrace CFSE (Invitrogen) for 20 minutes at 37°C. All monoclonal antibodies (mAbs) were from BD Biosciences, eBioscience, or Invitrogen.

In vitro stimulation for cytokine production

U-bottom tissue culture plates were coated for 90 minutes at 37˚C with 5 μg/mL anti-CD3 (2C11) and 1 μg/mL anti-CD28 (PV.1) (Fitch Monoclonal Facility). 500-2000 tracer TCR75 cells sorted from primary or secondary hosts were plated per well and incubated for 16-24 hours at 37˚C and 5% CO_2. Within each individual experiment, all wells were plated with the same number of cells. In some experiments, $1x10^5$ B6 splenocyte feeder cells per well were co-cultured with TCR75 cells. Unstimulated controls were plated in uncoated wells with 1ng/mL human IL-7 (PeproTech). 2 hours after plating, brefeldin A (BioLegend) was added to all wells. After stimulation cells were stained with fixable viability dye (Invitrogen) and then surface stained with anti-CD4, anti-CD8, anti-CD45.1, anti-CD45.2 and anti-CD44. Cells were then fixed and permeablized with the FoxP3 fixation permeabilization buffer kit (eBioscience) and stained with anti-IFN-γ (XMG1.2) and anti-TNF (MP6-XT22) for 30 minutes at RT or overnight at 4˚C before washing with permeablization and flow cytometry analysis.

Data analysis

Flow cytometry data were analyzed using FlowJo (Tree Star, Ashland, Oregon). Flow cytometry samples were gated on live single cells before analysis. Tetramer stained samples were also gated on B220- cells. Statistical analyses were performed where appropriate using GraphPad Prism (GraphPad, La Jolla, California). Each statistical test is listed in the figure legends.

Study Approval

The studies were performed in agreement with the University of Chicago's Institutional Animal Care and Use Committee, according to the NIH guidelines for animal use.

Results

T_{REG} depletion precipitates rejection in previously tolerant cardiac allograft recipients

We hypothesized that T_{REGs} were not only required to induce tolerance with anti-CD154/DST but that persistent T_{REG} activity was required to maintain tolerance long-term. To test this hypothesis, we induced tolerance to BALB/c heart allografts in WT and FoxP3^{DTR} mice on a B6 background by treating them with anti-CD154/DST. At least 30 days after transplantation, when tolerance had been established, we treated mice with DT to extensively deplete T_{REGs} specifically in recipient FoxP3^{DTR} mice (Fig. 3.1 A). Supporting our hypothesis, tolerized FoxP3^{DTR} mice swiftly rejected their grafts after administration of DT, whereas graft function in WT mice remained stable. DT-induced rejection was accompanied by the production of IgG specific for donor MHC Class I and Class II molecules, alloantibodies that were otherwise undetectable before DT (Fig. 3.1C). This result was unexpected as allospecific B cells have been shown to become intrinsically impaired in their ability to produce IgG (Khiew et al. 2020). That these alloantibodies were class-switched indicates that a productive allospecific T cell response also occurred, which was surprising since our previous data indicated that allospecific T cells become intrinsically dysfunctional during tolerance (Miller et al. 2019).

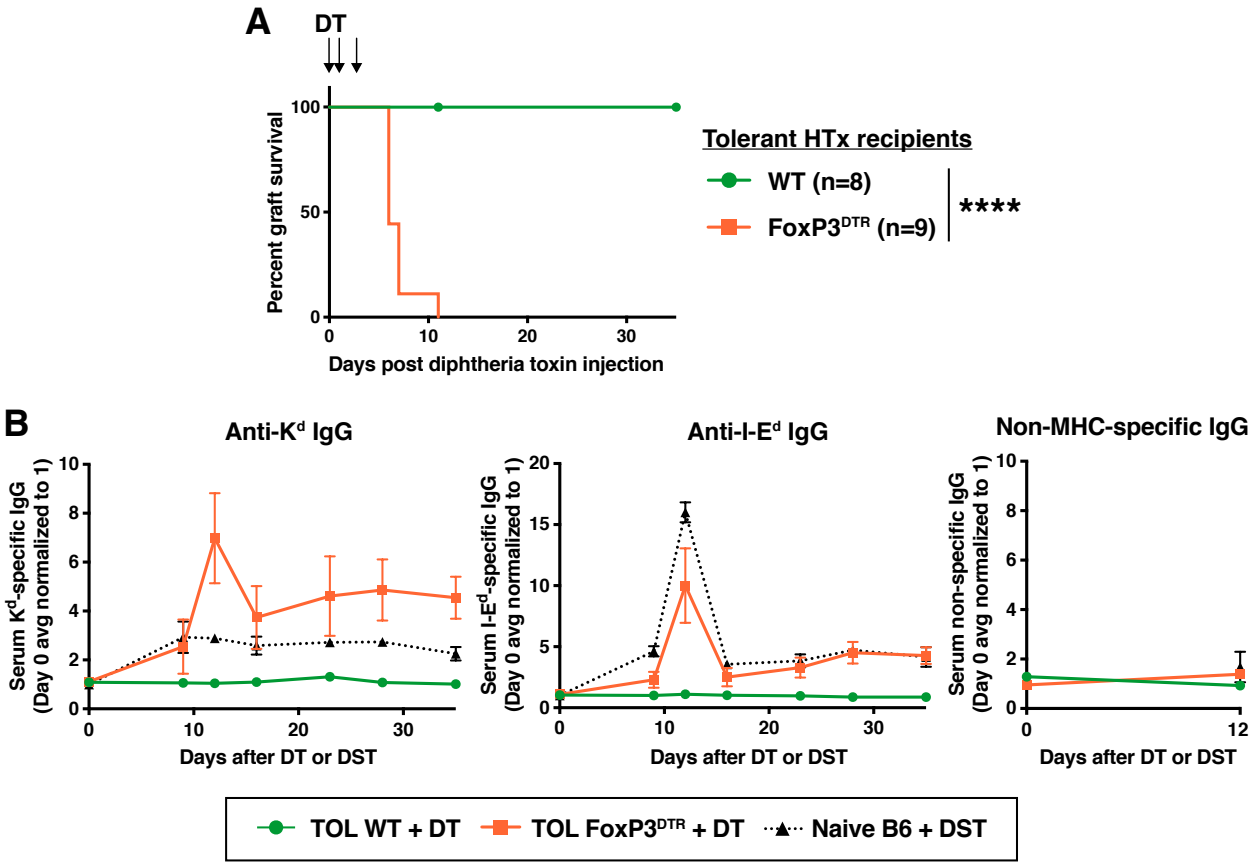

Figure 3.1 T_{REG} depletion causes rejection in previously tolerant heart recipients.

B6 WT and FoxP3^{DTR} mice were tolerized to BALB/c heart allografts with anti-CD154 and DST. ≥30 days after transplantation, tolerant mice were injected with DT. (A) Experimental design. (B) Heart allograft survival after diphtheria toxin treatment. (C) Detection of serum antibodies specific for donor MHC Class I (K^d), MHC Class II ($I\text{-}E^d$) or non-MHC-specific IgG binding uncoated ELISA plates in previously tolerant B6 WT and FoxP3^{DTR} heart recipients after DT and naïve B6 WT mice after immunization with BALB/c DST. Data were normalized within each experiment, with the average OD_{405} from samples at day 0 set to 1. Results were pooled from 2-3 independent experiments. Each time point represents data from 2-6 mice per experimental group with lines indicating average +/- SEM. Survival curves were analyzed by Log-rank (Mantel-Cox) text. ****p<0.0001.

To determine whether allospecific T cells responded during DT-induced rejection, we compared the expansion and expression of the proliferation marker Ki67 in allospecific T cells from the spleens of previously tolerant WT or FoxP3^{DTR} mice after DT injection. Consistent with previous reports (Kim, Rasmussen, and Rudensky 2007), T_{REG} depletion was accompanied by proliferation of $CD4^+$ and $CD8^+$ T cells, resulting in increased numbers of T cells overall (Figs.

3.2A, B). Surprisingly, allospecific CD4^{+} T cells identified by pK^{d}:I-A^{b} tetramer binding were also proliferating 12 days after DT treatment, having already expanded as compared to the pK^{d}:I-A^{b} tetramer^{+} population in stably tolerant WT mice (Fig. 3.2C).

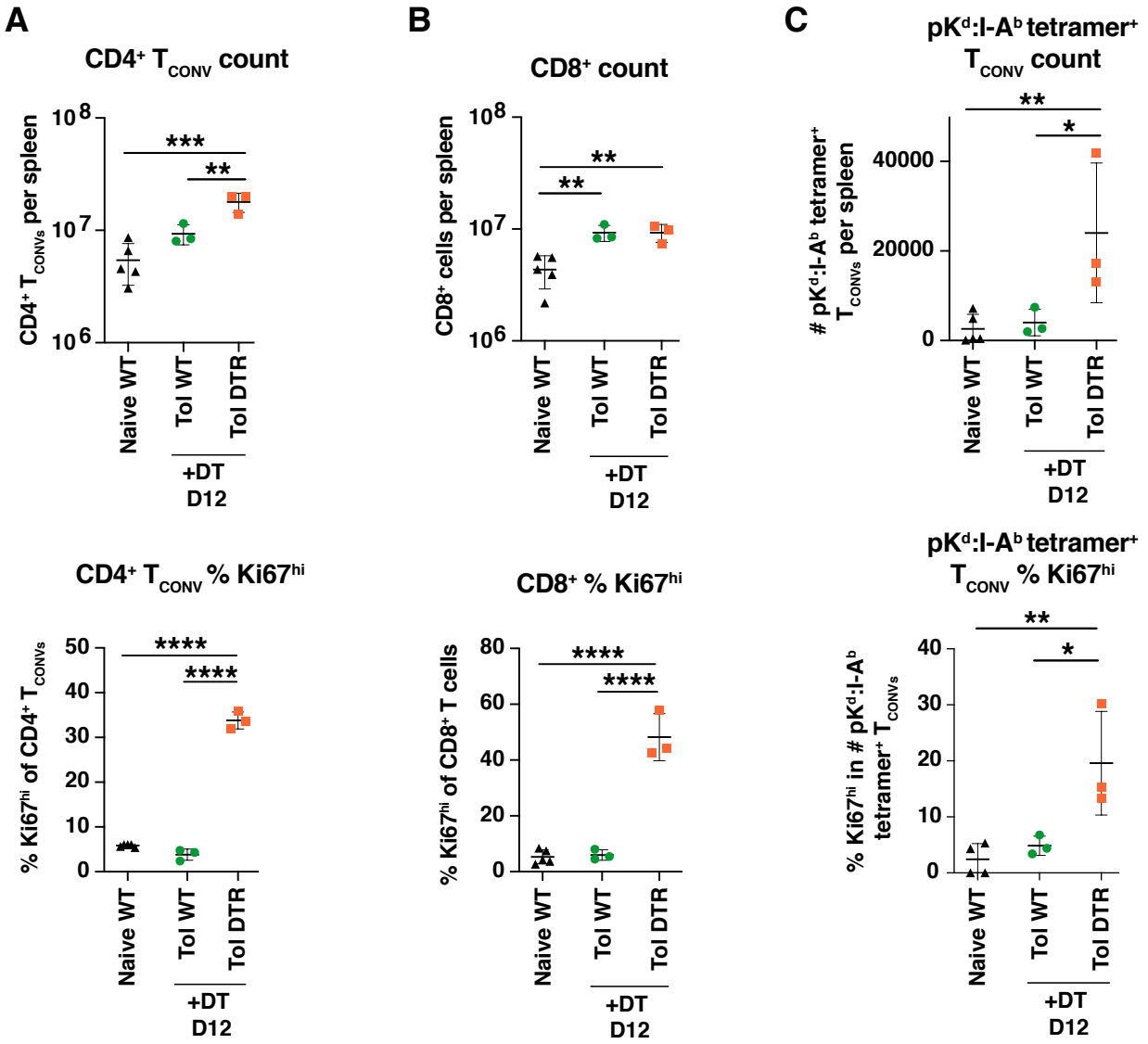

Figure 3.2 T_{REG} depletion in previously tolerant heart recipients results in expansion of allospecific T_{CONVs}.

Splenocytes were isolated from naïve B6 WT mice, WT or FoxP3^{DTR} heart recipients 12 days after DT. The quantity per spleen and percent and Ki67^{hi} were analyzed in (A) bulk CD4^{+} T_{CONVs}, (B) bulk CD8^{+} cells and (C) CD4^{+} pK^{d}:I-A^{b} tetramer^{+} cells. Results were pooled from 1-2 independent experiments. Each data point represents an individual mouse with lines indicating average +/- SD. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple pairwise comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Recovery of function in tolerant TCR75 cells in non-tolerant secondary hosts

We hypothesized that tolerized pK^d:I-A^b tetramer-binding T cells expanded after T_{REG} depletion because continuous cell-extrinsic signaling, including suppressive signals from T_{REGs}, are needed to maintain dysfunction in tolerant allospecific T_{CONVs}. To determine whether allospecific T cells recover function when removed from the tolerant environment, we isolated tracer TCR75 cells from tolerant or rejecting heart recipients ≥30 days after transplantation and compared their phenotype and functional profile 7 and 44 days after adoptive transfer into naïve B6 secondary host. Importantly, TCR75 cells rarely become T_{REGs} in tolerant graft recipients (Chai et al. 2015), thus suppression by TCR75 T_{REGs} co-transferred into the secondary host is unlikely to confound our results. Secondary host B6 mice have neither the enriched allospecific T_{REG} population nor the persistent source of donor antigen present in the tolerant primary host. Secondary hosts were immunized with BALB/c DST after adoptive transfer to expand TCR75 cells for improved cell recovery at late timepoints (Fig. 3.3A).

Tracer TCR75 cells and their progeny could be recovered for analysis by sorting $CD45.1^+$ $CD4^+$ cells from the secondary lymphoid organs of primary and secondary hosts (Fig. 3.3B). By day 7 post-transfer, the level of PD-1 expression on the surface of Tol TCR75 cells had declined to match that of rejecting TCR75 cells (Fig. 3.3C). CD127 expression decreased significantly in both Tol and Rej TCR75 cells 7 days after adoptive transfer (Fig. 3.3D), which was likely a consequence of their effector response to immunization with DST. We also found that Tol TCR75 cells expressed high levels of the anergy markers CD73 and FR4 and that expression of these markers decreased to match those of Rej TCR75 cells within 7 days of adoptive transfer (Fig. 3.3E, F). While studies have shown that autoreactive $CD73^{hi}$ $FR4^{hi}$ T cells convert into $FoxP3^+$ T_{REGs} after transfer into secondary hosts where their cognate antigen is not expressed as

self (Kalekar et al. 2016), Tol TCR75 cells did not convert into T_{REGs} after adoptive transfer into naïve secondary hosts (Fig. 3.3G).

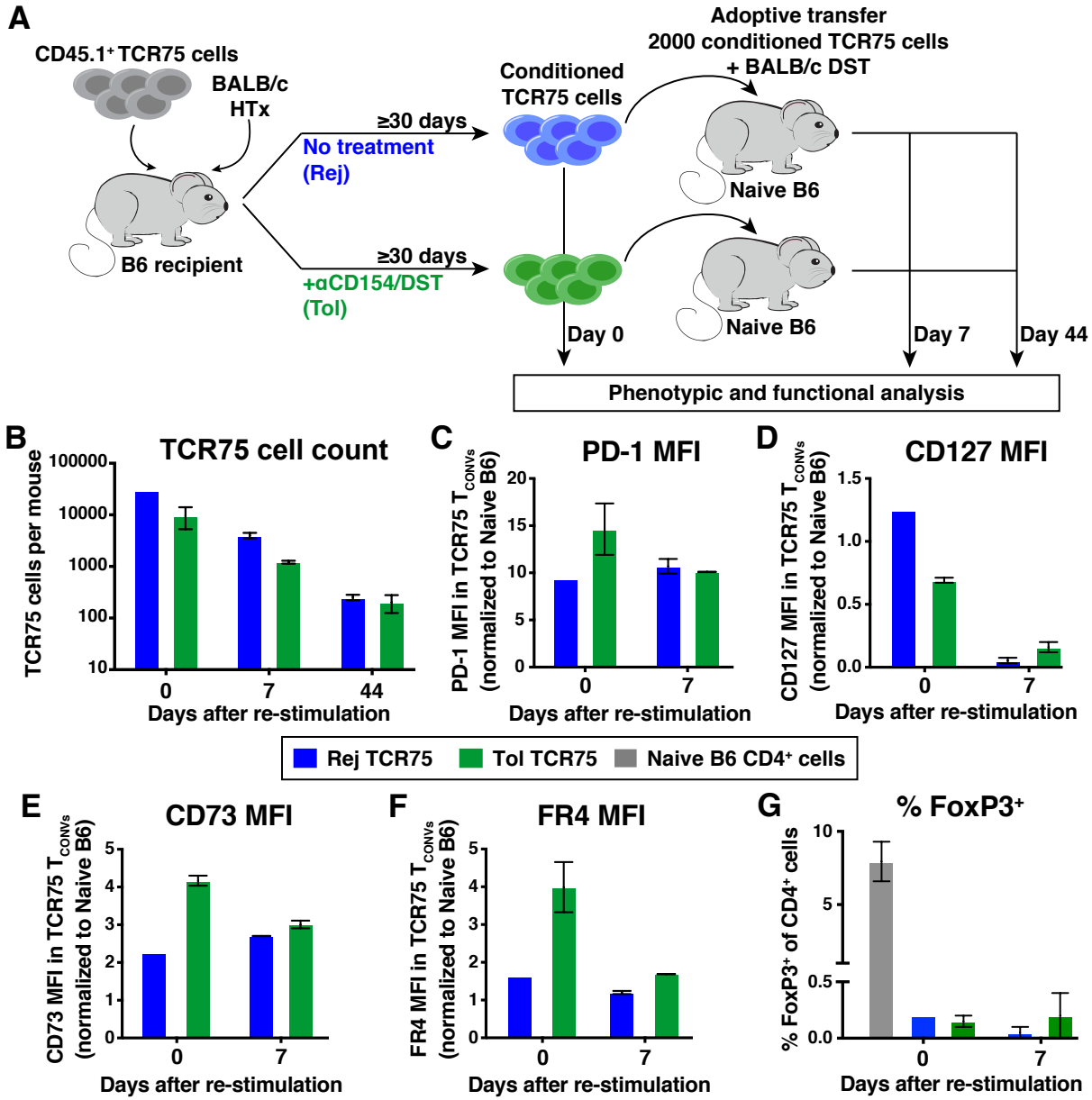

Figure 3.3 Tolerized T_{CONVs} lose their dysfunction-associated phenotype in non-tolerant secondary hosts.

B6 mice were seeded with tracer TCR75 cells prior to transplantation with BALB/c hearts with (Tol) or without (Rej) anti-CD154 DST. ≥30 days after heart transplantation $CD44^{hi}$ TCR75 cells were isolated from the spleens and lymph nodes of the transplanted mice, transferred into naïve B6 secondary hosts and expanded by immunizing the secondary hosts with BALB/c DST. (A) Experimental design. (B) Number of TCR75 cells recovered from the spleen and lymph nodes of primary hosts (day 0) or secondary hosts 7 and 44 days after adoptive transfer.

Figure 3.3, continued: Normalized MFI of PD-1 (C), CD127 (D), CD73 (E) and FR4 (F) in $CD44^{hi}$ $FoxP3^{-}$ tracer TCR75 cells isolated as in (B). MFI values were normalized within each time point, with the average MFI of $CD44^{lo}$ $CD4^{+}$ $FoxP3^{-}$ splenocytes from a naïve B6 mouse set to 1. (G) Percent $FoxP3^{+}$ in TCR75 cells isolated as in (B) and in $CD4^{+}$ cells from the splenocytes of naïve B6 mice. Results are from one experiment. Each sample was pooled from 1-3 mice before analysis. Lines indicate SD.

We next determined whether the reduction in expression of dysfunction-associated surface markers in Tol TCR75 cells after removal from the tolerant transplant recipient was accompanied by a return of function. Indeed, *in vitro* IFN-γ and TNF production by Tol TCR75 cells recovered in non-tolerant secondary hosts (Figs. 3.4A, B). Within 7 days of removal from the tolerant environment, Tol TCR75 cells had recovered some ability to produce IFN-γ and TNF and by day 44, their cytokine production was similar to that of memory TCR75 cells from rejecting mice.

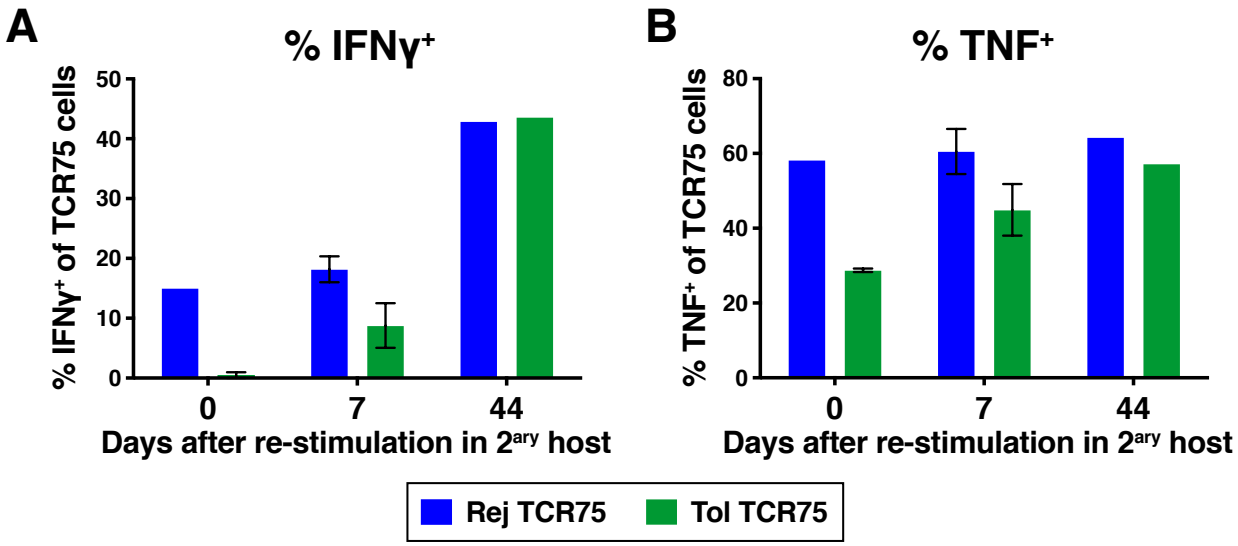

Figure 3.4 Tolerized T_{CONVs} recover function in non-tolerant secondary hosts.

Percent of TCR75 cells isolated from rejecting (Rej) or tolerant (Tol) BALB/c heart recipients (day 0) or non-transplanted secondary hosts (days 7, 44) that produced IFN-γ (A) or TNF (B) after *in vitro* re-stimulation with anti-CD3 + anti-CD28. Results are from one experiment. Cells isolated from 1-3 mice were pooled before *in vitro* re-stimulation. Lines indicate SD.

To determine whether Tol TCR75 cells recovered sufficient function to reject an allograft after removal from the tolerant host, we isolated tracer TCR75 cells from mice tolerized to

BALB/c heart allografts ≥30 days after heart transplantation, then adoptively transferred a minimal number of these cells into non-tolerant P14 Rag$^{-/-}$ secondary hosts. Hosts were then given a BALB/c skin allograft (Fig. 3.5A). By using P14 Rag$^{-/-}$ secondary hosts, which only bear T cells expressing an irrelevant LCMV-specific TCR, we could isolate the alloimmune response to the transferred TCR75 cells, while at the same time preventing the transferred cells from undergoing homeostatic proliferation, which would occur after adoptive transfer into T cell-deficient mice and has been shown to restore function to self-tolerant T cells (Schietinger et al. 2012). Tol TCR75 rejected skin grafts in secondary hosts with similar kinetics as the same number of TCR75 cells from rejecting heart recipients (Rej) or DST-immunized mice (Mem) (Fig. 3.5B, C). Forty-five days after skin transplantation, we confirmed that the TCR75 cells had recovered function in the secondary hosts by evaluating their ability to produce IFN-γ and TNF upon in vitro stimulation. Indeed, Tol TCR75 cells produced both cytokines to a similar extent as Rej TCR75 cells recovered from P14 Rag$^{-/-}$ skin graft recipients.

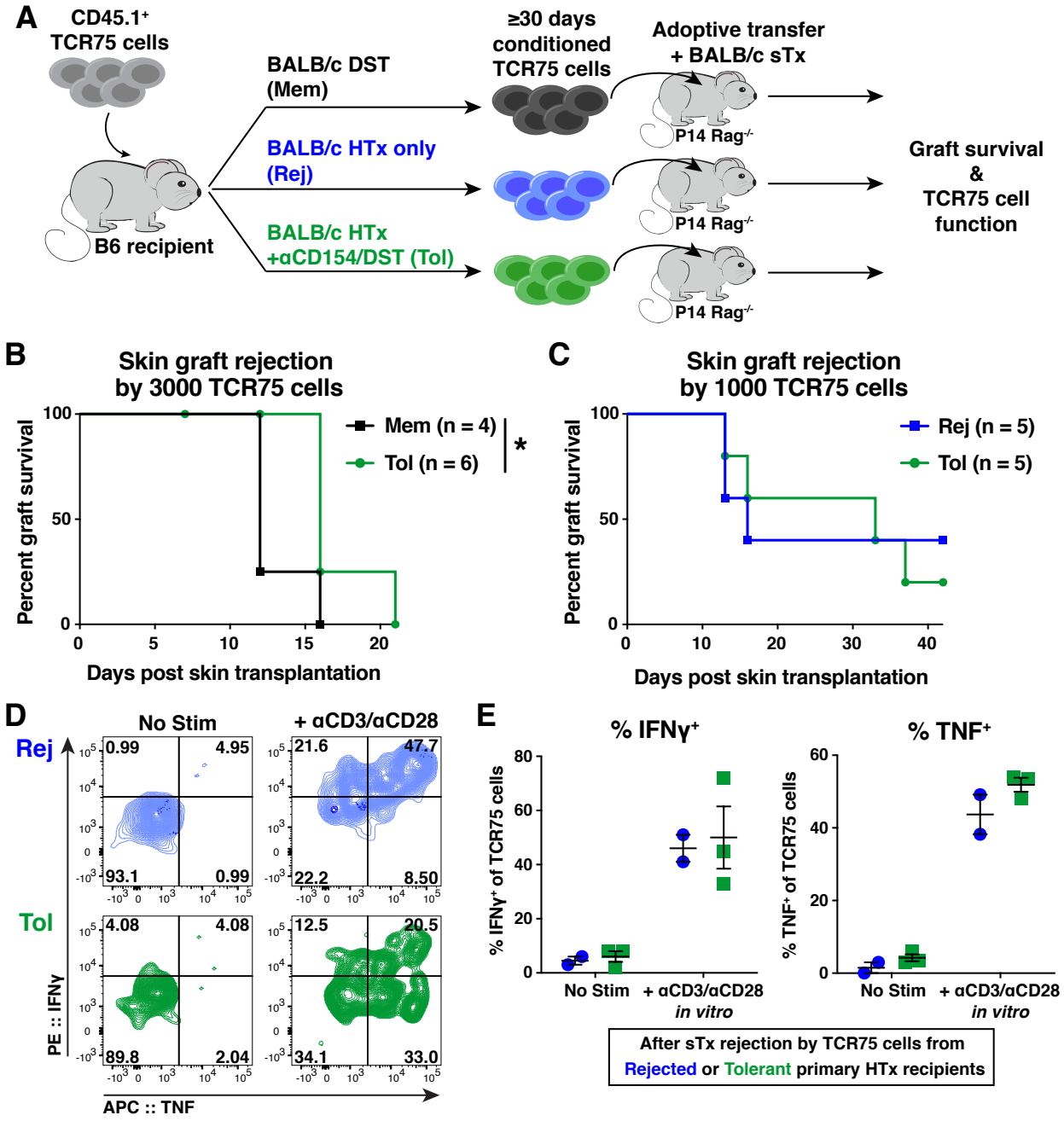

Figure 3.5 Tolerized allospecific T_{CONVs} reject skin grafts in non-tolerized secondary hosts.

(A) Experimental design. (B, C) BALB/c skin allograft rejection in P14 Rag$^{-/-}$ mice after adoptive transfer of (B) 3000 or (C) 1000 CD44^{hi} TCR75 cells from primary hosts that had received BALB/c heart allografts with (Tol) or without (Rej) anti-CD154/DST ≥30 days prior or were immunized with BALB/c DST (Mem) ≥30 days prior. (D, E) *In vitro* production of IFN-γ and TNF by TCR75 cells isolated from the spleen and lymph nodes of mice 45 days after skin transplantation as in (C). Results are from one experiment. Each data point and flow plot represents a sample pooled from 1-3 mice with lines indicating average +/- SD. Survival curves were analyzed by Log-rank (Mantel-Cox) test. *P<0.05.

Induction of T cell dysfunction requires signals from other lymphocytes

Having observed that tolerant TCR75 cells recover function over time in non-tolerant secondary hosts, where there are no other allospecific T cells, we sought to determine whether cell-extrinsic signals from other allospecific T cells are also necessary for programming T cell dysfunction during the induction of donor-specific tolerance. TCR75 cells ($4x10^4$) were adoptively transferred into B6 mice (containing endogenous T_{REGs} and B cells) or P14 $Rag^{-/-}$ mice (devoid of endogenous T_{REGs} and B cells) one day prior to heart transplantation with (Tol) or without (Rej) anti-CD154/DST treatment, or one day prior to DST immunization. At least 30 days after transplantation or immunization, TCR75 cells were re-isolated from the spleens and lymph nodes of transplanted mice and subjected to analysis of phenotype and function (Fig. 3.6A). A larger number of TCR75 cells was recovered from P14 $Rag^{-/-}$ heart recipients compared to WT recipients regardless of whether the hosts were tolerized (Fig. 3.6B). TCR75 cells were no more likely to develop into T_{REGs} in P14 $Rag^{-/-}$ hosts than in WT hosts (Fig. 3.6C). TCR75 cells in both B6 and P14 $Rag^{-/-}$ hosts similarly underwent a larger magnitude of expansion when immunized with DST rather than a heart allograft (Fig. 3.6B). These results suggest that other lymphocytes limit the magnitude of the T cell alloimmune response to solid organ transplants, but not to DST immunization.

We next evaluated the surface phenotype of tolerant and rejecting TCR75 T_{CONVs} from WT and P14 $Rag^{-/-}$ heart allograft recipients. Supporting our hypothesis that signals from other lymphocytes are necessary for inducing dysfunction in tolerant allospecific T_{CONVs}, TCR75 T_{CONVs} in tolerant P14 $Rag^{-/-}$ heart recipients failed to up-regulate CD73 and FR4 to the high levels seen in TCR75 T_{CONVs} from tolerant lymphoreplete mice (Fig. 3.6C). Tol TCR75 cells from P14 $Rag^{-/-}$ mice expressed a PD-1^{hi} $CD127^{lo}$ phenotype, similar to those in WT mice. This

finding dissociated the CD73^{hi} FR4^{hi} anergy-associated phenotype from the PD-1^{hi} CD127^{lo} exhaustion-associated phenotype. Our observations of phenotype in peripheral TCR75 cells were recapitulated within the graft. Indeed, larger numbers of TCR75 cells accumulated in the grafts of P14 Rag$^{-/-}$ recipients compared to WT recipients (Fig. 3.7A). Within the graft, TCR75 cells rarely expressed FoxP3 (Fig. 3.7B). The PD-1^{hi} CD127^{lo} exhaustion-associated phenotype was observed in TCR75 cells from Tol WT and P14 Rag$^{-/-}$ mice (Fig. 3.7C) but only in tolerized WT mice did graft infiltrating TCR75 cells up-regulate FR4 and CD73 expression (Fig. 3.7D).

Finally, we determined whether TCR75 cells retained function in tolerized P14 Rag$^{-/-}$ heart recipients. Indeed, while TCR75 cells in tolerized B6 mice lost much of their ability to produce IFN-γ and TNF, TCR75 cells in P14 Rag$^{-/-}$ mice were able to produce both cytokines (Fig. 3.6D). These results show that signals from other lymphocytes (T_{REGs} or other subsets of T cells or B cells) are needed for donor-specific T_{CONVs} to acquire dysfunction during tolerance induction.

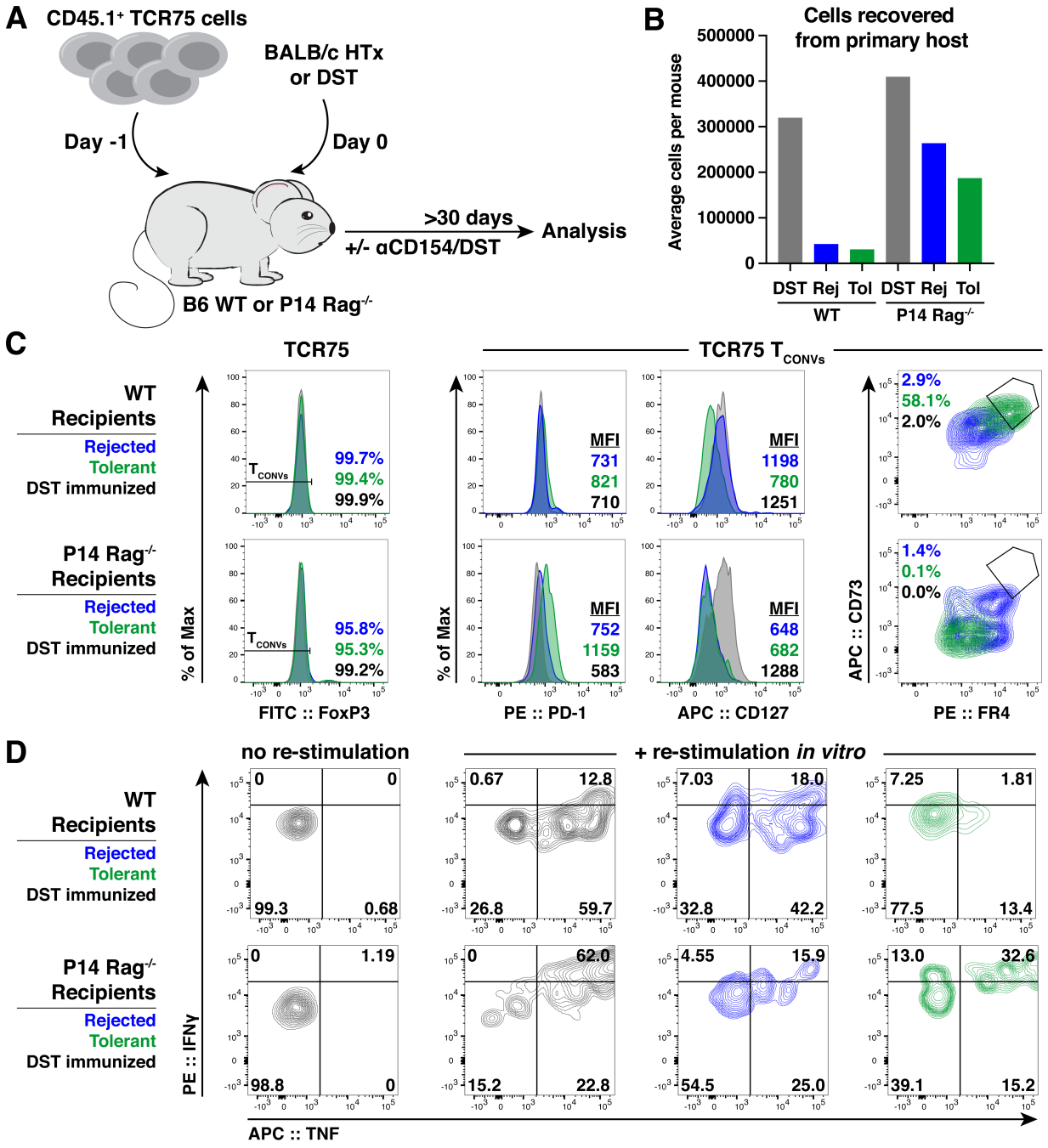

Figure 3.6 Allospecific T_{CONVs} fail to become dysfunctional after co-stimulation blockade in P14 Rag$^{-/-}$ heart recipients.

(A) Experimental design. (B) Number of tracer TCR75 cells recovered from the spleen and lymph nodes of WT or P14 Rag$^{-/-}$ mice ≥30 days after BALB/c DST or heart transplantation +/- anti-CD154/DST. (C) Left: flow plots showing FoxP3 expression in TCR75 cells. %FoxP3^{-} indicated inside plots; Right: flow plots showing expression of PD-1, CD127, FR4 and CD74 in TCR75 T_{CONVs} recovered as in (B) with MFI for each experimental group indicated inside plots. (D) Production of IFN-γ and TNF by TCR75 cells recovered as in B and re-stimulated *in vitro*. Results are from one experiment. Data represent samples pooled from 2-3 mice.

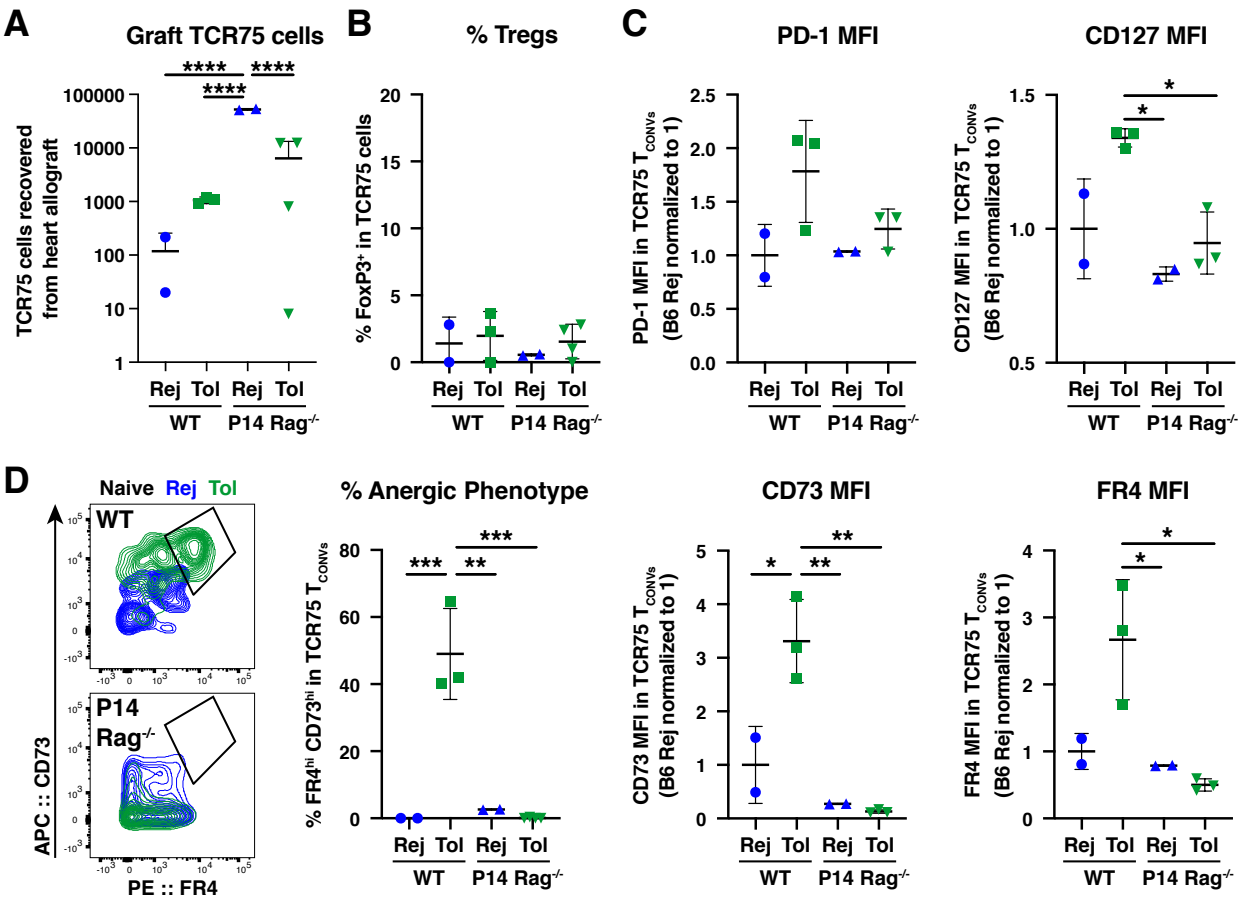

Figure 3.7 Graft-infiltrating allospecific T_{CONVs} fail to develop a dysfunction-associated phenotype after co-stimulation blockade in P14 Rag$^{-/-}$ heart recipients.

(A) Number of tracer TCR75 cells recovered from BALB/c heart allografts of B6 WT or P14 Rag$^{-/-}$ mice >30 days after transplantation +/- anti-CD154/DST. (B) Percent FoxP3^{+} in graft-infiltrating TCR75 cells, (C) PD-1 MFI and CD127 MFI in graft-infiltrating FoxP3^{-} TCR75 cells recovered as in A. (D) Representative flow plots for FR4 and CD73 staining, percent anergic (FR4^{hi} CD73^{hi}) cells, CD73 MFI and FR4 MFI in graft-infiltrating TCR75 T_{CONVs} recovered as in A. MFI values were normalized, with the average MFI for B6 Rej mice set to 1. Results are from one experiment. Each data point represents an individual mouse with lines indicating average +/- SD. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple pairwise comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Discussion

Many strategies for the induction of donor-specific tolerance result in long-term establishment of a high allospecific T_{REG}:T_{CONV} ratio. While it is appreciated that T_{REGs} are necessary for the induction of donor-specific tolerance with anti-CD154/DST, other immune

mechanisms such as T cell-intrinsic dysfunction are thought to prevent rejection during the maintenance phase of tolerance, possibly providing redundancy that would allow tolerance to persist even in the absence of T_{REG}-mediated suppression. In this study, we find that impairment in T_{REG} function results in breakage of established transplantation tolerance.

Here, we have found that T_{REG} depletion in FoxP3^{DTR} mice treated with DT can break established tolerance. However, in addition to eliminating allospecific T_{REGs}, DT treatment in FoxP3^{DTR} mice also eliminates autospecific T_{REGs}, resulting in severe autoimmunity and systemic inflammation. Thus, breakage of tolerance after DT treatment could result from impairing T_{REG}-mediated suppression of alloimmunity but may also result from systemic autoimmunity, through mechanisms involving the abundance of pro-inflammatory cytokines or activation of autospecific T_{CONVs} with cross-reactivity to the donor. Specifically eliminating allospecific T_{REGs} without impairing autospecific T_{REGs} would allow us to differentiate between these mechanisms, though techniques to accomplish this are not yet available.

While our findings indicate that T_{REGs} are required for the maintenance of tolerance, prior studies have shown that more mild depletion of T_{REGs} with anti-CD25 could not break established tolerance, though treatment with anti-CD25 at the time of transplantation did prevent the induction of tolerance, indicating that there is differential sensitivity to incomplete T_{REG} depletion during tolerance induction and maintenance (Miller et al. 2015, Miller et al. 2016a, Jiang et al. 2011). This may be because at the time of transplantation, the T_{REG}:T_{CONV} ratio has not yet increased to the level seen during stable tolerance, thus small aberrations in this ratio may have a larger physiological impact. It is also possible that T_{REGs} become more potent in suppressing T_{CONVs} during the maintenance phase of tolerance. Indeed, inflammation at the time of transplantation resulting from surgery and ischemia-reperfusion injury may inhibit T_{REG}

function. Finally, the differential efficacy of anti-CD25 and DT may have more to do with the compartments where T_{REGs} are depleted rather than the overall percentage of T_{REGs} depleted systemically. DT is more effective than anti-CD25 at depleting T_{REGs} within tissue (Li et al. 2010), thus during the maintenance phase of tolerance, graft-infiltrating T_{REGs} may protect the allograft from rejection even when circulating T_{REGs} are depleted by anti-CD25, whereas immediately after transplantation, recipient T_{REGs} have not yet accumulated in the graft, thus depletion of graft infiltrating T_{REGs} is not necessary.

That T_{REG} depletion precipitated T cell- and B cell-mediated alloimmunity was surprising considering prior findings that allospecific T cells and B cells become intrinsically dysfunctional during tolerance (Miller et al. 2019, Khiew et al. 2020). The findings that T_{REGs} are required for inducing and maintaining anergy in T cells tolerized to self or fetal antigens suggested that allospecific T cells in our model of transplantation tolerance may have recovered function after T_{REG} depletion, allowing them to expand, reject the graft and provide help to B cells. Indeed, we found that tolerized T_{CONVs} could recover function after removal from the tolerant environment and adoptive transfer into non-tolerant secondary hosts. Future studies will determine whether co-transfer of allospecific T_{REGs} into non-tolerant secondary hosts preserves dysfunction in tolerized allospecific T_{CONVs}. T_{CONVs} also encounter significant inflammation in T_{REG}-depleted primary hosts, as well as DST-immunized or skin transplanted secondary hosts. If co-transfer of T_{REGs} preserves dysfunction in tolerized T_{CONVs} transferred into skin grafted secondary hosts, it will also indicate that inflammation is not sufficient to restore function to tolerized T_{CONVs}.

It is also unclear how dysfunctional tolerized B cells recovered sufficient function to produce alloantibodies after T_{REG} depletion. Unlike tolerant T cells, tolerant B cells have been shown to remain dysfunctional for weeks after adoptive transfer into non-tolerant secondary

hosts, and even suppress responses by naïve B cells in the secondary host (Khiew et al. 2020). Thus, it is unlikely that T_{REGs} or other suppressive signals specific to the tolerant environment are required to maintain tolerant B cell dysfunction. Severe systemic inflammation may provide the signals necessary to re-program these B cells, though treatment with CpG and provision of help to tolerized B cells with agonist anti-CD40 did not rescue their function. More research is needed to understand the signals required for recovering function in tolerant allospecific T and B cells.

In vitro and *in vivo*, recovered tolerized T cells were functionally indistinguishable from memory T cells. As memory cells are more resistant to suppression, tolerance induction and conventional immunosuppression than naïve T cells, rejection after T_{REG} depletion is likely to permanently sensitize the host against a secondary transplant from a donor strain expressing alloantigens in common with the initial donor. Pre-existing alloantibodies also undermine tolerance, further preventing successful re-transplantation (Burns and Chong 2011). This would contrast with our previous findings that donor-specific tolerance spontaneously recovers after Lm-induced rejection, allowing acceptance of a second heart allograft from the same donor strain once the infection has resolved (Miller et al. 2015). Whether a second donor heart would be accepted or rejected following DT-dependent rejection of a primary tolerized graft remains to be determined.

We also found that TCR75 cells remained functional in anti-CD154/DST-treated P14 $Rag^{-/-}$ heart allograft recipients. Future studies will investigate whether co-adoptive transfer of T_{REGs} is sufficient to enable the development of alloreactive T_{CONV} dysfunction in P14 $Rag^{-/-}$ graft recipients treated with the tolerogenic protocol. Despite retaining functionality and infiltrating the allograft, cardiac allografts remained functional in these mice. This is consistent with previous reports that monoclonal $CD4^+$ T cells are inefficient at rejecting heart allografts

(Honjo et al. 2004, Brennan et al. 2008). Experiments to determine whether T_{REG} deficiency allows allospecific T_{CONVs} in the endogenous repertoire to retain sufficient function to reject the allograft are complicated by the necessity for T_{REGs} to prevent autoimmunity by autoreactive T cells persisting in the endogenous T cell repertoire.

Contrasting with our results, one study has found that recipient T_{REG} depletion with DT did not break tolerance to islet allografts (Besançon et al. 2017). There are several notable differences between this experimental model and our own, which may explain the discrepancy in results following T_{REG} depletion. First, this study achieved transplant tolerance by treating recipients with anti-CD3. Similar to anti-CD154/DST, anti-CD3 induces a tolerant state that includes allospecific T cell dysfunction and accumulation of allospecific T_{REGs}, though there are likely to be differences in the tolerant states induced by either treatment, potentially including the extent or stability of T_{CONV} dysfunction. It is also possible that reduced immunogenicity or antigen availability from islet allografts compared to cardiac allografts may allow allospecific T cells to ignore islet allografts, but not cardiac allografts, after T_{REG} depletion. T_{REGs} were also depleted for 2 days fewer in this study than in our own. We have found that allospecific T_{CONVs} progressively re-gain function over time in non-tolerant secondary hosts, thus longer T_{REG} disruption may be required for allospecific T cells to recover sufficient function to reject an allograft. Better understanding the factors that explain differential susceptibility of different transplantation tolerance models to T_{REG}-mediated suppression may identify methods to protect allografts from rejection in tolerized hosts that encounter a disruption in T_{REG} function.

Together, this study and findings described in a previous chapter indicate that T_{REGs} are required for maintaining transplantation tolerance, including multiple downstream immune mechanisms that prevent alloimmunity during transplantation tolerance. These include

constraining the size and avidity of the allospecific $CD4^+$ T_{CONV} and $CD8^+$ T cell populations and possibly the programming and maintenance of dysfunction in allospecific T_{CONV}s. Thus, T_{REG}s serve as a keystone mechanism for maintaining tolerance, explaining why tolerance fails when T_{REG}s are depleted. The tolerant state induced with anti-CD154/DST is likely vulnerable to any insults that severely impair T_{REG} function. While Lm infection is known to impair T_{REG} function, it is not yet clear whether this is the main mechanism responsible for Lm-induced rejection in previously tolerant graft recipients. Strategies to protect T_{REG} function during tolerance may improve the robustness of tolerance during inflammatory challenges, an important goal for clinical translation of tolerogenic therapies. Additionally, tracking T_{REG}-mediated suppression in transplant recipients may be an important biomarker for the robustness of transplantation tolerance. As Lm impacts T_{REG} function without reducing T_{REG} number, readouts of T_{REG} functionality are likely to be more useful early markers for stable tolerance than the T_{REG}:T_{CONV} ratio, especially at early timepoints before T_{CONV}s escaping suppression have had time to expand. It may also be possible to detect a dysfunction-associated phenotype in allospecific T_{CONV}s as a biomarker for effective T_{REG}-mediated suppression. In this study, we found that the $FR4^{hi}$ $CD73^{hi}$ phenotype was a better predictor of T cell dysfunction than the PD-1^{hi} $CD127^{lo}$ phenotype that has been associated with T cell exhaustion. Indeed, we found that in the absence of T_{REG}s, T cells became PD-1^{hi} $CD127^{lo}$ but retained functionality. Our results highlight a central role of T_{REG}s in maintaining tolerance through multiple downstream effects. Thus, therapies that support T_{REG} function, especially in the presence of infections or other inflammatory challenges, are likely to improve the robustness of tolerance.

Chapter 4. T cells specific for transiently expressed alloantigens retain function in tolerant allograft recipients.

Abstract

Achieving stable, robust donor-specific tolerance in transplant recipients has been challenging. While advances have been made to therapeutically induce tolerance in mouse models of transplantation and a limited number of clinical trials, resulting tolerant graft recipients are still vulnerable to inflammatory challenges such as infections. Understanding the mechanisms by which previously tolerized grafts are rejected after the establishment of donor-specific tolerance may identify strategies for improving the robustness of tolerance. While rejection after infection in previously tolerant graft recipients is T cell-dependent, it is unclear which specificity of alloreactive T cells mediate rejection since prior studies have indicated that allospecific T cells persisting during tolerance are intrinsically dysfunctional due to chronic stimulation in the tolerant environment. In this study, we found that alloreactive T cells specific for donor MHC Class II, a transiently expressed alloantigen, retain functionality during tolerance as they are not

chronically stimulated. Thus, there is heterogeneity in the fate of alloreactive T cells with distinct specificities persisting during tolerance, and cells retaining function during tolerance may pose a greater threat to the graft if their antigen is re-expressed after graft acceptance. These findings suggest that treatments to induce dysfunction in a broader array of allospecific T cells, such as prolonging exposure to alloantigens expressed transiently in the allograft, may improve the robustness of tolerance.

Introduction

Achieving stable donor-specific tolerance would immensely improve graft survival and quality of life for transplant recipients by eliminating the life-long exposure to drug toxicity, adverse effects and high risk of malignancy and infection associated with conventional immunosuppression. In mice, treatment with anti-CD154 and DST produces a particularly robust state of graft-specific tolerance (Miller et al. 2016a) and similar strategies to block CD40-CD154 signaling are being developed for clinical applications. However, studies in animal models have shown that susceptibility to rejection following infection remains a critical vulnerability in anti-CD154/DST-induced tolerance. Indeed, Lm infection induces T cell-dependent rejection in previously tolerant allograft recipients. Lm-induced rejection was dependent on IL-6 and type I IFN. Further, the combination of IL-6 + IFN-β broke established tolerance without infection, suggesting that tolerance may be vulnerable to any inflammatory setting eliciting these cytokines (Wang et al. 2010b). There is also evidence that infection poses a threat to clinical transplantation tolerance. A subset of patients who spontaneously developed operational tolerance rejected their grafts after years of stability, often after an infection (Brouard et al. 2012). Reports of patients made tolerant to renal allografts with concurrent hematopoietic stem

cell or bone marrow transplantation who later rejected their allografts following an infection further suggest that even the most effective strategies currently available for clinical tolerance induction are vulnerable to inflammatory challenges (Kawai et al. 2008, Leventhal et al. 2012). To become a reliable standard for clinical use, donor-specific tolerance must resist common insults such as infections.

Understanding the mechanisms of rejection after tolerance may identify strategies to improve the robustness of tolerance. While many studies have focused on the mechanisms of allograft rejection in otherwise untreated mice, little is known about rejection in previously tolerant mice. Lm-induced rejection was dependent on $CD4^+$ and $CD8^+$ T cells, which is surprising considering there are multiple immune mechanisms preventing T cell-mediated alloimmunity during transplantation tolerance. Anti-CD154/DST-mediated tolerance is associated with induction of alloreactive T_{REGs} (Ferrer et al. 2011) and abortive proliferation of alloreactive T_{CONVs}, resulting in a high T_{REG}:T_{CONV} ratio (Young et al. 2018). Our group previously found that T_{CONVs} specific for a donor MHC Class I-derived peptide presented on host APCs (TCR75 cells) that persist in tolerant mice become intrinsically dysfunctional, exhibiting poor effector function even after removal from the tolerant graft recipient (Miller et al. 2019). If all allospecific T_{CONVs} become intrinsically dysfunctional during tolerance, they should not be able to mediate rejection even after disruption of T_{REGs}.

As discussed in a previous chapter, T_{REG} depletion does break established tolerance, resulting in T cell-mediated alloimmunity. However, work from our group previously found that allospecific T cells did not expand or recover function after Lm infection (Miller et al. 2019), thus if T_{CONV} recovery does occur during Lm infection, it is likely to be short-lived and may involve a small quantity of T_{CONVs} compared to rejection in non-tolerant hosts, where expansion,

avidity maturation and strong effector responses are involved. Here, we investigated another potential source of T cell-mediated alloimmunity that could promote rejection after disruption of stable tolerance: T_{CONVs} that retain functionality and are controlled by other means, such as T_{REGs} or ignorance.

We previously identified markers of T cell dysfunction in monoclonal tracer allospecific T cells as well as endogenous allospecific T cells identified with allogeneic pMHC multimers (Miller et al. 2019), ensuring that our results were not specific to one T cell clone or specificity. However, the T cells observed thus far are unlikely to be representative of the entire allospecific T cell population, which is estimated to contain over 1% of mature T cells (Suchin et al. 2001). Variables including avidity for alloantigen and specificity for a particular alloantigen and allorecognition pathway may impact the fate of allospecific T cells during tolerance. Duration of antigen presentation has recently become appreciated as a key variable modulating alloreactive T cell responses (Ali et al. 2016b). Having determined that chronic alloantigen exposure is necessary for the development of dysfunction in allospecific T_{CONVs} during anti-CD154/DST-induced tolerance (Miller et al. 2019), we investigated whether duration of alloantigen expression impacts the development of T cell dysfunction.

Previous findings from our group indicated that the graft must persist for >1 week for tracer TCR75 cells to acquire dysfunction as TCR75 cells were not impaired functionally if grafts were surgically removed 1 week after transplantation with anti-CD154/DST treatment (Miller et al. 2019). However, some alloantigens are not expressed persistently in the graft. Unlike responses to donor MHC Class I, molecules constitutively expressed by all nucleated cells, direct responses to donor MHC Class II and indirect responses to peptides derived from donor MHC Class II are transient. Expression of MHC Class II antigens is thought to be short-

lived because donor APCs die shortly after transplantation (Ali et al. 2016a). Thus, there is potentially a large population of allospecific T cells specific for transiently expressed alloantigens whose fate during tolerance remains to be investigated.

Here, we investigated the functionality of donor MHC Class II peptide-reactive T cells persisting during transplantation tolerance. We found that these cells retained greater functionality than donor MHC Class I peptide-reactive T cells that were persistently activated during tolerance. Thus, T cells specific for transiently expressed alloantigens may remain functional but controlled by T_{REGs} and/or ignorance of the graft in tolerance because levels of their cognate antigen are low. Donor endothelial cells can upregulate MHC Class II in response to IFN-γ (Scott et al. 2018, Lian et al. 1996) produced, for example, during infection (O'Connell et al. 2004) or autoimmunity (Nyström et al. 2014). Re-expression of donor MHC Class II in endothelial cells or impairment in T_{REG}-mediated suppression observed after infection, or both, may contribute to rejection by alloreactive T_{CONVs} that remain functional in tolerance. Importantly, donor MHC Class II peptide-specific T cells could be made profoundly dysfunctional with repeated antigen stimulation over >4 weeks. Prolonging exposure to alloantigen through repeated donor blood transfusions during tolerance may represent a therapeutic approach to improve the robustness of tolerance by inducing dysfunction in a broader array of allospecific T cells.

Materials and Methods

Mice

B6 and BALB/c mice were purchased from Envigo RMS. TCR75 TCR-Tg mice obtained from R. Pat Bucy (University of Alabama) and TEa TCR-Tg mice obtained from Alexander Rudensky (when at the University of Washington) were crossed with $Rag^{-/-}$ mice to generate TCR75 $Rag^{-/-}$ (TCR75) and TEa $Rag^{-/-}$ (TEa) mice, respectively. Mice were housed under specific pathogen-free conditions.

DST was prepared by homogenizing and isolating a single cell suspension of splenocytes from BALB/c mice. Each injection contained splenocytes from one-quarter to one-sixth spleen in 200 µL PBS. In all experiments where mice were treated with DST, day 0 injection was i.v. In mice treated with repeated DST injections every 48 hours, all injections after day 0 were i.p.

Heart transplantation

Cardiac transplantation was performed using a technique adapted from Corry et al (Corry, Winn, and Russell 1973). For induction of tolerance, mice were treated with 500-600 µg of anti-CD154 (MR1, BioXCell) on days 0, (i.v.) 7 and 14 (i.p.) post-transplantation and DST (i.v.) on day 0.

Lm infection

An overnight culture of Lm engineered to express GFP (Lm-GFP) (Ertelt et al. 2009) was diluted 1:50 and grown until OD_{600} was within the log-phase of our Lm-GFP growth curve. OD_{600} was used to calculate colony forming units (CFU)/mL. Mice were infected i.p. with $1x10^6$ CFU Lm-GFP in 400 µL per mouse as this dose resulted in the highest rejection rate with minimal

lethality. Lm-GFP was plated to confirm dose. Graft survival was monitored twice per week after infection.

ELISA to detect donor MHC-specific antibodies

Clear 96-well high binding microplates (Corning) were bound overnight at 4˚C with 5 μg/mL streptavidin (Jackson Immunoresearch) in pH 9.63 carbonate-bicarbonate coating buffer. Plates were washed then bound with biotinylated SYIPSAEKI:K^d or FIEWNKLRFRQGLEW:I-E^d monomers (NIH Tetramer Facility) diluted to 8.65 nM in wash buffer (PBS + 0.05% Tween-20) for 1 hour at RT. After pMHC monomer binding, plates were washed, blocked with PBS + 1% bovine serum albumin (BSA), washed again and then incubated for 1 hour at RT with serum diluted 1:100 in wash buffer. Plates were washed then bound with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch) diluted 1:10,000 in wash buffer for 1.5 hours at RT. After washing, SigmaFAST para-nitrophenyl phosphate solution (Sigma) was added to detect alkaline phosphatase activity. OD_{405} was measured 15-30 minutes after addition of substrate. Background OD_{405} detection in wells without bound serum was subtracted from OD_{405} measurement in other wells before analysis.

Adoptive cell transfer

In experiments where TCR-Tg tracer cells were seeded before transplantation or immunization, cells were isolated from the spleen and lymph nodes (inguinal, axillary, brachial, cervical and mesenteric) of naïve TCR75 or TEa mice and counted with an Accuri C6 flow cytometer (BD Biosciences). $5x10^4$ cells were injected i.v. in 200 μL of PBS up to 1 day before transplantation or the first DST injection.

Isolation of tracer TCR-Tg cells from primary hosts

Spleen and lymph nodes from primary hosts were harvested and homogenized ≥30 days following transplantation or first DST injection. Single cell isolates were stained with anti-CD45.1-biotin (eBioscience) and incubated with streptavidin magnetic beads (Miltenyi) for magnetic enrichment with LS columns (Miltenyi) or an AutoMACS machine (Miltenyi). In some experiments, cells form mice within the same experimental group were pooled after magnetic enrichment. Magnetically enriched cells were then stained with fluorophore conjugated anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD44 (IM7) and streptavidin and sorted for $CD45.1^+$ $CD4^+$ $CD44^{hi}$ cells on a FACSAria cell sorter (BD Biosciences). Cells were sorted into FBS, then washed and re-suspended in PBS and subjected to further staining or functional analysis *in vitro* or *in vivo*.

Flow cytometry

Sorted TCR-Tg cells or unenriched spleen and lymph node cells were stained with a fixable live/dead stain (Invitrogen). For tetramer staining $5x10^6$ cells followed by phycoerytherin (PE)- or allophycocyanin (APC)-coupled pK^d:I-A^b tetramer (NIH Tetramer Core Facility) for 1 hour in a room temperature (RT) water bath, washed, and then stained with PE- or APC-coupled H60:K^b tetramer (NIH Tetramer Core Facility) for 20 minutes in a room temperature (RT) water bath. In some experiments, tetramer-stained cells were then magnetically enriched for T cells. Cells were then stained with fluorophore conjugated anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD44 (IM7), anti-PD-1 (J43), anti-CD127 (A7R34), anti-FR4 (12A5), anti-CD73 (TY/11.8) and anti-B220 (RA3-6B2). Surface-stained cells were then fixed with the FoxP3 fixation permeabilization buffer kit (eBioscience) for 15 minutes at RT and washed with 1x permeabilization buffer. Some

samples were intracellularly stained with anti-Ki67 (SolA15) or anti-FoxP3 (FJK-16s) for 30 minutes at RT; washed with permeabilization buffer; and analyzed by flow cytometry. CFSE labeling was performed by labeling cells with CellTrace CFSE (Invitrogen) for 20 minutes at 37°C. All monoclonal antibodies (mAbs) were from BD Biosciences, eBioscience, or Invitrogen.

In vitro stimulation for cytokine production

U-bottom tissue culture plates were coated for 90 minutes at 37˚C with 5 μg/mL anti-CD3 (2C11) and 1 μg/mL anti-CD28 (PV.1) (Fitch Monoclonal Facility). 500-2000 tracer TCR-Tg cells sorted from primary hosts were plated per well and incubated for 16-24 hours at 37˚C and 5% CO_2. Within each individual experiment, all wells were plated with the same number of cells. In some experiments, $1x10^5$ B6 splenocyte feeder cells per well were co-cultured with TCR-Tg cells. Unstimulated controls were plated in uncoated wells with 1 ng/mL human IL-7 (PeproTech). 2 hours after plating, brefeldin A (BioLegend) was added to all wells. After stimulation cells were stained with fixable viability dye (Invitrogen) and then surface stained with fluorophore conjugated anti-CD4, anti-CD8, anti-CD45.1, anti-CD45.2 and anti-CD44. Cells were then fixed and permeablized with the FoxP3 fixation permeabilization buffer kit (eBioscience) and stained with fluorophore conjugated anti-IFN-γ (XMG1.2) and anti-TNF (MP6-XT22) for 30 minutes at RT or overnight at 4˚C before washing with permeablization and flow cytometry analysis.

Evaluation of recall expansion

500-2000 sorted TCR-Tg cells were injected i.v. into naïve B6 mice. After one day, secondary hosts were immunized with BALB/c DST i.v. Within each experiment, the number of transferred cells was consistent between mice. Cell concentration was confirmed by counting on an Accuri

C6 flow cytometer prior to injection. 5 days after DST, $5x10^6$ splenocytes were isolated from secondary hosts then stained with a viability dye and fluorophore conjugated anti-CD4, anti-CD8, anti-CD45.1 and anti-CD45.2. The number of $CD45.1^+$ $CD4^+$ cells was calculated per mouse.

Data analysis

Flow cytometry data were analyzed using FlowJo (Tree Star, Ashland, Oregon). Flow cytometry samples were gated on live single cells before analysis. Tetramer stained samples were also gated on $B220^-$ cells. Statistical analyses were performed where appropriate using GraphPad Prism (GraphPad, La Jolla, California). Each statistical test is listed in the figure legends.

Study Approval

The studies were performed in agreement with the University of Chicago's Institutional Animal Care and Use Committee, according to the NIH guidelines for animal use.

Results

The alloimmune response to MHC Class II is temporary

We hypothesized that in mice receiving MHC Class I and II mismatched heart allografts, recipient T cells continue to respond to donor MHC Class I as it is persistently expressed by most donor cells, while the alloimmune response to donor MHC Class II is transient, as passenger APCs, a large source of donor MHC Class II, die shortly after transplantation. Graft endothelial cells can also up-regulate expression of MHC Class II in response to IFN-γ (Pober et al. 1983, Lapierre, Fiers, and Pober 1988), though we hypothesized that endothelial cells would

not provide sufficient antigen to persistently stimulate T cells specific for donor MHC Class II-derived antigens late after transplantation when inflammation due to ischemia-reperfusion injury had resolved. We adapted a previously established protocol to detect T cell responses to indirectly presented peptides derived from donor MHC Class I and II during the maintenance phase of tolerance (Ali et al. 2016b) by adoptively transferring CFSE-labeled TCR-Tg T cells with known specificity for each alloantigen into tolerant BALB/c heart recipients ≥35 days after transplantation with anti-CD154/DST treatment. CFSE dilution was observed after 4 days as a measure of cell proliferation. To detect the T cell response to donor MHC Class I late after the establishment of tolerance, we used TCR75 TCR-Tg cells, which are specific for a donor K^d-derived peptide presented indirectly on host I-A^b. TEa TCR-Tg cells specific for a donor I-E^d-derived peptide presented indirectly on host I-A^b were used to track T cell-mediated autoimmunity to donor MHC Class II (Figs. 4.1A, B).

As expected, almost all TCR75 cells proliferated sufficiently to fully dilute CFSE after adoptive transfer into B6 mice that had received BALB/c heart allografts with anti-CD154/DST 35 days prior (Fig. 4.1C left), reflecting the persistence of donor MHC Class I expression. Conversely, the majority of naïve CFSE-labeled TEa cells failed to proliferate after adoptive transfer into similarly tolerant mice (Fig. 4.1C center left), suggesting low expression and/or presentation of donor MHC Class II in the tolerant hosts. Importantly, TEa cells proliferated to a similar extent as TCR75 cells after transfer into naïve B6 mice immunized with BALB/c DST, indicating that naïve TEa cells are not intrinsically impaired in their ability to proliferate.

We next investigated whether provision of additional antigen in the form of BALB/c DST could rescue naïve TEa proliferation after transfer into stably tolerant heart recipients. Indeed, the addition of DST allowed nearly all TEa cells to proliferate after transfer into tolerant

heart recipients (Fig. 4.1C center right), suggesting that the TEa response is limited by donor MHC Class II antigen quantity 35 days after transplantation in tolerant hosts. Nevertheless, TEa cells underwent fewer divisions on average in these DST-immunized tolerant hosts than in DST-immunized naïve mice, indicating that the magnitude of the TEa response was suppressed in the tolerant animals. Overall, antigen did appear to be limiting for TEa cells in tolerant mice, but not for TCR75 cells in tolerant mice.

It was also possible that, in our model, heart allografts do not contain sufficient MHC Class II to consistently stimulate TEa cells. In this case, TEa cells would not proliferate well even in recently transplanted mice. We tested this hypothesis by transferring CFSE-labeled TEa cells into a mouse transplanted with a BALB/c heart allograft 3 days prior. We found that shortly after transplantation, BALB/c heart allografts contain sufficient antigen to stimulate a response by nearly all transferred TEa cells (Fig. 4.1C right).

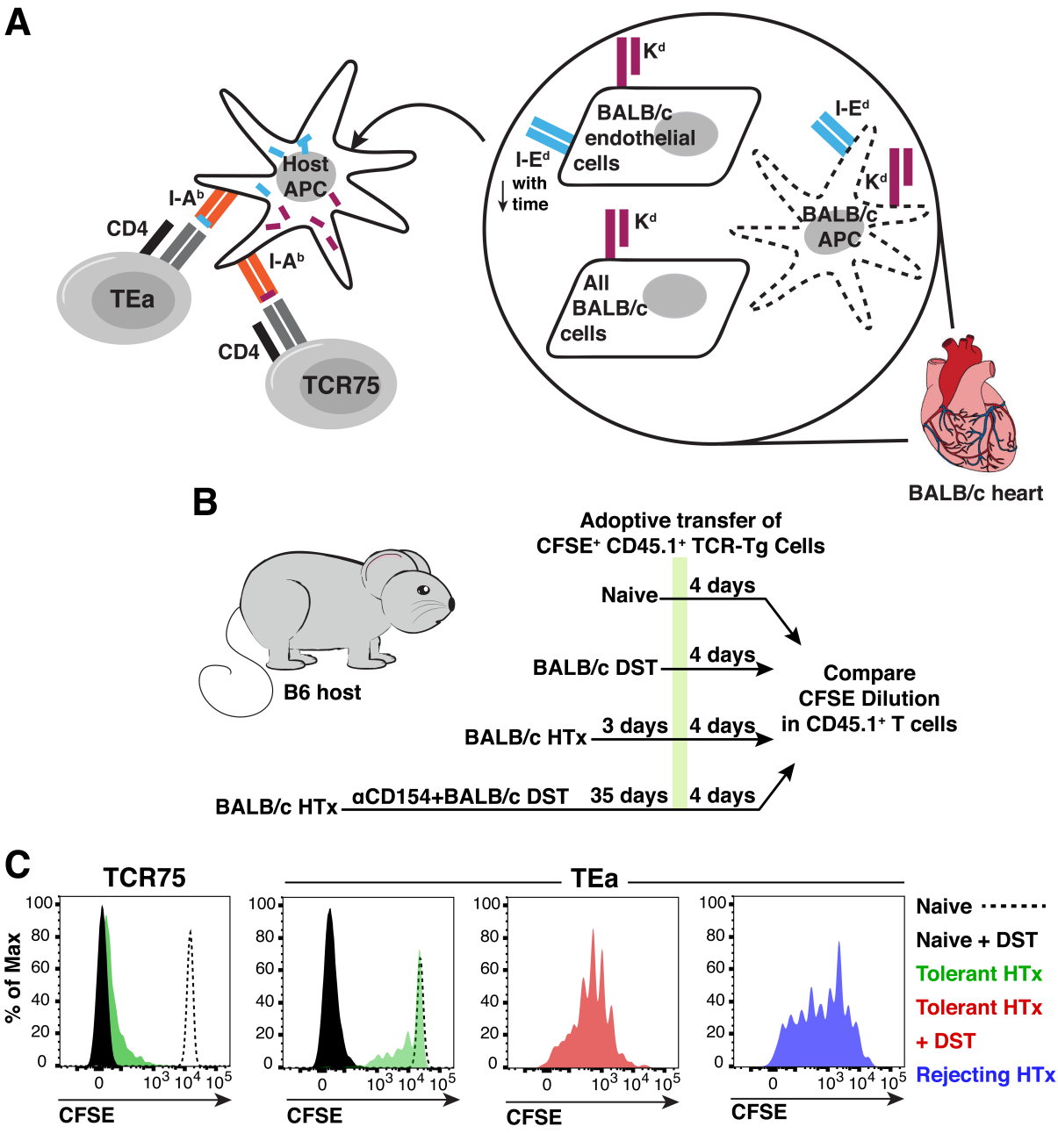

Figure 4.1 T cell response to donor MHC Class II declines after heart transplantation.

(A) Model of antigen persistence and presentation to TEa and TCR75 cells after BALB/c to B6 heart transplantation. (B) Experimental design for (C). (C) Histograms showing CFSE dilution in TCR75 Rag$^{-/-}$ or TEa Rag$^{-/-}$ T cells 4 days after adoptive transfer into naïve B6 mice or rejecting or tolerant B6 recipients of BALB/c heart allografts +/- BALB/c DST at the time of adoptive transfer. Results are representative of 2-3 mice per experimental group.

T cells specific for donor MHC Class II retain more functionality than T cells specific for donor MHC Class I during the maintenance phase of tolerance.

Having observed that the majority of TEa cells specific for donor MHC Class II fail to proliferate during the maintenance phase of tolerance, and considering our previous findings that persistent antigen stimulation is required for the induction of tolerance (Miller et al. 2019), we hypothesized that even antigen experienced TEa cells would fail to become dysfunctional during tolerance as they would not be chronically stimulated. To test this hypothesis, we compared the phenotype and functionality of $CD44^{hi}$ tracer TCR75 versus tracer TEa cells isolated from rejecting or anti-CD154/DST-treated BALB/c heart recipients ≥35 days after transplantation (Fig. 4.2A). Fewer TEa cells than TCR75 cells were isolated from the spleen and lymph nodes of tolerant mice (Fig. 4.2B), the majority of which were $CD44^{hi}$. Importantly, many TEa cells differentiated into T_{REGs} in tolerant mice, whereas TCR75 cells rarely develop into T_{REGs} (Fig. 4.2C). Later analyses were performed only on $CF44^{hi}$ $FoxP3^{-}$ tracer TCR75 and TEa T_{CONVs}. We found that while Tol TCR75 cells had higher expression of PD-1, a marker of recent stimulation, than naïve TCR75 cells, Tol TEa cells expressed a low level of PD-1 similar to naïve cells (Figs. 4.2 D, E), further supporting our findings that TEa cells are not stimulated well during the maintenance phase of tolerance. Interestingly, both Tol TCR75 and Tol TEa cells down-regulated CD127 expression compared to cells from rejecting mice (Figs. 4.2D, F), suggesting that while donor MHC Class II antigen may be limiting at late time points after transplantation in tolerant hosts, Tol TEa cells do not form the canonical memory cells ($CD127^{hi}$) found in rejecting mice, as would be expected if the donor MHC Class II antigen had been eliminated from the tolerant host. Finally, we compared expression of the anergy markers FR4 and CD73 on Tol TCR75 and Tol TEa cells. While Tol TCR75 cells expressed very high levels of both FR4

and CD73, TEa cells only expressed high levels of FR4. Instead of the high expression of CD73 seen on Tol TCR75 cells, Tol TEa cells expressed an intermediate level of CD73 similar to that seen on the surface of memory cells (Figs. 4.2D, G, H). There were no differences in the rejecting or naïve cell phenotypes between TEa and TCR75 T_{CONVs} with regard to FoxP3, PD-1, CD127, FR4 and CD73 expression (Fig. 4.2).

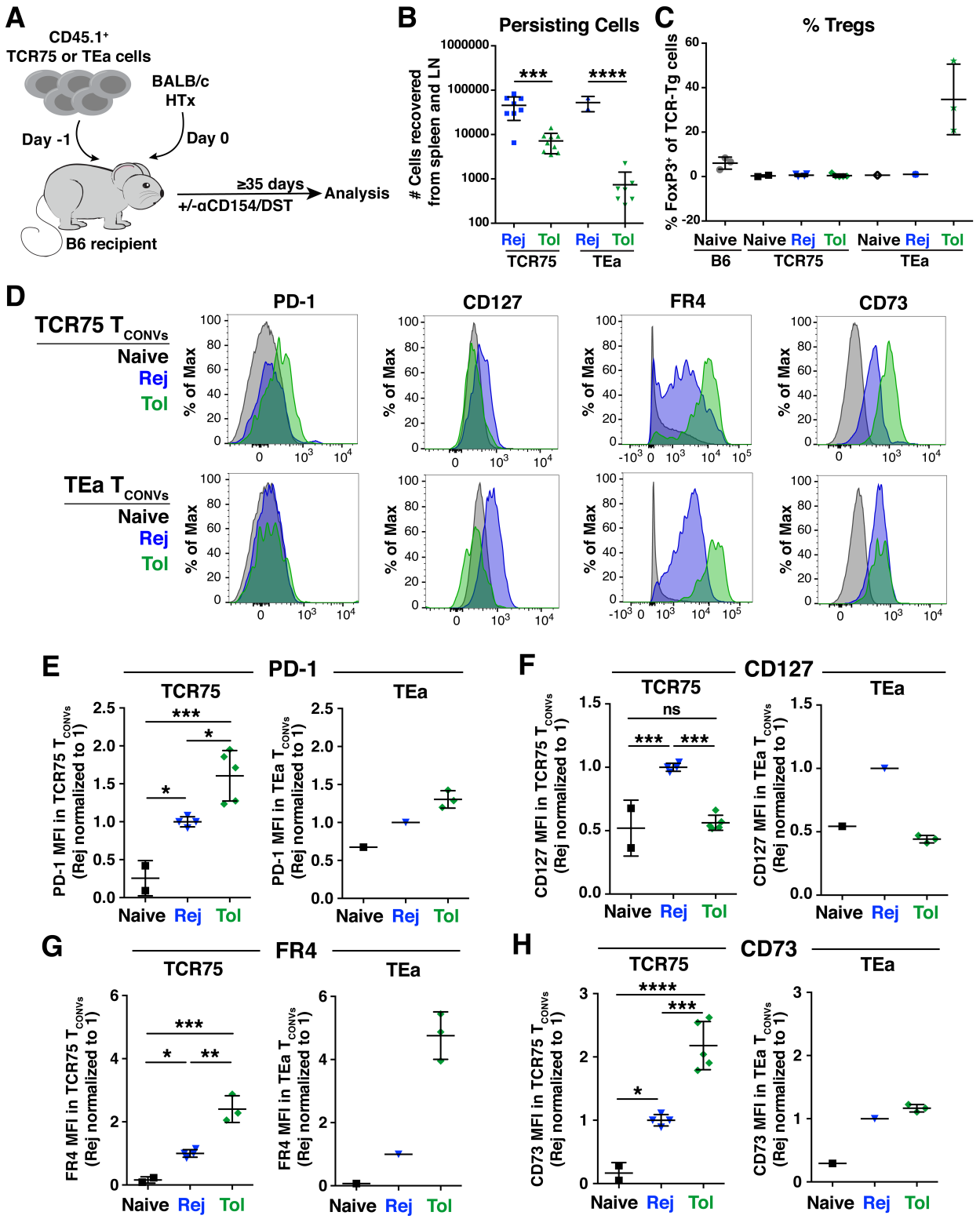

Figure 4.2 T cells specific for donor MHC Class II express a partial dysfunction-associated surface phenotype during the maintenance of tolerance.

(A) Experimental design. (B) Number of tracer TCR75 or TEa cells recovered from the spleens and lymph nodes of tolerant (Tol) and rejecting (Rej) B6 recipients of BALB/c heart allografts.

Figure 4.2, continued: (C) Percent FoxP3^{+} in naïve B6, TCR75 and TEa cells and CD44^{hi} tracer TCR-Tg cells recovered as in (B). (D) Representative flow plots for PD-1, CD127, FR4 and CD74 staining in naïve Tol and Rej TCR75 and TEa T_{CONVs}. (E) PD-1, (F) CD127, (G) FR4 and (H) CD73 MFI in naïve, Tol and Rej TCR75 and TEa T_{CONVs}. Results were pooled from 1-4 independent experiments. All results are representative of at least 2 independent experiments. Each data point or flow plot represents a sample pooled from 1-5 mice with lines indicating average +/- SD. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple pairwise comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Having found that TEa cells expressed some but not all markers of T cell dysfunction, we next investigated whether TEa cells failed to lose function during tolerance by isolating tracer TEa and TCR75 cells from tolerant and rejecting heart allograft recipients ≥35 days after transplantation and subjecting them to functional analyses. Consistent with prior findings from our group, Tol TCR75 cells proliferated less than Rej TCR75 cells upon re-stimulation in secondary hosts. Conversely, TEa cells from Tol mice were not impaired in their expansion in secondary hosts when compared to Rej TEa cells (Fig. 4.3A). We next evaluated the ability of Tol TEa cells to produce effector cytokines. As memory cells, both Rej TCR75 and Rej TEa cells were able to produce IFN-γ and TNF upon *in vitro* stimulation. Tol TCR75 cells were significantly impaired in their production of both IFN-γ and TNF. Tol TEa cells, on the other hand, fully retained their ability to produce TNF but were impaired in IFN-γ production to a similar extent as Tol TCR75 cells (Figs. 4.3B, C). This is consistent with work in exhausted T cells, where effector functions are progressively lost during chronic viral infection, with the ability to produce IFN-γ lost before the ability to produce TNF (Wherry and Kurachi 2015), and suggests an intermediate dysfunction of Tol TEa cells.

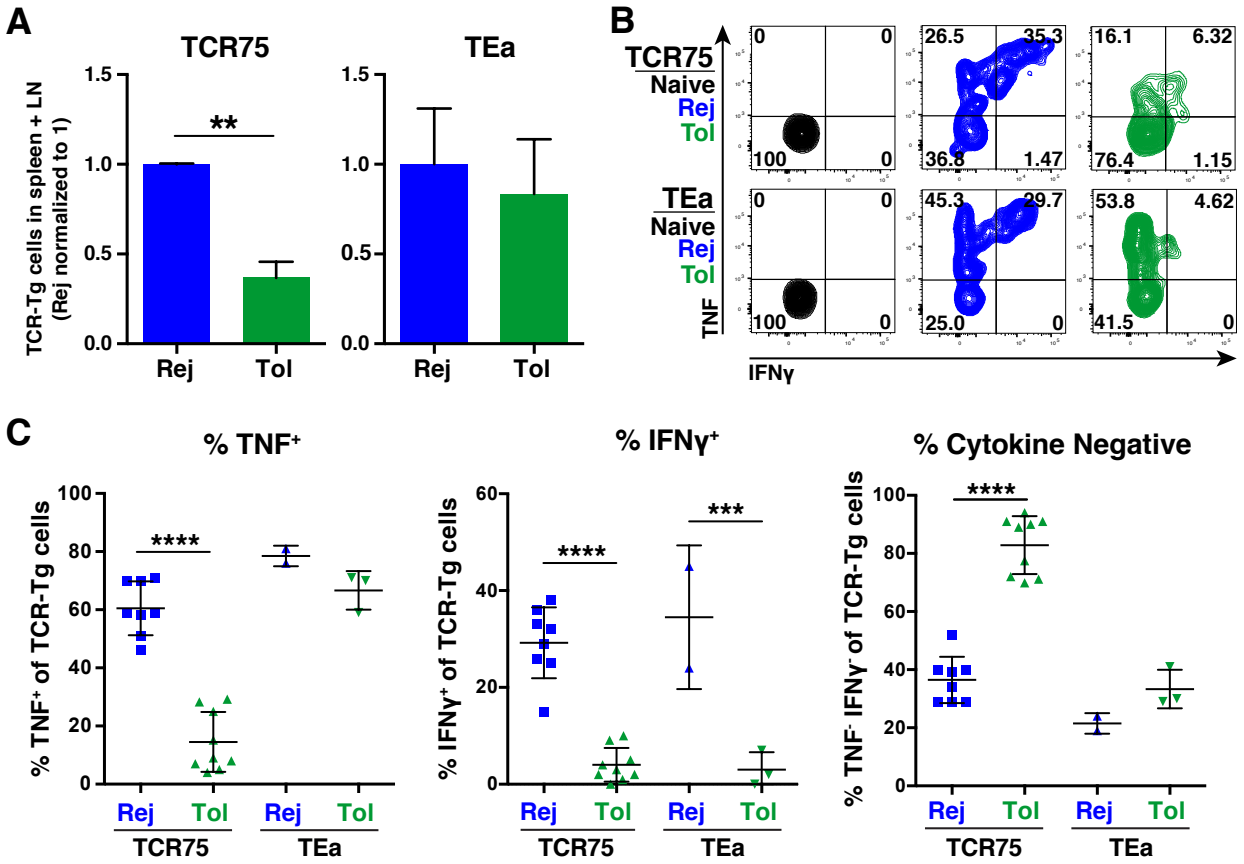

Figure 4.3 T cells specific for donor MHC Class II retain greater functionality than T cells specific for donor MHC Class I during the maintenance of tolerance.

(A) CD44^{hi} tracer TCR75 Rag$^{-/-}$ and TEa Rag$^{-/-}$ cells were sorted from the spleens and lymph nodes of rejecting (Rej) or tolerized (Tol) B6 recipients of BALB/c heart allografts ≥30 days post-transplantation and then adoptively transferred into naïve B6 secondary hosts. One day later, secondary hosts were immunized with BALB/c DST and recall expansion was measured by quantifying the number of TCR-Tg cells that had accumulated in the spleen 5 days later. Data were normalized, with the average cell recovery from Rej mice set to 1 for each experiment. (B, C) *In vitro* production of IFN-γ and TNF by TCR-Tg cells recovered as in (A). Results were pooled from 1-2 independent experiments. All results are representative of at least 2 independent experiments. Each data point or flow plot represents a sample pooled from 1-5 mice with lines indicating average +/- SD. Data were analyzed by t-test (A) or one-way ANOVA with Bonferroni correction for multiple pairwise comparisons (C). **p<0.01, ***p<0.001, ****p<0.0001.

Both donor MHC Class I- and MHC Class II-specific T cells can be made dysfunctional by the addition of repeated injections of donor splenocytes to co-stimulation blockade.

Our comparison of tolerant TCR75 versus tolerant TEa cells suggested that prolonged exposure to alloantigen was associated with greater dysfunction in allospecific T cells. Thus, we determined whether TEa cells could be made similarly dysfunctional to TCR75 cells if they were exposed to their alloantigen for a longer period of time. To accomplish this, we seeded naïve B6 mice with tracer TCR75 or TEa cells and then either immunized the mice with one injection of BALB/c DST or treated them with anti-CD154 and repeated injections of BALB/c DST, which contains abundant MHC Class I and II, every 48 hours for 35 days prior to functional analysis of the persisting tracer TCR-Tg cells. Indeed, we found that anti-CD154 combined with prolonged antigen exposure in the form of repeated DST injections led to profound impairment in recall proliferation of tracer TCR75 and TEa cells (Fig. 4.4A). TCR75 and TEa cells were also similarly impaired in production of both IFN-γ and TNF (Figs. 4.4B, C). These results indicate that increasing the persistence of alloantigen can lead to a loss of function in more allospecific T cells, and also show that TEa cells do not intrinsically resist the development of dysfunction. These data also suggest a therapeutic avenue to increase the robustness of donor-specific T cell dysfunction and potentially transplantation tolerance.

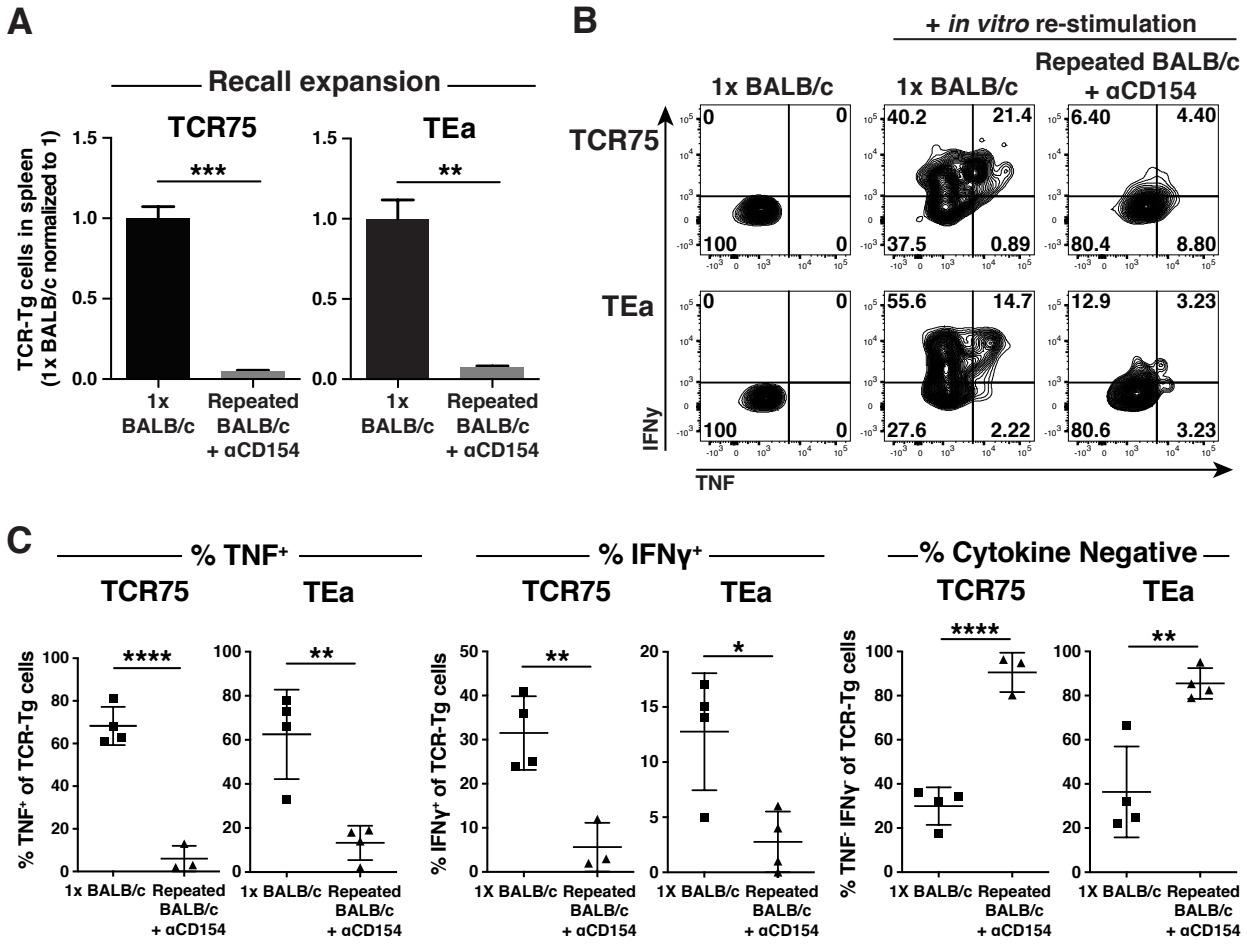

Figure 4.4 Donor MHC Class I- and Class II-specific T cells become dysfunctional with prolonged exposure to their cognate antigen.

B6 mice were adoptively transferred with TCR75 Rag$^{-/-}$ or TEa Rag$^{-/-}$ cells then either immunized with a single injection of BALB/c DST (1x BALB/c) or given anti-CD154 (days 0, 7 and 14) and repeated injections of BALB/c DST every 48 hours until sacrifice on day 35 (Repeated BALB/c + anti-CD154). (A) CD44^{hi} tracer TCR-Tg cells were sorted from spleens and lymph nodes. Recovered cells were then adoptively transferred into naïve B6 secondary hosts. One day later, secondary hosts were immunized with BALB/c DST and recall expansion was measured by quantifying the number of TCR-Tg cells that had accumulated in the spleen 5 days later. Data were normalized, with the average cell recovery from 1x BALB/c mice set to 1 for each experiment. (B, C) *In vitro* production of IFN-γ and TNF by TCR-Tg cells recovered as in (A). Results were pooled from 2 independent experiments. Each data point represents a sample pooled from 1-2 mice with lines indicating average +/- SD. Data were analyzed by t-test. **p<0.01, ***p<0.001, ****p<0.0001.

Discussion

In this study, we determined that allospecific T_{CONVs} develop varied levels of dysfunction during anti-CD154/DST-induced transplantation tolerance. Our results suggest that the kinetics of expression of different alloantigens contribute to this heterogeneity. Allospecific T cells experiencing persistent, chronic stimulation during tolerance, modeled by TCR75 cells, lose more function than transiently expressed alloantigen-specific T cells, including tracer TEa cells. Cells that retain more functionality may pose a greater threat to graft survival during tolerance, especially if they escape suppression by T_{REGs}.

The population of endogenous T cells recognizing alloantigens expressed transiently during tolerance is likely to expand beyond cells specific for indirectly presented MHC Class II-derived peptides. T cells recognizing donor MHC Class II presented directly on donor APCs are likely to experience similarly transient kinetics of stimulation as cells recognizing donor MHC Class II-derived peptides presented indirectly on host APCs. It is also possible that some minor histocompatibility antigens are expressed transiently in the graft, potentially including polymorphic stress-induced molecules whose expression is expected to decline after resolution of ischemia-reperfusion injury. Additionally, T cells with low avidity for alloantigens that are persistently expressed or T cells specific for alloantigens persistently presented in low quantities may not be chronically stimulated if they rarely encounter APCs presenting a sufficient quantity of their cognate antigen to activate their TCR. Thus, there is potentially a large population of allospecific T cells that would not experience the chronic stimulation needed to program dysfunction during tolerance. Observing the diversity of T cell clones from a naïve mouse that are activated after adoptive transfer into recently transplanted versus stably tolerant mice may provide an estimate of the number of transiently stimulated allospecific T cells.

It is not yet clear whether TCR75 and TEa cells represent the full range of dysfunctional states in allospecific T cells during tolerance. It is possible that there are allospecific T cells that retain even more function than TEa cells during tolerance, including IFN-γ production. Previous results from our group showed that in mice without of a heart allograft to provide chronic alloantigen exposure, anti-CD154/DST treatment induced TCR75 cells to differentiate into memory cells instead of developing dysfunction (Miller et al. 2019). TEa cells did not up-regulate expression of the canonical memory cell marker CD127 during tolerance, possibly because they experience an intermediate duration of alloantigen exposure. There may be other allospecific T cells stimulated even more briefly than TEa cells, which form long-lived memory cells during tolerance. The persistence of allospecific memory T cells during tolerance is of particular concern as memory T cells have been shown to resist T_{REG}-mediated suppression (Yang et al. 2007).

Little is known about the programming of dysfunction in tolerized allospecific T cells beyond the necessity for chronic alloantigen exposure. That TEa cells have lost the ability to produce IFN-γ but are fully capable of recall expansion and TNF production, rather than displaying a partial impairment in all three functions, suggests that there is a sequential loss of function and potentially discrete stages of dysfunction in tolerized cells. Up-regulation of the surface markers of anergy also appears to occur in stages, with CD73 up-regulated to an intermediate level, similar to that observed in memory T cells, followed by strong up-regulation of FR4, and then a second up-regulation of CD73 expression. Expression of each of these markers may correlate with loss of particular effector functions, making them useful biomarkers for the extent of dysfunction in T cells. For example, FR4 up-regulation appears to occur

alongside loss of IFN-γ production, while loss of TNF production and recall proliferation appear to occur later, with the second up-regulation of CD73.

We have evaluated the functionality of tolerized allospecific T_{CONVs} by examining their ability to perform multiple central functions: expansion and production of effector cytokines. However, T_{CONVs} play a multi-faceted role during allograft rejection, which includes providing help to B cells and $CD8^+$ T cells. Indeed, while expansion and production of IFN-γ and TNF are important for T_{CONV} function, it is possible that even the most dysfunctional allospecific T_{CONVs} retain sufficient functionality during tolerance to reject an allograft and that rejection is controlled fully by T_{REGs}. As shown in the previous chapter, experiments to evaluate whether tolerized allospecific T_{CONVs} are sufficient to reject a graft in the absence of other lymphocytes are complicated by the recovery of T_{CONVs} outside the tolerant environment, including other lymphocytes. Preliminary RNAseq data from our group do indicate that tolerant TCR75 cells are highly distinct from memory TCR75 cells at the transcriptional level beyond the effector functions evaluated in this study.

For functional transiently activated T cells to pose a threat to the allograft, they must be exposed to their cognate alloantigen at a later time. Thus, T cells specific for alloantigens irreversibly down-regulated shortly after transplantation are likely harmless. For T cells directly and indirectly recognizing donor MHC Class II, we hypothesize that secondary exposure would occur during an inflammatory challenge, when MHC Class II expression is up-regulated on graft endothelial cells in response to IFN-γ. Of course, this would only be possible for vascularized grafts containing donor-derived endothelial cells that survive long-term after transplantation. Similarly, expression of hypothesized transiently-expressed alloantigens derived from stress-induced minor histocompatibility antigens would be up-regulated during injury or inflammation.

Alternatively, T cells specific for alloantigens that are expressed persistently at levels too low to induce chronic stimulation may, on rare occasion, encounter sufficient alloantigen to become activated. This may occur by chance, or at a time when a large amount of their alloantigen is shed from the graft, processed and presented, for example following injury and death of graft cells.

The functional allospecific T cells we have identified may play an important role in rejection late after transplantation that is observed commonly in clinical transplant recipients. Prior work from our lab showed that chronically stimulated allospecific T cells also developed dysfunction in graft recipients treated with conventional immunosuppression rather than anti-CD154/DST (Miller et al. 2019). However, alloimmune responses still occur late after transplantation, resulting in chronic rejection and episodes of acute rejection. Allospecific T cells retaining functionality long-term during tolerance may provide the source of alloreactivity needed for rejection late after transplantation. Indeed, alloantibodies produced *de novo* after transplantation are often directed towards alloantigens predicted to exhibit transient expression. For example, de novo alloantibody production is more frequently directed towards donor MHC Class II than MHC Class I, and is predictive of graft failure (Ntokou et al. 2011, Haririan et al. 2009). More recently, alloantibodies specific for the polymorphic stress-induced molecule MICA have been detected in transplant recipients, and are associated with poor graft outcome (Zou et al. 2007).

Understanding how allospecific T_{CONVs} can circumvent or reverse the mechanisms of tolerance and which cells drive rejection during infection will inform strategies to prevent disruption of tolerance. Overall, we have identified a previously unappreciated source of functionality in the allospecific T cell population that may pose a significant risk to graft survival

during donor-specific tolerance or possibly also following transplantation with conventional immunosuppression. We have also determined that repeated injections of donor splenocytes could provide the chronic antigen exposure needed to induce dysfunction in TEa cells. If functionality retained by allospecific T cells during transplantation tolerance contributes to rejection late after transplantation, then prolonging exposure to alloantigens expressed transiently in the graft may increase the robustness of donor-specific tolerance by promoting the development of more severe dysfunction in a wider array of allospecific T cells.

Chapter 5. Discussion

Introduction

This work identifies T_{REG}-mediated suppression as a central mechanism required for maintaining transplantation tolerance. Indeed, disruption of T_{REG}-mediated suppression swiftly resulted in rejection in previously tolerant graft recipients, accompanied by allospecific T cell expansion, avidity maturation and alloantibody production. While cell-intrinsic dysfunction in tolerized allospecific T cells should protect the graft from rejection, we find that some allospecific T cells retain functionality during tolerance, and those which do become profoundly dysfunctional may recover, potentially posing a threat to graft survival. Our results indicate that the robustness of tolerance may be improved by strategies to protect T_{REG} function, and may be enhanced by treatments that promote more profound dysfunction in a broader population of allospecific T cells. In transplant recipients who achieve tolerance, monitoring allospecific T_{REG} function or T_{CONV} dysfunction may be a useful strategy to detect disruptions to the tolerant state, prompting treatments to restore tolerance and prevent rejection.

Working Model

We propose a working model of the mechanisms required to maintain transplantation tolerance and describe how tolerance may be disrupted following insults such as Lm infection. Treatment with the combination of anti-CD154 and DST drives allospecific T_{REG} induction and expansion paired with abortive proliferation of allospecific T_{CONV}s. The resulting high T_{REG}:T_{CONV} ratio is maintained long-term during tolerance, promoting continuous suppression of persisting allospecific T_{CONV}s as well as new thymic emigrants (Fig. 5.1). As a result, allospecific T_{CONV}s fail to expand during tolerance, even though they continue to encounter their cognate antigen. As avidity maturation requires T cell expansion, suppressing productive T cell proliferation through co-stimulation blockade, and later T_{REG}-mediated suppression, prevents avidity maturation of allospecific T_{CONV}s. It is not yet clear whether any individual downstream effect of T_{REG}-mediated suppression is most important for maintaining tolerance. Transfer of a large number of allospecific T_{CONV}s can induce rejection in previously tolerant hosts (Miller et al. 2016b), though it is not known whether reversing T_{CONV} dysfunction without direct impairment of T_{REG}s can break established tolerance.

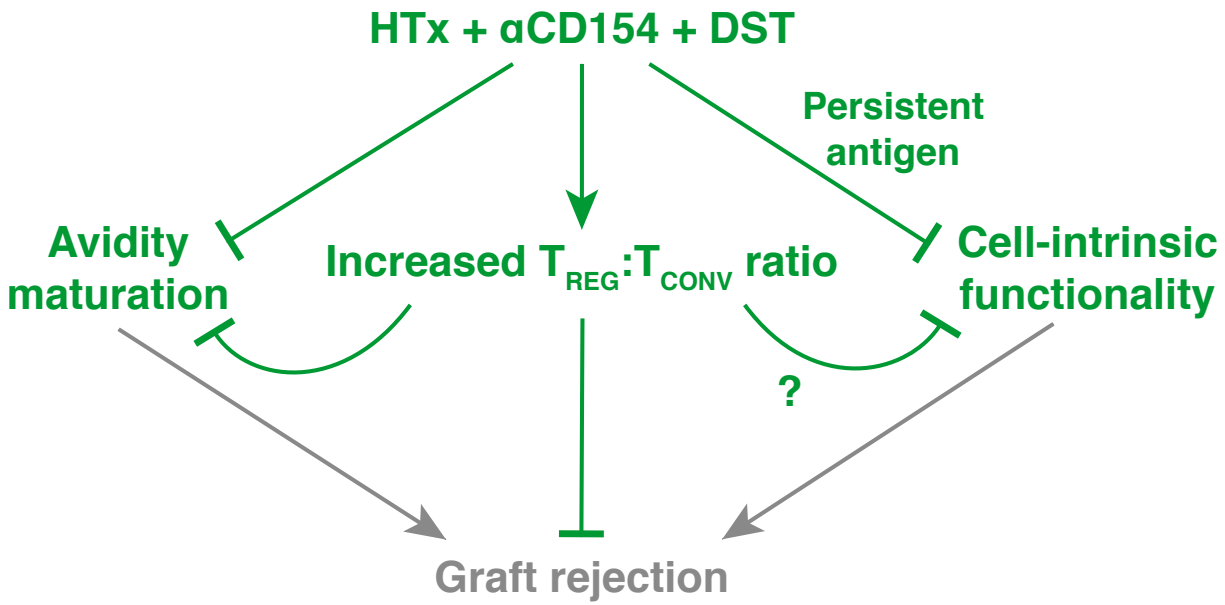

Figure 5.1 Changes to the allospecific T cell repertoire that support allograft acceptance after anti-CD154/DST treatment.

In our model describing the mechanisms of transplantation tolerance, anti-CD154/DST treatment induces long-term changes to the allospecific T cell repertoire, which support allograft survival. First, anti-CD154/DST treatment restricts avidity maturation within the allospecific T cell population, limiting accumulation of highly potent high avidity T cells. Second, treatment with anti-CD154/DST increases the ratio of allospecific T_{REGs}:T_{CONVs}, promoting T_{REG}-mediated suppression of alloimmunity. Finally, anti-CD154/DST treatment induces cell-intrinsic dysfunction in persisting allospecific T_{CONVs} by promoting survival of the graft as a source of chronic antigen stimulation and inducing additional dysfunction-promoting signals. There may also be interplay between these mechanisms, as impairment in T_{REG} function led to avidity maturation of some allospecific T cells and T_{REGs} may provide signals required for programming T_{CONV}-intrinsic dysfunction during tolerance.

Allospecific T_{CONVs} persisting within the tolerant graft recipient encounter chronic stimulation and dysfunction-inducing signals, which prevent them from developing into highly functional memory cells. As cells are chronically stimulated, they first lose the ability to produce IFN-γ, then later lose potential for recall expansion and TNF production. As they lose functionality, chronically stimulated cells also up-regulate markers of anergy, first up-regulating expression of CD73 to an intermediate level, then increasing FR4 expression, and finally undergoing a second up-regulation of CD73. Intrinsic dysfunction may prevent T_{CONVs} from expanding or performing effector functions if they are stimulated while a T_{REG} is not

immediately present to suppress their activation, as we observed in tolerant TCR75 cells that were isolated from the tolerant environment and stimulated either *in vitro* or in naïve secondary hosts. Cell-intrinsic dysfunction may also explain why some allospecific T cells do not avidity mature when T_{REGs} are depleted during the maintenance phase of tolerance, though it is unclear why some T cells do remain dependent on T_{REGs} for preventing avidity maturation.

We have found that the dysfunctional state is reversible. While the signals required to reverse T cell dysfunction have not been identified, they likely include an absence of suppressive signals (potentially deriving from T_{REGs}), cessation of TCR signaling, encounter of pro-inflammatory molecules or some combination of these factors. T cells whose cognate alloantigens are expressed only transiently within the graft may retain functionality during tolerance either because they were not stimulated with enough chronicity to induce the dysfunctional state or because they recovered function after they stopped encountering TCR signaling (Fig. 5.2). While we have focused our study on an indirectly presented donor MHC Class II-derived peptide as a transiently expressed alloantigen, there are likely other transiently expressed alloantigens in both murine and human allografts, including stress-induced molecules and directly presented donor MHC Class II. Low avidity allospecific T cells or T cells specific for poorly presented alloantigens additionally may not encounter chronic TCR signaling of sufficient intensity to program dysfunction to the same extent as we have observed in TCR75 cells during tolerance.

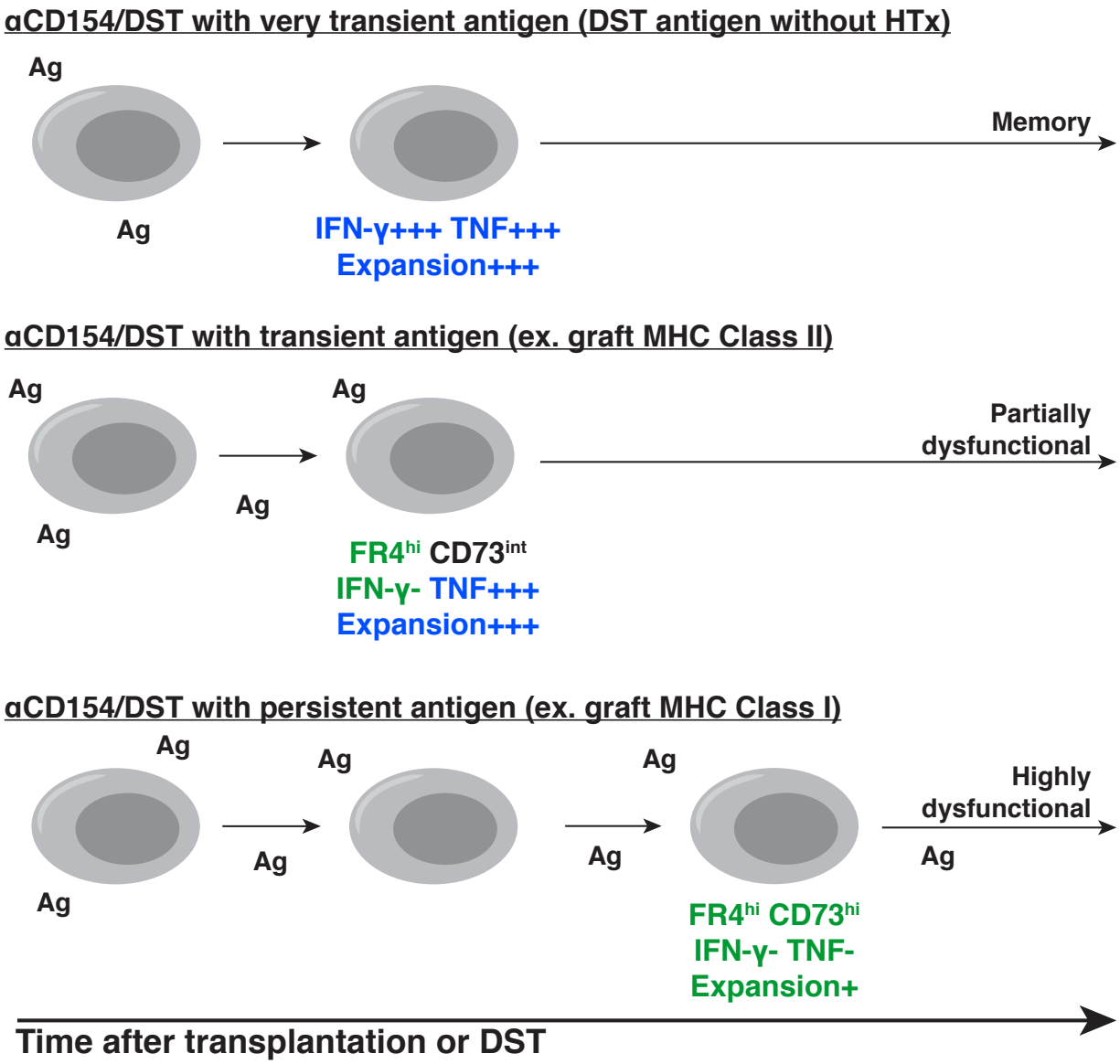

Figure 5.2 Development of dysfunction in allospecific T_{CONVs} specific for antigens with various expression kinetics.

In our model, the duration of alloantigen (Ag) exposure influences the fate of allospecific T cells during tolerance, with T cells specific for very transiently expressed antigens (such as those encountered after anti-CD154/DST without heart transplantation) developing memory, T cells encountering slightly more persistent antigens (such as donor MHC Class II within a heart allograft) losing some effector function and T cells specific for the most persistent alloantigens (such as donor MHC Class I within a heart allograft) losing the greatest amount of function.

We hypothesize that T_{REGs} suppress alloimmune responses by T_{CONVs} that have retained or recovered function when encountering their cognate antigen during transplantation tolerance. Thus, functional allospecific T cells may only pose a threat to graft survival if T_{REG} function is

impaired. While we have not identified allospecific memory cells persisting during tolerance, that very transient alloantigen encounter in untransplanted anti-CD154/DST-treated mice led to the development of allospecific memory T cells indicates that alloantigens expressed very transiently within an allograft may also induce the development of persistent memory cells that are resistant to suppression. We do not yet understand the breadth of functional states T cells can develop during tolerance or the relative abundance of allospecific T cells falling into these different states.

Impairment of T_{REG} function through T_{REG} depletion or other insults potentially including Lm infection, disrupts several downstream immune mechanisms maintaining tolerance, leading to activation of alloimmunity and rejection. Due to loss of T_{REG}-mediated suppression and/or systemic autoimmunity, allospecific T cells expand after T_{REG} impairment in previously tolerant hosts. For some allospecificities, highly potent high avidity T cell clones expand preferentially, leading to avidity maturation. Considering our data showing that tolerant T_{CONVs} do not immediately recover functionality when removed from the tolerant environment, it is possible that cells specific for transiently expressed alloantigens, which retain functionality, are the first to mediate rejection when released from T_{REG}-mediated suppression. Chronically stimulated allospecific T cells may recover function over time and then join the alloimmune response. Allospecific B cells also appear to recover functionality in this environment. If the disruption in T_{REG} function is brief, chronically stimulated T cells and B cells may not have sufficient time to recover function. Future studies will investigate the relationship between the duration of a challenge to tolerance and the quality of alloimmune response.

Given this model, methods to prevent disruption of T_{REG}-mediated suppression are expected to promote the robustness of donor-specific tolerance. Additionally, inducing

dysfunction in T cells specific for transiently expressed alloantigens may make tolerance more robust, as even though dysfunctional cells can recover under some circumstances, they do remain dysfunctional in a cell-intrinsic manner for several days. Cell-intrinsic dysfunction in T_{CONVs} may represent a mechanism to prevent unwanted immune responses given that it cannot be guaranteed that a T_{REG} will be present during every interaction between an auto- or allo-reactive T_{CONV} and its cognate pMHC. Perhaps this mechanism plays a particularly critical role in maintaining tolerance during infections, when self- or donor-specific T_{REGs} are intrinsically impaired or overcrowding with T cells specific for the pathogen limits T_{REG}:APC interactions. Dysfunction may therefore act as a memory of suppression until the cell is reminded by another encounter with its suppressive signal. Future studies will investigate whether programming dysfunction in a broader population of allospecific T_{CONVs} is able to stabilize the tolerant state during challenges such as Lm infection.

Future Directions

Generating TCR-retrogenic (Rg) $CD4^+$ T cells with varied avidity for alloantigen.

We have found that high avidity $CD8^+$ T cells are more potent in mediating skin allograft rejection than lower avidity $CD8^+$ T cells with the same specificity. Whether avidity for alloantigen also impacts the strength of rejection mediated by $CD4^+$ T cells remains to be determined. The factors that influence the strength of $CD4^+$ T cell-mediated immunity are key as $CD4^+$ T cells are thought to be critical contributors to clinical allograft rejection. $CD4^+$ T cells also play distinct and diverse roles in alloimmunity, including cytokine production, and help to T cells, B cells and other immune cells. Determining which, if any, of these functions are impacted

by $CD4^+$ T cell avidity will provide a greater understanding of the processes of rejection and basic immune functions of T_{CONVs}. Additionally, because skin allografts are resistant to tolerance induction, studying the role of avidity in tolerance requires the use of other transplant models, such as heart transplantation, where $CD4^+$ T cells are necessary for rejection (Krieger, Yin, and Fathman 1996).

While the TCR-Tg mice expressing monoclonal $CD8^+$ OT-I and OT-3 T cells are important for studying the role of avidity in shaping alloimmunity mediated by $CD8^+$ T cells, TCR-Tg mice expressing $CD4^+$ T cells with varied avidity for the same alloantigen have not yet been described. We have generated "TCR-retrogenic (Rg)" mice that received bone marrow virally transduced to express TCRs of varied avidity for the same alloantigen. TCR-Rg mice offer several advantages compared to TCR-Tg mice that express a TCR of interest due to germline incorporation of that TCR sequence as a transgene. TCR-Tg mice must be bred continuously to generate new mice and propagate the line, making comparison of multiple TCR-Tg mouse lines very costly. In contrast, TCR-Rg mice are created as needed by transfecting bone marrow from a single line of bone marrow donor mice with any TCR of interest. This makes feasible the comparison of multiple TCR sequences, which, for example, would allow us to draw stronger conclusions about the association between T cell avidity for alloantigen and alloimmunity or susceptibility to the mechanisms of tolerance. Generation of new TCR-Tg mice is also a slow process, often taking greater than 6 months, whereas TCR-Rg mice are typically ready for experimentation 6 weeks after BMT. Finally, comparison of TCR-Tg mice is complicated by the random insertion of the transgene, which can disrupt endogenous gene functions in all progeny (Bettini, Bettini, and Vignali 2012, Holst et al. 2006). Thus, TCR-Rg mice are ideal for comparing T cells with varied avidities for the same alloantigen.

We chose to generate TCR-Rg T cells specific for 2W:I-A^b as rejection of minor mismatched 2W-mOva B6 grafts and haploidentical 2W-mOva F1 allografts in B6 mice has been well characterized by our group and others. Fully mismatched 2W-mOva BALB/c donor mice are also available and would limit presentation of the alloantigen to the indirect allopresentation pathway. Using 2W:I-A^b pMHC multimers, we have also observed a strong response to 2W:I-A^b in the polyclonal endogenous T cell population of B6 mice after transplantation with a $2W^+$ graft. Thus, monoclonal 2W:I-A^b-specific T cells would be valuable for studying alloimmunity and tolerance in a variety of settings. To identify TCR sequences from T_{CONVs} specific for 2W:I-A^b we first expanded the endogenous 2W:I-A^b-specific population in B6 Vβ3-Tg FoxP3^{GFP} mice with 2W-mOva F1 DST. We chose to fix the Vβ sequence of the B6 mice so that only TCRα would need to be sequenced and transduced into the TCR-Rg mice. Eight days after immunization, we sorted $CD4^+$ $CD44^{hi}$ $FoxP3^-$ 2W:I-A^b tetramer-binding splenocytes then sequenced the TCRα in those cells for sequencing (Fig. 5.3A). Because 2W:I-A^b tetramer MFI correlated with functional avidity (Fig. 2.1B), we collected 2W:I-A^b bright (Hi) and 2W:I-A^b dim (Lo) cells separately from within the 2W:I-A^b tetramer-binding population (Fig. 5.3B), hypothesizing that higher avidity T cells would be found uniquely in the tetramer^{hi} population, lower avidity cells would be uniquely in the tetramer^{lo} population and cells with intermediate avidity would be in both populations. We collected tetramer^{hi} and tetramer^{lo} cells from 10 mice, which were pooled into 3 groups of 3-4 mice each before TCR sequencing. Distribution of cells into each gate was fairly consistent between mice (Fig. 5.3C).

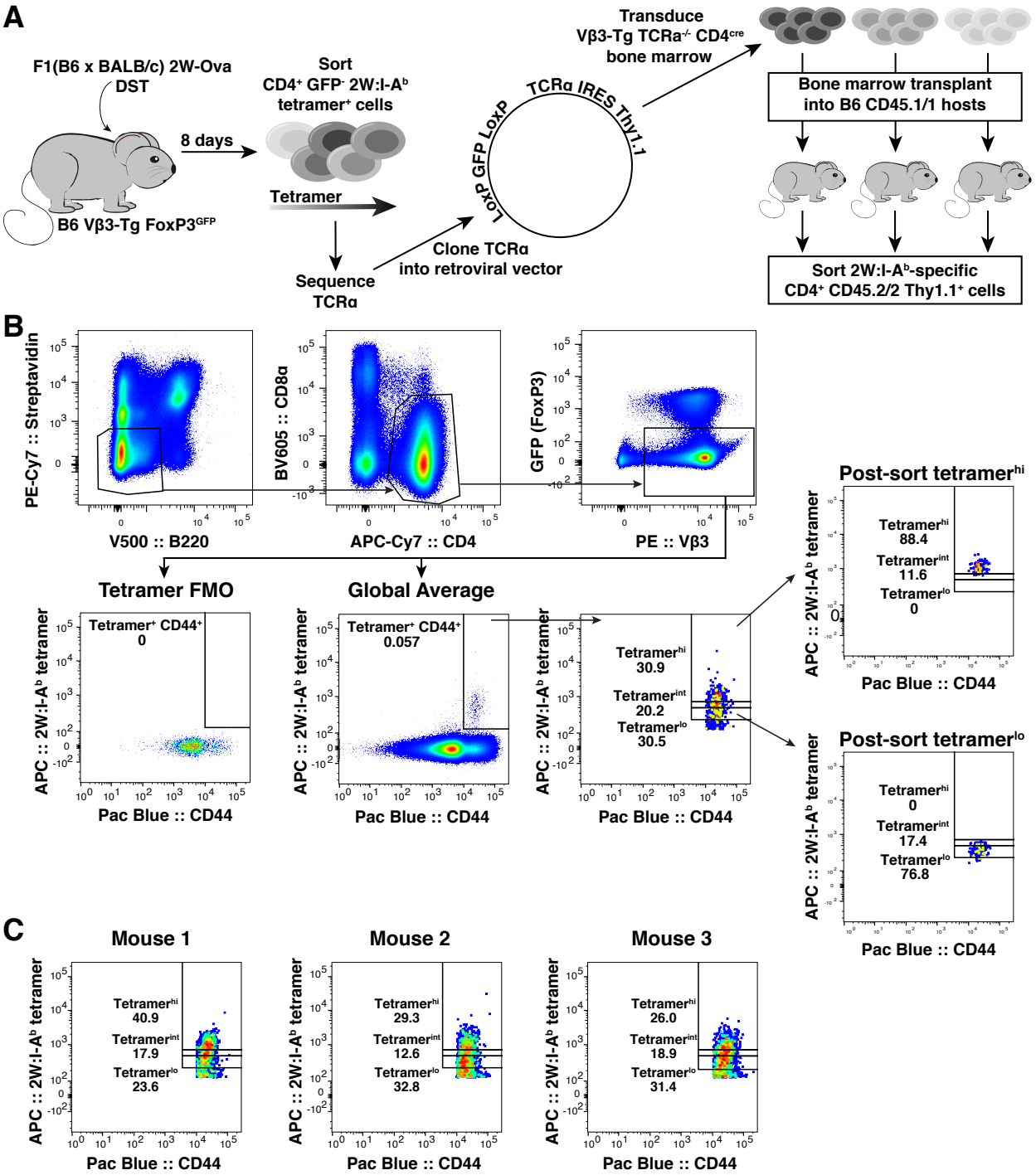

Figure 5.3 Generation of TCR-retrogenic T cells with varied avidities for 2W:I-A^b.

(A) Experimental design for generation of TCR-retrogenic T cells with varied avidities for 2W:I-A^b. (B) Left: gating strategy for sorting 2W:I-A^b tetramer$^+$ cells of varied structural avidities from B6 Vβ3-Tg FoxP3^{GFP} mice after BALB/c DST immunization. Right: tetramer staining intensity in previously sorted tetramer^{hi} and tetramer^{lo} cells. (C) Distribution of tetramer staining intensity in cells from three independent mice gated as shown in (B).

We identified 570 unique CDR3 sequences from the tetramer^{hi} and tetramer^{lo} samples analyzed. We narrowed our selection to the 40 CDR3 sequences identified in ≥2 samples of the same tetramer binding intensity to exclude non-2W:I-A^{b}-specific sequences that may have been erroneously included in the samples. Seven CDR3 sequences (TRAVs 1-7) with relatively high prevalence but different distribution patterns across tetramer^{hi} and tetramer^{lo} samples were chosen for TCRα cloning and generation of TCR-Rg mice (Fig. 5.4A, Table 5.1). TRAVs 1 and 2 were highly abundant and predicted to have intermediate avidity as they were found in both tetramer^{hi} and tetramer^{lo} samples. TRAVs 3 and 4 were predicted to have high avidity as they were exclusively found in tetramer^{hi} samples. TRAV5 was predicted to have intermediate-high avidity as it was more representative of the tetramer^{hi} population than the tetramer^{lo} population. TRAVs 6 and 7 were found exclusively in the tetramer^{lo} population, suggesting that they were of low avidity. TCR-Rg mice have successfully been made for TRAVs 1, 5 and 6. TRAV6 cells were found to likely be autoreactive as they were very rarely observed in the peripheral T cell repertoire and the majority of those present in the spleen and lymph nodes were CD44^{hi} in naïve mice. We tested the specificity and functional avidity of CD44^{lo} CD4^{+} CD45.2/.2 Thy1.1^{+} spleen and lymph node cells from TRAV1 and TRAV5 TCR-Rg mice. Both proliferated in response to B6 APCs presenting 2W peptide *in vitro*. We also determined that they were not reactive to other alloantigens present in 2W-mOva F1 DST, which would also be present as alloantigens in our mouse models of transplantation, by stimulating them with B6 APCs presenting an MHC Class II restricted Ova peptide (OT-2) or F1 APCs *in vitro* (Fig. 5.4B). We then compared the functional avidity of TRAV1 and TRAV5 by stimulating CTV-labeled cells with B6 APCs and a dose titration of 2W peptide *in vitro*. The avidities of TRAV1 and TRAV5 were very similar though TRAV1 may be of slightly higher avidity (EC_{50} within 10 μg/mL of TRAV5) (Fig. 5.4C).

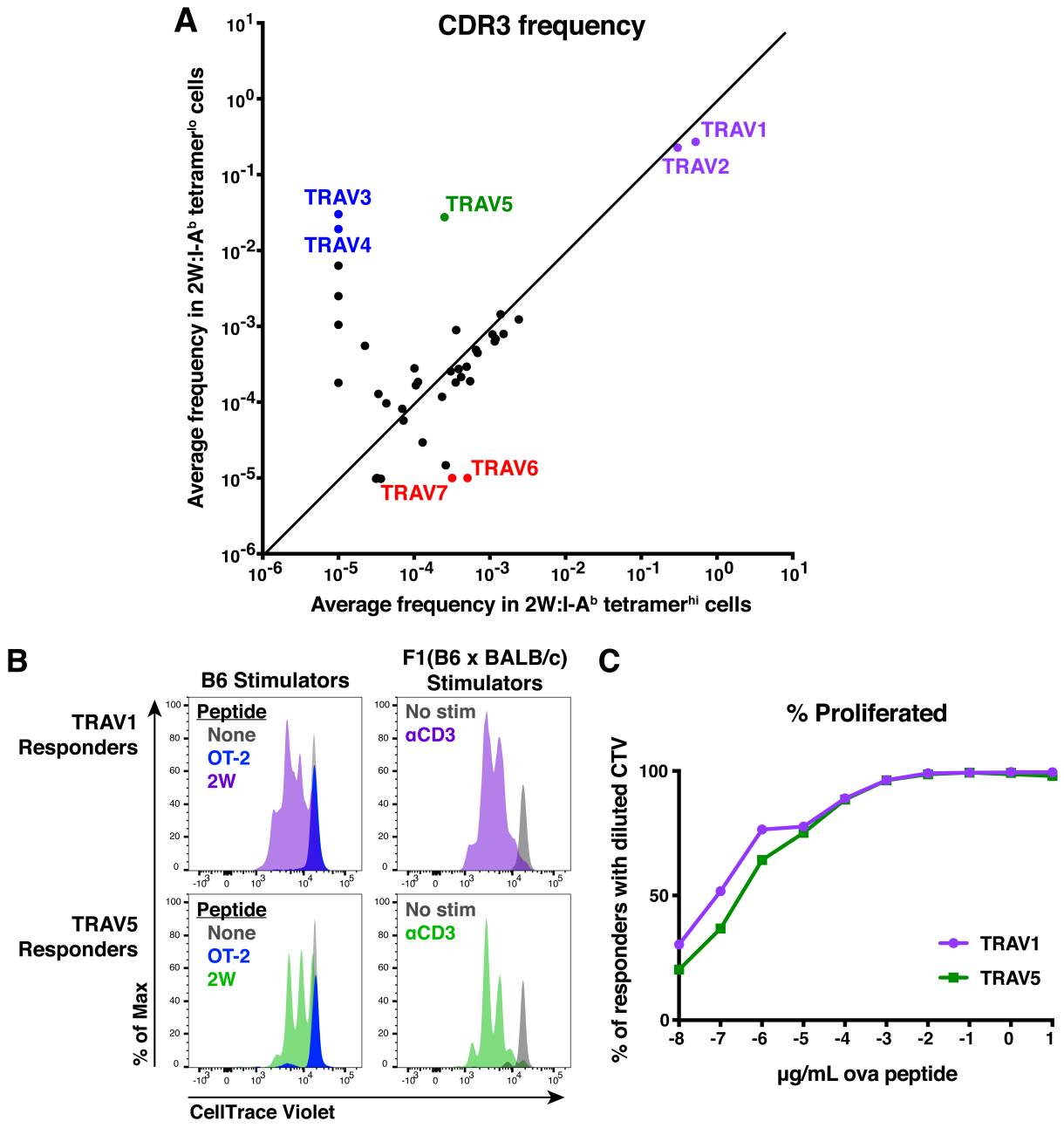

Figure 5.4 TCR-Rg T cell specificity and relative avidity for 2W:I-A^b.

(A) Frequency of CDR3 sequences in CD44^{hi} FoxP3$^-$ 2W:I-A^b tetramer^{lo} and tetramer^{hi} cells sorted from B6 Vβ3-Tg FoxP3^{GFP} mice immunized with F1 (B6 x BALB/c) 2W-Ova DST. CDR3 sequences cloned for generation of Rg T cells are labeled. Predicted T cell avidities are color coded in blue (low), green (low-intermediate), purple (intermediate) and red (high). Only the 40 CDR3 sequences found in ≥ 2 samples of the same tetramer staining intensity are shown out of 570 CDR3 sequences identified. Line at x=y. (B) Histograms showing CTV dilution in TRAV1- and TRAV5-Rg T cells after *in vitro* stimulation by B6 or F1 (B6 x BALB/c) APCs +/- OT-2 peptide, 2W peptide or anti-CD3 (C) CTV dilution in TRAV1- and TRAV5-Rg T cells stimulated *in vitro* by B6 APCs with a dose titration of 2W peptide. Results in (B) and (C) are representative of 1 experiment for TRAV5 and 2 independent experiments for TRAV1.

Table 5.1 CDR3 sequences of TCR-Rg T cells with contributing Vα and Jα segments.

Identifier	CDR3 amino acid sequence	Vα	Jα
TRAV1	ATERPNKVV	8D-2*01	34*02
TRAV2	ATDRGSALGRLH	8D-2*02	18*01
TRAV3	ALRDTNAYKVI	6D-5*01	30*01
TRAV4	ATERPHNVGDNSKLI	8D-2*01	38*01
TRAV5	ATERPYNVGDNSKLI	8D-2*01	38*01
TRAV6	AVSMRNNNAPR	7-6*02	43*01
TRAV7	ASNNYAQGLT	13D-1*02	26*01

It is possible that 2W:I-A^b tetramer binding requires relatively high avidity, and that the CDR3 sequences identified thus far reflect a narrow range of avidities. If that is the case, we will attempt to identify lower avidity T cells for sequencing by staining them with higher-order pMHC dodecamers, which are better able to bind low avidity cells than pMHC tetramers or pentamers (Huang et al. 2016). After identifying TCR-Rg T cells with a broad range of avidities for 2W:I-A^b, we will test whether high avidity $CD4^+$ T cells are more potent in mediating graft rejection and which alloimmune functions enhanced in high avidity cells contribute to their increased efficacy. Limiting the number of high avidity T cells may also support tolerance if high avidity cells are resistant to suppression or other mechanisms of tolerance. Thus, we will determine whether adoptive transfer of high versus low avidity allospecific T cells prevents induction or maintenance of tolerance to heart transplants. If either cell population is deleterious to tolerance, we can investigate which mechanisms of tolerance they resist by comparing suppression, anergy induction or deletion of high avidity versus low avidity cells. We may also compare transcriptional profiles of high and low avidity allospecific tracer TCR-Tg cells after immunization, rejection or tolerance by RNAseq to identify markers of avidity as well as functions and expression patterns that correlate with avidity.

Pairing structural avidity and transcriptomic analysis through DNA barcoded pMHC dextramers.

Monoclonal T cells from TCR-Tg and TCR-Rg mice are important tools for studying the function of allospecific T cells with varied avidities during rejection and tolerance. However, the endogenous polyclonal allospecific T cell repertoire is vast. Indeed, we identified over 500 CDR3 sequences from 2W:I-A^b tetramer-binding cells even without TCRβ rearrangement contributing to TCR diversity. It is not feasible to compare the function of nearly these many TCR-Rg mice and a small number of selected TCRs is unlikely to represent the behavior of such a diverse population. However, DNA-barcoded pMHC multimers (Immudex) have recently been developed to identify T cells of a given antigen specificity in parallel with single cell RNA sequencing (scRNAseq). We hypothesize that staining intensity and barcode read count will correlate with T cell functional avidity, as we have found for pMHC multimers in the past (Figs. 2.1 and 2.2). Additionally, because barcoded pMHC dextramers can contain a higher number of pMHC molecules per multimer than the tetramers and pentamers we have used in the past, they may also be able to identify T cells of lower avidity than we have been able to study thus far.

If we find that pMHC dextramer staining intensity and barcode read count correlate with functional avidity, we will perform scRNAseq paired with allogeneic pMHC dextramer binding in $CD4^+$ and $CD8^+$ T cells from rejecting and tolerant mice. Using these data, we will identify transcripts whose expression levels correlate with avidity, potentially identifying biomarkers that could be used to track high avidity allospecific T cells in transplant patients. We will determine whether higher avidity cells produce more effector molecules on a per-cell basis or whether high and low avidity cells perform distinct functions during rejection or tolerance. We will determine whether high avidity T_{REGs} express more suppressive molecules or a different subset of

suppressive molecules than lower avidity T_{REGs} during tolerance and observe whether high or low avidity T_{CONVs} retain more effector function during tolerance, indicating resistance to mechanisms of tolerance. We will then confirm these findings *in vivo* using TCR-Rg T cells of varied avidities. Further, single cell pMHC dextramer barcode sequencing and RNA sequencing can be combined with TCR sequencing to identify additional sequences for generating TCR-Rg mice of different specificities or broader avidities.

Are suppressive signals from T_{REGs} sufficient to induce and maintain transplantation tolerance?

Our findings that tracer TCR75 cells remained functional and failed to develop an anergy-associated phenotype in P14 $Rag^{-/-}$ hosts after transplantation with anti-CD154/DST treatment indicate that P14 $Rag^{-/-}$ mice are missing one or more components required for programming T_{CONV} dysfunction during transplantation tolerance. Dysfunctional tolerized TCR75 cells from lymphoreplete heart allograft recipients also recovered function in P14 $Rag^{-/-}$ secondary hosts, indicating that P14 $Rag^{-/-}$ hosts lack signals needed to maintain dysfunction in tolerized T_{CONVs}. In future studies, we will identify these necessary signals and the cells supplying them in the tolerant environment.

P14 $Rag^{-/-}$ mice lack all lymphocytes except for a single clone of $CD8^+$ T cells recognizing an LCMV-derived antigen. Thus, P14 $Rag^{-/-}$ mice are devoid of B cells as well as allospecific $CD4^+$ and $CD8^+$ T cells, including suppressive T cell subsets. Because we have found that T_{REGs} are necessary to prevent T cell-mediated rejection during tolerance, and T_{REGs} are required to maintain dysfunction in autoreactive T cells, we hypothesize that T_{REGs} provide the signal required for inducing and maintaining dysfunction that is missing from P14 $Rag^{-/-}$ mice. We will test whether adoptive transfer of T_{REGs} into P14 $Rag^{-/-}$ primary hosts prior to

transplantation with anti-CD154/DST is sufficient to allow the development of dysfunction in tracer TCR75 cells. We will also test whether tolerized TCR75 cells remain dysfunctional and fail to reject skin grafts after adoptive transfer into P14 $Rag^{-/-}$ secondary hosts when T_{REGs} are co-transferred with them. These protocols may be adapted to co-transfer other lymphocyte subsets that are missing in P14/$Rag^{-/-}$ mice if we find that T_{REGs} do not restore the induction or maintenance of dysfunction in tolerized TCR75 cells. Identifying the cell subset responsible for the dysfunctional state we have observed will improve our understanding of the mechanisms contributing to transplantation tolerance, and suggest which molecular signals are provided to the T_{CONV} to induce dysfunction. Further experiments to better understand the signaling pathways involved in programming dysfunction may inform improved strategies for tolerance induction and a better understanding of T cell dysfunction in other physiological contexts.

Prolonging alloantigen exposure through multiple transfusions of donor splenocytes to induce a tolerant state that is resistant to systemic infection with Listeria monocytogenes.

We have found that some allospecific T cells retain greater functionality than others during transplantation tolerance. We hypothesize that the enhanced functionality of this subset increases their potency in rejecting previously accepted allografts after Lm infection. Having found that prolonging exposure to alloantigen through repeated injections of DST promotes dysfunction in a wider array of allospecific T cells than does a single DST injection, we will test whether adding repeated injections of DST to our existing tolerization protocol enhances the robustness of tolerance. We will determine whether graft recipients tolerized with anti-CD154 and multiple repeated injections of DST experience more stable graft function after Lm infection than mice tolerized to their graft with anti-CD154 and a single DST injection.

Our results showing recovery of function in tolerized TCR75 cells after transfer into non-tolerant secondary hosts may reflect a requirement for continuous stimulation to maintain the dysfunctional state. Thus, if prolonging alloantigen exposure does enhance the robustness of donor-specific tolerance, it will be important to determine whether DST injections are required perpetually, whether an extended but defined period of treatment permanently increases the robustness of tolerance, or whether occasional DST injections would be sufficient to reinforce T cell dysfunction after the induction of tolerance. To test whether continuous injections are required to improve the resistance of tolerance to Lm infection, we will provide multiple repeated injections of DST over the course of 30 days, and then cease injections for ≥30 days prior to Lm infection. These findings will have important implications for the clinical translation of prolonged exogenous alloantigen exposure to enhance tolerogenic therapies.

Investigating the necessity for persistent alloantigen exposure to prevent development of alloimmunity in clinical transplant recipients.

We have established a collaboration with clinicians at Hôpital Erasme in Brussesls, Belgium to collect serum samples from renal and combined renal/pancreas transplant recipients who underwent conventional immunosuppression and later required transplantectomy due to graft failure. In these patients, transplantectomy removes the source of alloantigen, thus ceasing chronic stimulation to allospecific T cells. We hypothesize that alloreactive cells will recover functionality after transplantectomy. Our study will compare alloantibody production in the serum from these patients before and after transplantectomy to determine whether alloreactivity is enhanced after removal of the graft. These results will provide important clinical translation of our findings that chronic antigen exposure is required to maintain dysfunction in alloreactive lymphocytes.

Conclusion

In summary, we have shown that T_{REG}-mediated suppression is responsible for multiple downstream immune mechanisms acting on allospecific T_{CONVs} to support transplantation tolerance. Further, we showed that T_{REGs} are required for maintaining tolerance. We have also identified a population of functional allospecific T cells persisting long-term in tolerant graft recipients, which may represent a larger population of functional allospecific T cells posing a threat to long-term graft survival. Finally, we have developed an approach to reduce functionality of these cells. These findings highlight previously unappreciated vulnerabilities of tolerance, mechanisms of rejection after tolerance and identify strategies that may improve the robustness of transplantation tolerance.

Appendix. Impact of donor and recipient microbiome composition on transplant outcomes.

Note: the following section titled “Impact of donor and recipient microbiome composition on transplant outcomes” is reproduced with minor editing and re-numbering of figures from reference (McIntosh et al. 2018).

Abstract

Background

Solid organ transplant recipients show heterogeneity in the occurrence and timing of acute rejection episodes. Understanding the factors responsible for such variability in patient outcomes may lead to improved diagnostic and therapeutic approaches. Rejection kinetics of transplanted organs mainly depends on the extent of genetic disparities between donor and recipient, but a role for environmental factors is emerging. We have recently shown that major alterations of the microbiota following broad-spectrum antibiotics, or use of germ-free animals, promoted longer skin graft survival in mice. Here, we tested whether spontaneous differences in

microbial colonization between genetically similar individuals can contribute to variability in graft rejection kinetics.

Results

We compared rejection kinetics of minor mismatched skin grafts in C57BL/6 mice from Jackson Laboratory (Jax) and Taconic Farms (Tac), genetically similar animals colonized by different commensal microbes. Female Tac mice rejected skin grafts from vendor-matched males more quickly than Jax mice. We observed prolonged graft survival in Tac mice when they were exposed to Jax mice microbiome through co-housing or fecal microbiota transplantation (FMT) by gastric gavage. In contrast, exposure to Tac mice did not change graft rejection kinetics in Jax mice, suggesting a dominant suppressive effect of Jax microbiota. High-throughput sequencing of 16S rRNA gene amplicons from Jax and Tac mice fecal samples confirmed a convergence of microbiota composition after cohousing or fecal transfer. Our analysis of amplicon data associated members of a single bacterial genus, *Alistipes*, with prolonged graft survival. Consistent with this finding, members of the genus *Alistipes* were absent in a separate Tac cohort, in which fecal transfer from Jax mice failed to prolong graft survival.

Conclusions

These results demonstrate that differences in resident microbiome in healthy individuals may translate into distinct kinetics of graft rejection, and contribute to interpersonal variability in graft outcomes. The association between *Alistipes* and prolonged skin graft survival in mice suggests that members of this genus might affect host physiology, including at sites distal to the gastrointestinal tract. Overall, these findings allude to a potential therapeutic role for specific gut microbes to promote graft survival through the administration of probiotics, or FMT.

Introduction

Solid organ transplantation is a common treatment for end-stage organ failure. However, most transplant recipients need to remain on lifelong immunosuppression to prevent immune-mediated acute rejection of the donor organ, leaving them susceptible to infections (Fishman 2017), malignancies (Doycheva, Amer, and Watt 2016), and drug toxicity (Naesens, Kuypers, and Sarwal 2009). In the absence of immunosuppression, the transplant recipient's immune system recognizes the donor organ as non-self and mounts an immune response, termed the alloimmune response (Gurley, Lowry, and Forbes 1983). Even in patients taking immunosuppressive drugs, an alloimmune response can occur and cause acute graft rejection leading to permanent damage to and loss of the transplanted organ. Importantly, patients who are successfully treated for episodes of acute rejection experience worse long-term graft survival than patients who never experience an acute rejection episode (Koo et al. 2015).

Recipients of solid organ transplants show heterogeneity in the occurrence and timing of acute rejection episodes. While some patients experience acute rejection within the first year after transplantation, others retain their grafts long-term despite similar immunosuppression (Hart et al. 2017, Kim et al. 2017), and a small subset spontaneously develops operational tolerance, the ability to maintain allografts without rejection after withdrawal of immunosuppression (Roussey-Kesler et al. 2006, Mazariegos et al. 1997). Understanding the factors responsible for such heterogeneity may lead to improved screening protocols for patients and the development of therapeutics to prevent or treat acute rejection.

The likelihood and intensity of acute rejection episodes is mainly determined by the extent of genetic disparities between the donor and the recipient of the allograft. However, a role for environmental factors is emerging. High-fat (Molinero et al. 2016) and high-salt diet (Safa et

al. 2015) have been shown to lead to accelerated transplant rejection in mice. Additionally, infection has been associated with increased incidence of acute rejection in kidney (Abbott et al. 2004, Reinke et al. 1994) and lung (Husain et al. 2007, Vilchez et al. 2003) transplant patients. In mice, infection with *S. aureus* (Ahmed et al. 2011) or *Listeria monocytogenes* (Wang et al. 2008) prevented the induction of graft-specific tolerance and *L. monocytogenes* infection could also break transplantation tolerance after it had been established (Wang et al. 2010b). Given its role in the development and function of the immune system (Smith, McCoy, and Macpherson 2007), and the molecular similarity between pathogens and commensal microbes, the microbiota may also contribute to the intensity of alloimmunity and the kinetics of acute rejection.

Our group has previously shown that germ-free and antibiotic-pre-treated mice exhibit dampened alloimmunity, and prolonged survival of skin grafts, and that gastric inoculation of germ-free mice with FMT from conventional mice is sufficient to accelerate skin graft rejection (Lei et al. 2016). These findings indicate that the microbiota is an environmental factor that can causally affect alloimmunity and that massive alterations in the microbiota can translate into measurable differences in graft outcome. However, the comparison between sterile and colonized mice is not representative of the differences in microbiota composition between individuals. In this study, using a murine model of skin transplantation, we set out to determine whether differences in resident microbiota between healthy individuals at steady state may impact graft survival.

Investigating communities of commensal microbes associated with different skin transplant outcomes may allow identification of specific members of the microbiota contributing to transplant rejection. Individual microbial taxa have been shown to promote varied immune phenotypes in their hosts. For example, *Bacteroides fragilis* and some *Clostridium* species have

been shown to promote the development of T_{REGs} (Atarashi et al. 2011, Ochoa-Repáraz et al. 2010), and segmented filamentous bacteria have been shown to promote differentiation of naïve T cells into T_{H17} cells (Ivanov et al. 2009) in the gut mucosa. With the recent discovery of specific microbes modulating anti-tumor immunity (Sivan et al. 2015, Vétizou et al. 2015, Iida et al. 2013), susceptibility to rheumatoid arthritis (Scher et al. 2013, Marietta et al. 2016), experimental autoimmune encephalomyelitis (Ochoa-Repáraz et al. 2010), and other immune-mediated diseases distal to the gut in mouse models (Belkaid and Hand 2014), we sought to identify members of the gut microbiota associated with improved or worsened transplant survival. Here, we provide evidence that members of the genus *Alistipes* are associated with improved survival of transplanted skin in mice.

Materials and Methods

Mice

We obtained C57BL/6 (B6) from Jackson Laboratory or Taconic Farms and performed all experiments using 6–8-week-old male or female mice. We fed mice with Harlan Teklad 2018 diet, maintained them on distilled water, and housed them in individually ventilated cages in a specific pathogen-free animal facility at the University of Chicago. We collected fecal samples directly into autoclaved tubes, avoiding contact with mouse skin or urine, and stored them at − 20°C until DNA isolation.

Skin transplantation

We transplanted tail skin from male B6 mice onto the flank of female B6 recipients as previously described (Kellersmann and Zhong 1998) and removed bandages after 7 days, while monitoring

the graft survival every other day thereafter. We reported “rejection” when less than 20% of transplanted skin was viable.

Cohousing and FMT experiments

For cohousing experiments, we kept two or three B6 mice from each Jax and Tac cohort in the same cage (five mice/cage) for 3 weeks prior to transplantation. Cohousing experiments prior to transplantation included both skin transplant donors and recipients grouped based on their gender. For FMT experiments, male mice received oral FMT from male mice, while female mice received oral FMT from female mice. FMT donor mice arrived from Jax or Tac in the same shipments as FMT recipient mice, but were housed separately from FMT recipients. We prepared a separate fecal suspension from each of three FMT donor mice by suspending one fecal pellet in 1 mL sterile phosphate buffered saline using a syringe and 18-gauge needle (Becton Dickinson) until all volume could pass through the needle. We allowed particulates to settle for 30 s before drawing supernatant into a syringe attached to a 22-gauge gavage needle (Fine Science Tools). We pooled together 500 μL of the supernatants from each of the three 1 mL suspensions and deposited 200 μL of this pooled supernatant into the stomach of each recipient mouse by gavage. Each FMT recipient was gavaged 7, 4, and 1 days prior to skin transplantation, with donor feces freshly harvested from the same three Jax or Tac FMT donors immediately prior to gavage, and the first FMT obtained upon arrival of the Jax or Tac mice to our facility. We cleaned gavage needles with 70% ethanol between mice, using different gavage needles for each treatment group. We cleaned gavage needles with 70% ethanol and autoclaved them after the final gavage on each experimental day.

Microbial DNA isolation, sequencing library preparation, and analysis

QIAamp DNA Stool Mini Kit (QIAGEN) extracted the DNA from fecal samples homogenized with 0.1 mm zirconia/silica beads in 1.4 mL ASL buffer (QIAGEN) in a Mini-Beadbeater (Biospec). The Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory (Argonne, IL, USA) performed library preparation and sequencing of our DNA isolates. Thirty-five cycles of amplification were performed using primers (Caporaso et al. 2012) that target the V4-V5 region of the 16S rRNA gene to generate our amplicons from purified DNA, and Illumina MiSeq paired-end sequencing (2 × 300) was used to sequence our amplicon libraries at the High-Throughput Genome Analysis Core in the Argonne National Laboratory. Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al. 2010) joined paired-end sequences and de-multiplexed raw sequencing data into samples. We identified ASVs in our dataset to infer high-resolution microbial community structures using Minimum Entropy Decomposition (MED) (Eren, Morrison, et al. 2015) via the previously described oligotyping pipeline (Eren et al. 2013). We used Global Alignment for Sequence Taxonomy (GAST) (Huse et al. 2008) to infer the taxonomic affiliation of each ASV. After identifying ASVs, QIIME calculated alpha diversity estimates with Shannon entropy and inverse Simpson index. We performed principal coordinate analysis (PCoA) to infer similarities between samples based on their microbial community structures using the relative abundances of ASVs and the Bray-Curtis distance with the R package phyloseq (McMurdie and Holmes 2013) after removing samples with less than 2500 reads. We generated heat map visualizations of ASV percent relative abundances using anvi'o v3 (Eren, Esen, et al. 2015).

Statistical analysis

Kaplan-Meier plots and log-rank (Mantel-Cox) tests compared graft survival curves, and unpaired Mann-Whitney tests with Sidak-Bonferroni correction compared the relative abundance of microbial taxa across samples. We used Prism 6 (Graphpad) for statistical analyses and considered $P < 0.05$ to be statistically significant denoting levels of significance with increasing numbers of asterisks in our text and figures: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Results

Microbiota composition differs in Jax and Tac mice of the same genetic background

To determine whether specific commensal microbes could impact skin transplantation outcomes, we obtained mice of the same B6 genetic background from different vendors as they have been shown to be colonized by different communities of commensal microbes (Ivanov et al. 2009, Sivan et al. 2015). We performed 16S rRNA gene amplicon sequencing of fecal samples from Jax and Tac mice upon their arrival to our facility. We inferred highly resolved microbial community structures in our amplicon data with Minimum Entropy Decomposition (MED). MED iteratively decomposes a given amplicon dataset using highly variable nucleotide positions identified by Shannon entropy, until the variation within the population of amplicon sequences that resolve to the same oligotype, or “amplicon sequence variant” (ASV), is minimal (Eren, Morrison, et al. 2015). This strategy allows the identification of closely related but distinct taxa at a single-nucleotide resolution, better explaining micro-diversity compared to clustering

strategies that rely on arbitrary similarity thresholds (Callahan, McMurdie, and Holmes 2017, Needham, Sachdeva, and Fuhrman 2017, Eren et al. 2014, Eren, Sogin, and Maignien 2016).

MED identified 195 ASVs present in female Jax or Tac mice. Female Jax and Tac mice were both colonized almost entirely by members of the phyla Firmicutes and Bacteroidetes and a small proportion of the phylum Tenericutes (Fig. A.1a left), which agrees with previous studies (Sivan et al. 2015).Tac mice were also colonized by a small proportion of ASVs assigned to the phylum Verrucomicrobia. While these ASVs made up only 2.1% of the Tac mice microbiota on average, this was a relative abundance 700-fold greater than in Jax mice. This lack of Verrucomicrobia members in Jax mice is consistent with our previous findings (Sivan et al. 2015).

To gain an understanding of the differences between Jax and Tac microbiota composition beyond taxonomic annotations, we compared the most abundant ASVs in Jax and Tac mice that made up at least 50% of the overall composition of each mouse (Fig. A.1a right). The distribution of the 12 ASVs that matched this criterion differed remarkably between Jax and Tac mice except two. One of the two ASVs that were similarly abundant in both groups (ASV_10871) could not be assigned taxonomy beyond the order Bacteroidales, while the other (ASV_66) resolved to the genus *Lactobacillus*. Five of the remaining ten ASVs (8132, 10739, 12136, 12072 and 12134) were specific to Tac mice, as they were detected in every Tac mouse while being virtually undetected in Jax mice. None of the Tac-specific ASVs could be assigned taxonomy at a level higher than Bacteroidales order. Remaining five ASVs were specific to Jax mice, three of which (ASVs 1857, 1858, and 4421) were not assigned beyond the order Bacteroidales, and the last two were assigned to *Allobaculum* (ASV_100) and *Alistipes* (ASV_5969). Some lower abundance ASVs were also differentially abundant between Jax and

Tac mice. Relative abundance of ASVs_122, 5039, and 5968 were all over 1000-fold higher in Tac than in Jax mice and were assigned to family Prevotellaceae, genus *Lactobacillus*, and genus *Parabacteroides*, respectively. Conversely, the Bacteroidales-assigned ASV_1859 was over 1000-fold more abundant in Jax than in Tac.

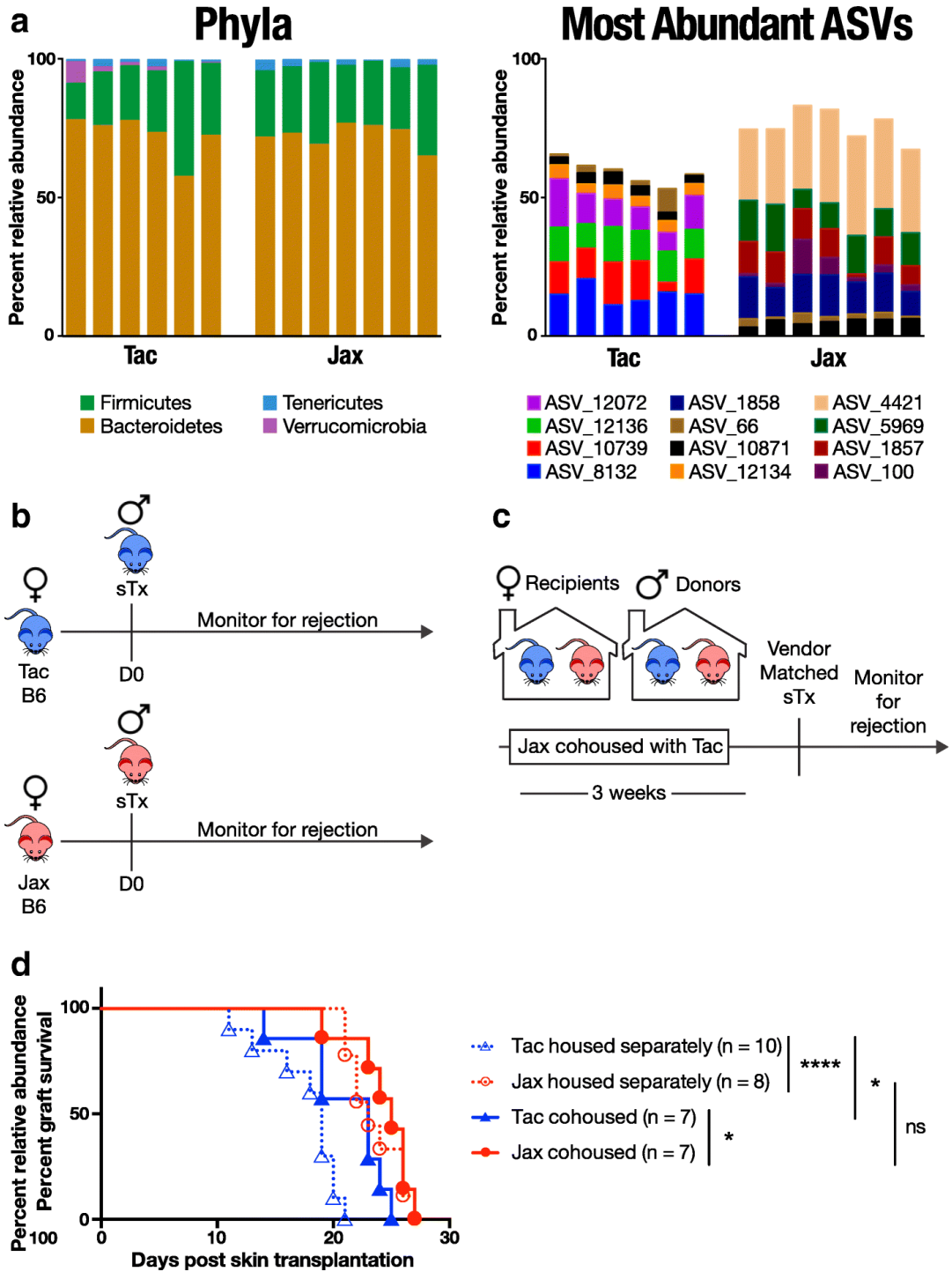

Figure A.1 Cohousing with Jax B6 mice results in prolonged skin transplant survival in Tac B6 mice.

(a) Representation of phyla (left) or the 12 ASVs with highest average percent relative abundance across pre-cohousing Jax and Tac samples (right) after 16S rRNA gene sequencing and clustering of amplicons into ASVs using MED in female Tac and Jax B6 mice.

Figure A.1, continued: Each column represents an individual mouse. All phyla contributing to at least 1% of overall microbial composition are labeled and shown in color. (b-d) Survival of vendor-matched male B6 skin transplants in female Tac and Jax B6 mice housed separately or following cohousing of Jax and Tac skin graft donors and cohousing of Jax and Tac recipients for 3 weeks prior to transplantation. *$P < 0.05$, ****$P < 0.0001$; ns = not significant

Skin graft rejection kinetics in Tac mice are affected by cohousing or FMT

Following our observation of differences in microbiota composition between Jax and Tac mice arriving at our facility, we compared the kinetics of rejection of skin grafts bearing sex-dependent minor (non-major histocompatibility complex) mismatches, as skin from male mice expresses H-Y antigens encoded by the Y chromosome (Billingham, Silvers, and Wilson 1965, Scheid et al. 1972). H-Y antigens are recognized following transplantation by the immune system of otherwise genetically identical female recipients, resulting in rejection. We grafted skin from male B6 Jax and Tac mice to their female B6 counterparts from the same vendor (Fig. A.1b) and monitored animals to determine the length of time until each graft was rejected (Fig. A.1d) Female Tac mice rejected male skin grafts significantly faster (MST 18 ± 3 days, $n = 10$) than did female Jax mice (MST 24 ± 2 days, $n = 8$; $P < 0.001$). To eliminate the possibility that genetic drift in B6 mice at Jax or Tac facilities had contributed to the observed differences in rejection kinetics, we cohoused Jax and Tac skin graft donor mice together for 3 weeks prior to transplantation, and Jax and Tac skin graft recipients together for 3 weeks prior to transplantation (Fig. A.1c), to allow transfer of some environmental factors, including commensal microbes, between individuals from different vendors, while preserving any existing host genetic differences. In support of an environmental but not host genetic cause for the delayed rejection in Jax mice, Tac mice rejected grafts with slower Jax-like kinetics after cohousing (Fig. A.1d) (MST 21 ± 4 days, $n = 7$; $P = 0.02$ when compared to Tac mice housed separately from Jax mice). In contrast, rejection kinetics in Jax mice remained unchanged (MST 24 ± 3 days, $n = 7$)

when compared with Jax mice housed separately from Tac mice, suggesting that Jax mice were transferring a dominant inhibitory factor to Tac mice by cohousing, rather than Jax mice losing a graft-protective factor or gaining a rejection-accelerating factor by cohousing with Tac mice.

Because components of the fecal microbiota are transferred between cohoused mice, we sought to determine whether the environmental factor responsible for differences in skin transplantation outcomes in Jax mice could be found in Jax feces. To this end, skin graft donor and skin graft recipient Tac mice received gastric FMT from Jax mice, prior to skin transplantation (Fig. A.2a). Indeed, Jax FMT into Tac mice promoted slower skin graft rejection by Tac mice when compared to Tac skin graft recipients that had received Tac FMT (MST 21 ± 1 days, n = 5 versus 15 ± 3 days, n = 4; $P < 0.005$) (Fig. A.2b). In contrast, Tac or Jax FMT into Jax mice did not change graft rejection kinetics in Jax mice (MST 20 ± 4 days, n = 4 versus MST 22 ± 2 days, n = 3). This confirmed the presence of a component of Jax feces that dominantly prolonged skin graft survival and could be transferred to Tac mice by FMT.

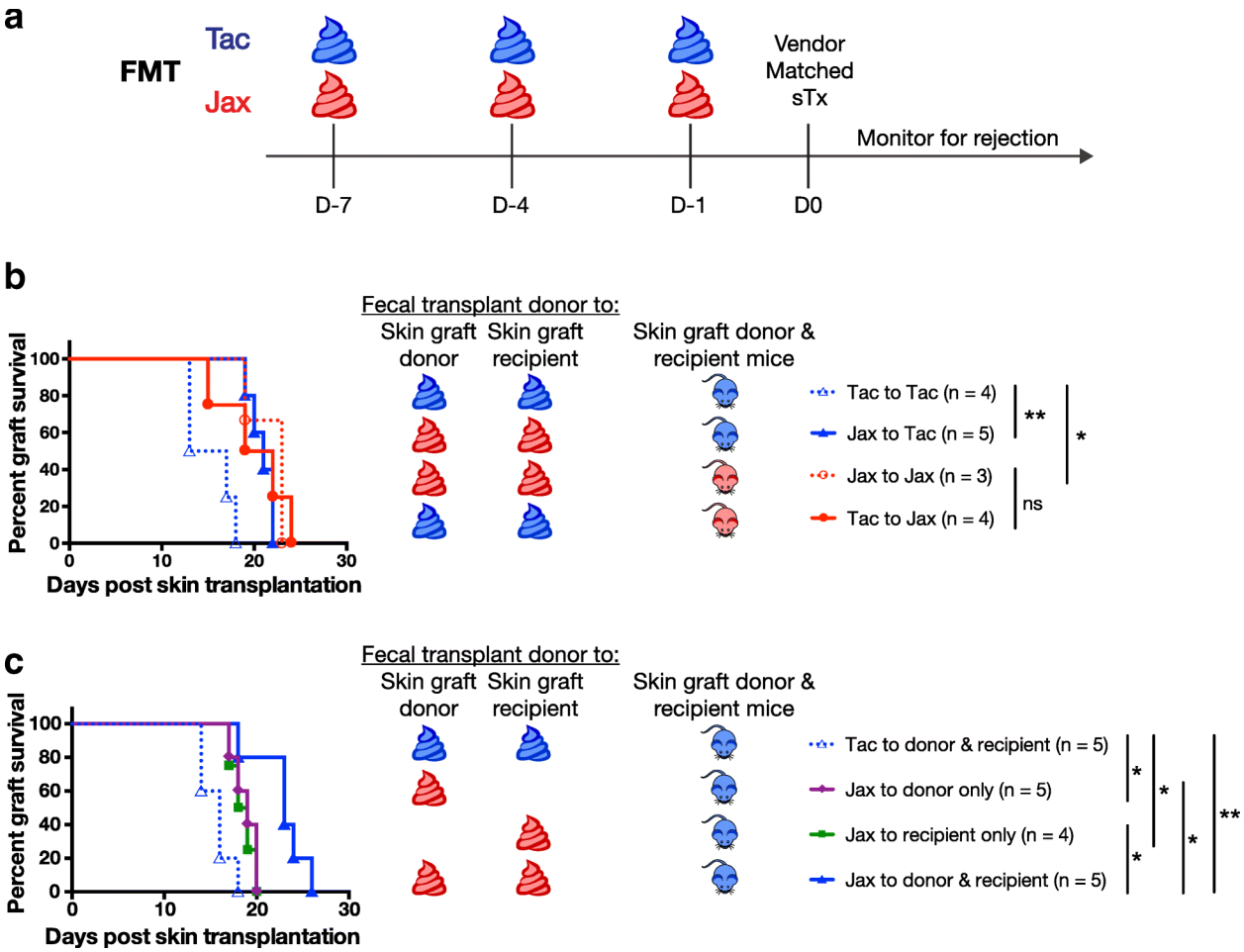

Figure A.2 Jax FMT into both Tac skin transplant donor and recipient mice delays skin transplant rejection.

(a) Treatment schedule for mice receiving Jax or Tac FMT. (b) Survival kinetics of vendor-matched male B6 skin transplants in female Jax or Tac B6 graft recipients following Jax or Tac FMT into both skin transplant donor and recipient. (c) Survival of male Tac B6 skin transplants in female Tac B6 recipients following Jax or Tac FMT into skin transplant donor and/or recipient. *P < 0.05, **P < 0.01; ns = not significant

Jax FMT to both Tac skin graft donor and Tac skin graft recipient is required for maximal prolongation of graft survival

In organ transplantation, genetic or environmental factors associated with either the donor or the recipient could influence the rejection of the transplanted organ. For instance, polymorphisms of both the transplant donor (Ge et al. 2014, Kamei et al. 2013) and recipient (Kamei et al. 2016,

Thude et al. 2016, Mandegary et al. 2015) have been shown to impact graft rejection, and we have previously demonstrated that antibiotic pre-treatment needs to be administered to both the donor and the recipient to result in prolonged skin graft survival (Lei et al. 2016). In the cohousing and fecal transfer experiments described in Figs. AII.1 and AII.2, skin donors and recipients were both cohoused or given FMT prior to transplantation, so the effect of Jax feces on graft survival could have occurred through changes to the donor and/or recipient. To determine whether the improvement in skin transplantation outcome in Tac mice after exposure to Jax microbiota occurred through changes to the donor, recipient, or both, only Tac skin graft donors or recipients received FMT from Jax mice prior to transplantation (Fig. A.2c). While Jax FMT into only the Tac skin graft donor (MST 19 ± 1 days, n = 5) or only the Tac skin graft recipient (MST 19 ± 1 days, n = 4) led to a slight improvement in skin transplant survival compared to Tac FMT into Tac mice (MST 16 ± 2 days, n = 5), Jax FMT into both Tac skin graft donor and recipient led to more significant prolongation in graft survival (MST 23 ± 3 days, n = 5; $P < 0.01$) when compared to Tac FMT. Thus, maximal skin graft survival improvement by Jax FMT required that both the Tac skin graft donor and the Tac skin graft recipient receive the Jax FMT.

Convergence of fecal microbiota composition in Jax and Tac mice following cohousing and FMT

To determine whether Jax fecal transfer led to changes in the microbiota of Tac mice, we compared microbial community profiles in samples from Jax and Tac mice before and after cohousing or FMT experiments. The distinct composition of Jax and Tac microbiota composition at baseline and their convergence after cohousing or cross-vendor FMT was clearly evident in the relative abundance of microbes at the ASV level (Figs. A.3a and A.4a). Principal coordinate analysis (PCoA) of these data also revealed a clear separation of Jax (n = 7) and Tac (n = 6) fecal

samples at baseline and a convergence of Jax (n = 7) and Tac (n = 7) microbiota composition following cohousing (Fig. A.3b). PCoA showed a separation between Jax and Tac mice following FMT from vendor-matched fecal donors, but similar to cohousing, cross-vendor-FMT resulted in a convergence of microbiota composition (Fig. A.4b). Alpha diversity as defined by Shannon entropy or inverse Simpson index was similar in Jax and Tac fecal samples, and longer graft survival did not correlate with differences in alpha diversity. These results suggest that one or more taxa are transferred from Jax to Tac by cohousing or FMT and that the transfer is associated with prolonged skin graft survival in Tac mice.

We then analyzed the microbial community structure between these cohorts to determine which taxa changed in Tac mice following cohousing with Jax mice or following Jax FMT that could explain the improvement in rejection kinetics in Tac mice. To gain a broad view of the efficiency of microbial transfer in our experiments, we determined the number of ASVs detected in the majority of mice from either Jax or Tac prior to cohousing or FMT and quantified how many of these ASVs were gained or lost post cohousing or FMT. Of 30 Jax-specific ASVs, 14 were gained by Tac mice post cohousing and 21 out of 27 Tac-specific ASVs were gained by Jax post cohousing. Fourteen of 60 Jax-specific ASVs were transferred to Tac by Jax FMT, and 8 of 15 Tac-specific ASVs were transferred to Jax by Tac FMT. Five ASVs were gained by Tac mice by both cohousing and Jax FMT. Three ASVs were gained by Jax mice by both cohousing and Tac FMT.

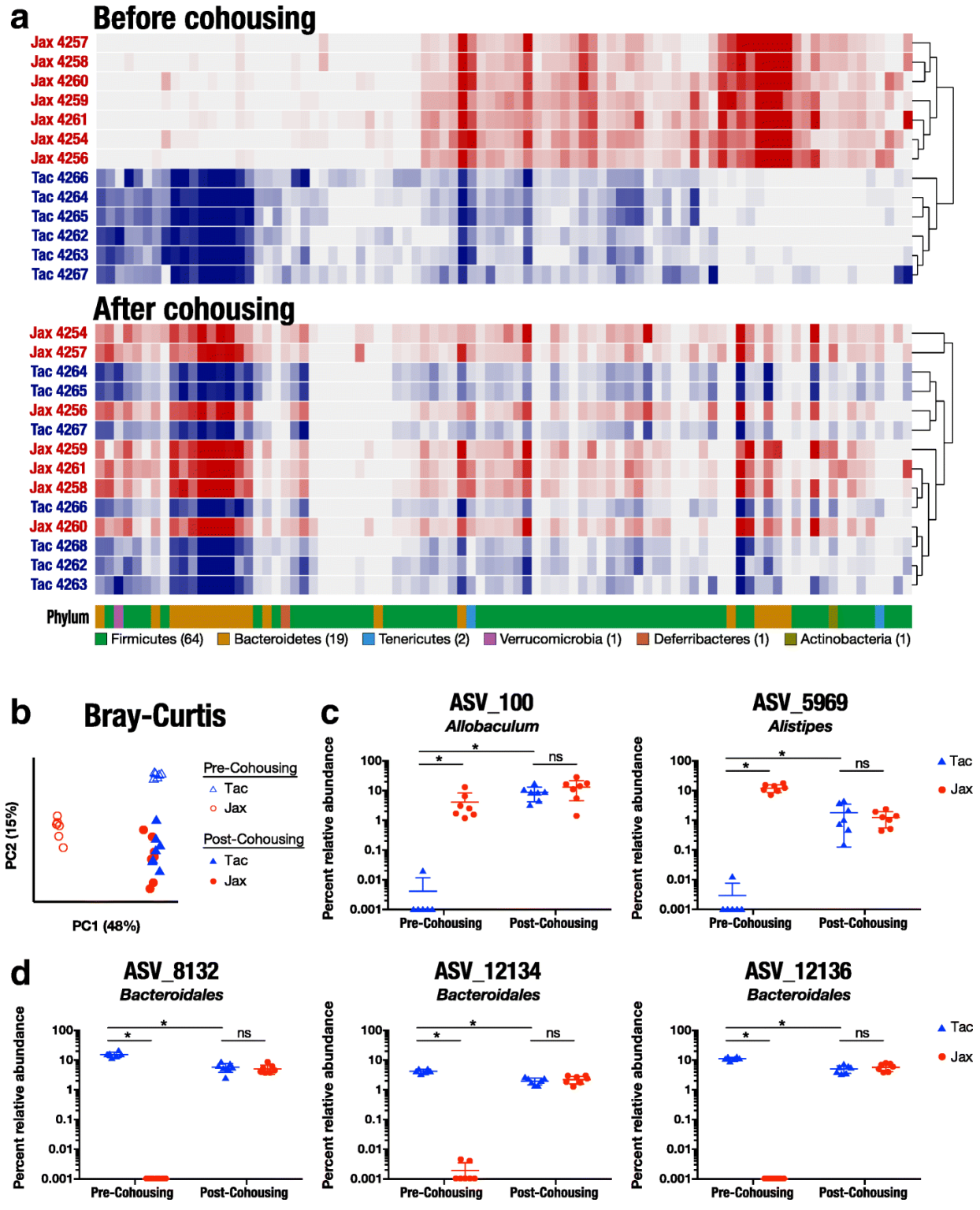

Figure A.3 Jax and Tac microbiota composition converges following cohousing.

(a) Heat map of percent relative abundance of ASVs in Jax and Tac mice before and after cohousing. Each row represents an individual mouse. Each column represents a single ASV.

Figure A.3, continued: Only ASVs with percent relative abundance of at least 1% in a minimum of two samples from the same vendor before cohousing were included. Arrangement of ASVs was kept constant between all rows. Bottom row displays the phylum to which the ASV in the same column was assigned. (b) Principal coordinate analysis of female B6 Jax and Tac fecal microbiota composition using Bray-Curtis distance prior to or following cohousing of Jax and Tac mice for 3 weeks. Each dot represents an individual mouse. (c, d) Percent relative abundance of all ASVs significantly differentially abundant between Tac and Jax before cohousing and between Tac before and after cohousing with Jax, but not significantly different between Jax and Tac after cohousing. Each dot represents an individual mouse. *$P < 0.05$, ns = not significant

We chose to focus further analysis on ASVs that had a relative abundance of at least 1% in a minimum of two mice from either Jax or Tac at baseline, reasoning that very low abundance microbes were more likely to represent sequencing errors. Of the 34 ASVs meeting our criteria for inclusion, our analysis revealed 24 ASVs that were differentially abundant between Jax and Tac mice upon arrival at our facility. Following cohousing, none of those 24 ASVs remained significantly different between Jax and Tac, and the relative abundance of only five (ASVs_3100, 5969, 8132, 12134, and 12135) had changed in Tac mice before and after cohousing with Jax (Fig. A.3c, d). ASVs_100 and ASV_5969 were present only in Jax mice at baseline and were transferred to Tac mice by cohousing, while remaining unchanged in Jax mice. Three ASVs that resolved to the Bacteroidales order (ASVs_8132, 12134, and 12136) were present only in Tac mice at baseline, and their abundance increased significantly in Jax mice after cohousing. They also decreased two to threefold in relative abundance in Tac mice after cohousing, though the increase in these ASVs in Jax mice was far greater than their decrease in Tac mice. Because ASVs_100 and ASV_5969 only changed in Tac following cohousing, we reasoned that the abundance of these ASVs tracked best with delayed skin transplant rejection and that these were the best candidates for ASVs promoting skin transplant survival.

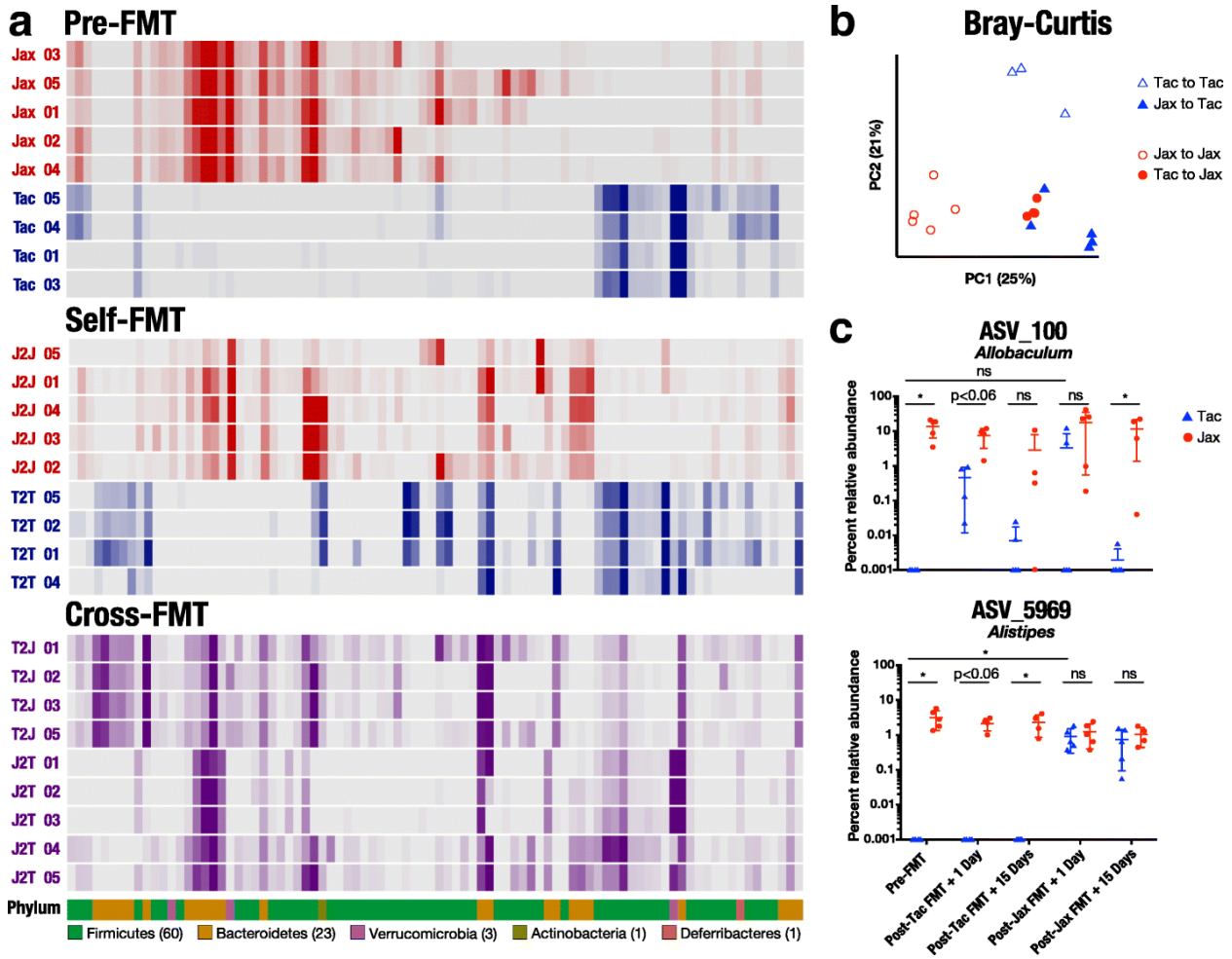

Figure A.4 Jax and Tac microbiota composition converges following FMT, including transfer of ASVs transferred by cohousing.

(a) Heat map of percent relative abundance of ASVs in Jax and Tac mice before FMT, or 1 day after the third FMT from either vendor-matched fecal donor (self-FMT) or fecal donor from different vendor (cross-FMT). Each row represents an individual mouse. Each column represents a single ASV. Only ASVs with percent relative abundance of at least 1% in a minimum of two samples from the same vendor were included. Arrangement of ASVs was kept constant between all rows. Bottom row displays the phylum to which the ASV in the same column was assigned. (b) Principal coordinate analysis of female B6 Jax and Tac fecal microbiota composition using Bray-Curtis distance following either self-FMT (Jax to Jax, Tac to Tac) or cross-FMT (Tac to Jax, Jax to Tac). (c) Percent relative abundance of ASVs associated with prolonged graft survival in Jax and Tac mice either pre-FMT, or 1 or 15 days after the third self-FMT or cross-FMT. Each dot represents an individual mouse. *P < 0.05, ns = not significant

Next, we analyzed taxonomic composition of the fecal microbiota in Jax and Tac mice prior to and following Jax or Tac FMT. ASVs_100 and ASV_5969 were again detected in Jax

(n = 5) but not Tac (n = 4) mice upon arrival at our facility (Fig. A.4c). Following Jax FMT into Tac (n = 5), relative abundance of ASV_100 rose in two mice to match relative abundance in Jax mice but returned to nearly undetectable levels within 15 days after FMT. Tac mice gavaged with Tac feces (n = 4) also acquired some ASV_100. Jax mice maintained relatively high levels of ASV_100 whether gavaged with Jax or Tac feces, though the relative abundance did drop in some Jax mice. Much better correlated with graft outcome was ASV_5969, which was consistently transferred to Tac mice given Jax FMT, reaching levels similar to those seen in Jax mice. Relative abundance of ASV_5969 remained consistently high 15 days following the last FMT, showing that colonization was stable over time and through the stress of surgery. Jax mice maintained consistently high levels of ASV_5969 whether given FMT from Jax (n = 5) or Tac (n = 4) mice. We also note that, slower or faster skin graft rejection was associated with the presence or absence of ASV_100 and ASV_5969, independent of their relative abundance in animals in which they were detected (data not shown).

ASV_100 and ASV_5969 were absent from an outlier cohort where Jax FMT did not prolong skin transplant survival in Tac mice

Importantly, in one experiment, Tac mice treated with Jax FMT did not display delayed rejection kinetics (MST 19 ± 2 days, n = 5) when compared with Tac FMT-treated Tac mice (MST 19 ± 1 days, n = 5) (Fig. A.5a), affording us the opportunity to compare microbiota composition between these Jax FMT-treated Tac mice with fast rejection kinetics (fast Jax to Tac) and Jax FMT-treated Tac mice with delayed graft rejection from prior experiments (slow Jax to Tac). We performed a PCoA of 16S rRNA gene sequencing data in fecal DNA isolates from Tac FMT-treated Tac mice (Tac to Tac 1, n = 3 and Tac to Tac 2, n = 4), slow Jax FMT-gavaged Tac mice (n = 5), and fast Jax FMT-gavaged Tac mice (n = 3) using Bray-Curtis distance. Our analysis

revealed that mice exhibiting delayed skin graft rejection (slow Jax FMT-gavaged Tac mice) clustered separately from mice rejecting grafts more quickly (Tac mice and fast-Jax FMT-treated Tac mice) (Fig. A.5b), supporting our hypothesis that microbiota composition impacts graft rejection kinetics. Consistent with a role for ASV_100 or ASV_5969 in prolonging skin graft survival, fast Jax FMT-treated Tac mice were nearly devoid of both of these ASVs (n = 2, Fig. A.5c).

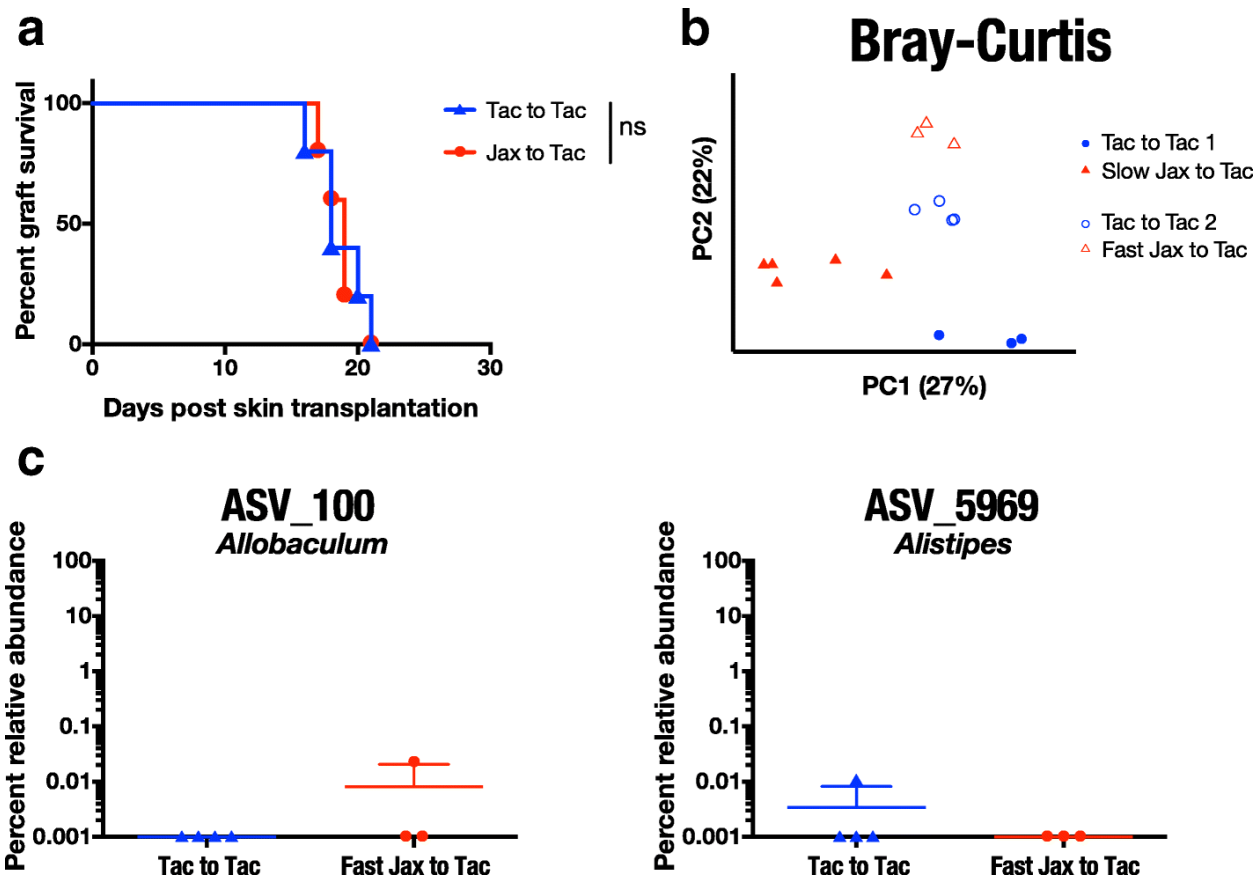

Figure A.5 Skin transplant survival-associated ASVs are missing from cohort of Jax to Tac mice with fast rejection.

(a) Male B6 Tac skin transplant survival kinetics in an outlier cohort of female B6 Tac mice that had received Jax or Tac FMT. (b) Principal coordinate analysis of microbiota composition using Bray-Curtis distance in female Tac B6 mice treated with Tac FMT (Tac to Tac 1 and Tac to Tac 2), with Jax FMT that delayed graft rejection (Slow Jax to Tac) or Jax FMT that did not delay graft rejection (Fast Jax to Tac). Each dot represents an individual mouse.

Figure A.5, continued: (c) Percent relative abundance of ASVs previously associated with prolonged graft survival (ASV_100 and ASV_5969) in Tac mice shown in a 1 day after the third Tac or Fast Jax FMT. ns = not significant

Discussion

In this study, we have determined that the composition of the fecal microbiota impacts the kinetics of skin transplant rejection in otherwise unmanipulated mice (i.e., not treated with antibiotics nor immunosuppressed). In the human population, there is a large diversity of microbiota composition between individuals, variability which can already be observed within the first year of life (Palmer et al. 2007). Even within an individual, large shifts in microbiota composition can occur through time following dietary changes (Palmer et al. 2007, Koenig et al. 2011). Our findings show that such differences in microbial community composition between individuals at steady state, independent of genetic differences between graft donor and recipient, can translate into varied kinetics of graft rejection and may therefore contribute to inter-patient variability in transplant outcomes.

We have identified a specific bacterial group that resolved to the genus *Alistipes* (ASV_5969), whose presence consistently correlated with delayed skin graft rejection in Jax mice or in Tac mice following cohousing with Jax mice or following Jax FMT pre-treatment. While transfer of ASV_5969 from Jax to Tac was consistently associated with prolonged graft survival, the mechanism by which members of this ASV could provide such an effect remains uncertain. Prolongation of skin graft survival could occur through directly or indirectly dampened adaptive alloimmunity, improved wound healing, or other processes impacting longevity of a transplanted organ. Alternatively, ASV_5969 may be displacing, after transfer into Tac mice, microbial species that promote faster graft rejection. Such displacement would need to

be broadly distributed across different genera and/or variable between individuals as we did not detect any microbial taxon consistently displaying a negative correlation with skin transplant survival. Members of ASV_5969 may also inhibit certain behaviors of other commensal microbes that promote graft rejection without limiting their colonization.

Interestingly, optimal prolongation of skin transplant survival in Tac mice required treating both the skin transplant donor and the skin transplant recipient with Jax FMT. As the female skin transplant recipient only receives skin from the male skin transplant donor, and the graft is disinfected with ethanol prior to transplantation, this finding suggests that the effect of Jax FMT in the transplant donor is localized to the skin tissue itself. The skin tissue may be affected, before its harvest for transplantation, by soluble factors circulating from the intestine to the skin, or by direct contact with the modified feces in the cage. Alternatively, we cannot eliminate the possibility that some Jax-derived microbes resist skin disinfection and modulate the immune system of the skin graft host after transplantation. Nevertheless, despite differences in microbiota between male and female mice evident in our data as well as in previous reports (Yurkovetskiy et al. 2013), ASV_5969 was transferred from Jax to Tac in both male skin graft donors and female skin graft recipients, supporting the hypothesis that the same immune modulation is at play in the graft donor and graft recipient.

While mechanisms behind these observations warrant further study, our data suggest a potential immunomodulatory role for the *Alistipes* population identified by ASV_5969. Not much is known about the immune impact of individual members of the *Alistipes* genus. One species, *Alistipes shahii*, has been associated with increased, rather than diminished, immune responses, as it has been shown to improve efficacy of anti-tumor immunotherapy in a mouse model (Iida et al. 2013). This effect was attributed to lipopolysaccharide (LPS) on the surface of

A. shahii acting as a toll-like receptor (TLR) 4 ligand, promoting tumor necrosis factor production and thus enhanced anti-tumor immunity. It is unlikely that *Alistipes* is promoting graft survival through LPS-TLR4 interactions, as it is polymorphisms associated with loss rather than gain of function in TLR4 that have been shown to be protective from acute rejection in humans (Palmer et al. 2003, Fekete et al. 2006), and signaling through TLR4 has been shown to promote rejection in a mouse skin graft model (Thornley et al. 2007).

An alloimmunity-dampening mechanism more consistent with our findings relates to the recent report that *Alistipes* members produce the anti-inflammatory metabolite sulfobacin B (Walker et al. 2017). *In vitro*, incubation of macrophages with sulfobacin B has been shown to inhibit production of TNF and NF-κB following stimulation with LPS (Maeda et al. 2010). *In vivo*, intraperitoneal injection with sulfobacin A lessened the inflammatory response to phorbol 12-myristate 13-acetate and LPS. In our *Alistipes*-colonized transplant recipients, it is conceivable that sulfobacin B might similarly impair NF-κB signaling and TNF production, leading to a reduction in the strength of the alloimmune response and thus delayed allograft rejection. Consistent with this, in preliminary results, we found reduced expression of TNF transcripts in the lymph nodes of skin transplanted Jax mice when compared to Tac mice.

While graft survival was only prolonged by several days in our slow-rejecting experimental groups, this modest survival increase may be biologically relevant. Indeed, we and our collaborators previously found similar differences in kinetics of tumor growth in mice obtained from different vendors and differences in tumor control were significantly amplified when the animals were treated with anti-PD-L1, a checkpoint blockade therapy (Sivan et al. 2015). We have since extended these data to the clinical setting and confirmed that differences in the fecal microbiota observed prior to initiation of checkpoint blockade therapy could predict

responsiveness to subsequent immunotherapy in melanoma patients (Matson et al. 2018). These findings as well as a recent study showing synergy of low-dose tacrolimus with FMT from mice treated with high-dose tacrolimus in delaying the rejection of major mismatched skin allografts in mice (Zhang, Liu, et al. 2018) suggest that small effects of the microbiota at steady state on tumor control or graft survival may be amplified by pharmacologic treatments. While our study has utilized a minor mismatch model of skin transplant rejection to provide a better window to observe either acceleration or delay in rejection kinetics without requiring immunosuppression, we have recently shown that antibiotic pre-treatment of skin graft donors and recipients can prolong survival of major mismatched skin grafts as well, supporting a wider role of the microbiota in various transplantation settings (Lei et al. 2016).

The shift in graft rejection kinetics observed between Tac mice treated with slow Jax-FMT and Tac mice treated with fast Jax-FMT, despite the Jax fecal donors originating from the same room in the same breeding facility at Jackson Laboratories, should provide a cautionary tale to investigators, as changes in microbial community composition that may occur over periods of time can impact the phenotype of interest.

One of the most noteworthy outcomes of our study is that there may be microbial populations that consistently colonize newly exposed mice and prolong graft survival. This observation suggests that whether patients undergoing organ transplantation may benefit from probiotic therapies or FMT warrants further research.

Our results also highlight the importance of understanding the effects of surgery and medications given to transplant recipients on microbiota composition, and the potential for them to induce colonization by microbes promoting or impairing graft survival. Studies have shown

changes to the microbiota of liver transplant recipients following transplantation, though no changes to relative abundance of *Alistipes* were observed (Sun et al. 2017). While the cause of microbiota changes in transplant recipients remains unknown, a recent study has shown that between multiple immunosuppressive drugs commonly used in transplantation, only steroids significantly impacted the composition of the mouse fecal microbiota, including a reduction in Bacteroidetes and an increase in Firmicutes (Tourret et al. 2017). Whether these post-transplantation changes to the microbiota have a meaningful impact on transplant outcomes remains to be investigated.

Conclusions

Our comparison of skin transplant rejection kinetics in mice of the same genetic background from different commercial vendors demonstrated that the composition of the resident microbiome in healthy individuals can impact the kinetics of transplant rejection, and may thus also contribute to inter-individual variability in graft outcomes in patients. We identified a single ASV that was consistently and stably transferred from the fecal microbiota of Jax to Tac and which was associated with prolonged skin graft survival in Tac mice. These observations suggest that we need to better understand transplantation-associated changes to patient microbiota composition as these changes may impact transplant outcomes.

References

"Research Pipeline - Sanofi Genzyme." Last Modified 5/2020, accessed 7/6/2020. https://www.sanofigenzyme.com/en/products-research/research-pipeline.

Abbott, Kevin C., S. John Swanson, Erich R. Richter, Erin M. Bohen, Lawrence Y. Agodoa, Thomas G. Peters, Galen Barbour, Robert Lipnick, and David F. Cruess. 2004. "Late urinary tract infection after renal transplantation in the United States¹." *American Journal of Kidney Diseases* 44 (2):353-362. doi: 10.1053/j.ajkd.2004.04.040.

Ahmed, E. B., T. Wang, M. Daniels, M. L. Alegre, and A. S. Chong. 2011. "IL-6 Induced by Staphylococcus aureus Infection Prevents the Induction of Skin Allograft Acceptance in Mice." *American Journal of Transplantation* 11 (5):936-946. doi: 10.1111/j.1600-6143.2011.03476.x.

Aiello, Sistiana, Federica Rocchetta, Lorena Longaretti, Silvia Faravelli, Marta Todeschini, Linda Cassis, Francesca Pezzuto, Susanna Tomasoni, Nadia Azzollini, Marilena Mister, Caterina Mele, Sara Conti, Matteo Breno, Giuseppe Remuzzi, Marina Noris, and Ariela Benigni. 2017. "Extracellular vesicles derived from T regulatory cells suppress T cell proliferation and prolong allograft survival." *Scientific Reports* 7 (1):11518. doi: 10.1038/s41598-017-08617-3.

Akamatsu, Masahiko, Norihisa Mikami, Naganari Ohkura, Ryoji Kawakami, Yohko Kitagawa, Atsushi Sugimoto, Keiji Hirota, Naoto Nakamura, Satoru Ujihara, Toshio Kurosaki, Hisao Hamaguchi, Hironori Harada, Guliang Xia, Yoshiaki Morita, Ichiro Aramori, Shuh Narumiya, and Shimon Sakaguchi. 2019. "Conversion of antigen-specific effector/memory T cells into Foxp3-expressing T_{reg} cells by inhibition of CDK8/19." *Science Immunology* 4 (40):eaaw2707. doi: 10.1126/sciimmunol.aaw2707.

Akkaya, Billur, Yoshihiro Oya, Munir Akkaya, Jafar Al Souz, Amanda H. Holstein, Olena Kamenyeva, Juraj Kabat, Ryutaro Matsumura, David W. Dorward, Deborah D. Glass, and Ethan M. Shevach. 2019. "Regulatory T cells mediate specific suppression by depleting peptide–MHC class II from dendritic cells." *Nature Immunology* 20 (2):218-231. doi: 10.1038/s41590-018-0280-2.

Al-Sheyyab, Ahmed, Laura Binari, Mohammed Shwetar, Everly Ramos, Meghan E. Kapp, Stefanie Bala, Nikita Wilson, Rachel C. Forbes, J. Harold Helderman, Khaled Abdel-Kader, and Beatrice P. Concepcion. 2019. "Association of medication non-adherence with short-term allograft loss after the treatment of severe acute kidney transplant rejection." *BMC Nephrology* 20 (1):373. doi: 10.1186/s12882-019-1563-z.

Alexander-Miller, M. A., G. R. Leggatt, and J. A. Berzofsky. 1996. "Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy." *Proc Natl Acad Sci U S A* 93 (9):4102-7. doi: 10.1073/pnas.93.9.4102.

Ali, Jason M., Margaret C. Negus, Thomas M. Conlon, Ines G. Harper, M. Saeed Qureshi, Reza Motallebzadeh, Richard Willis, Kourosh Saeb-Parsy, Eleanor M. Bolton, J. Andrew Bradley, and Gavin J. Pettigrew. 2016a. "Diversity of the CD4 T Cell Alloresponse: The Short and the Long of It." *Cell reports* 14 (5):1232-1245. doi: 10.1016/j.celrep.2015.12.099.

Ali, Jason M., Margaret C. Negus, Thomas M. Conlon, Ines G. Harper, M. Saeed Qureshi, Reza Motallebzadeh, Richard Willis, Kourosh Saeb-Parsy, Eleanor M. Bolton, J. Andrew Bradley, and Gavin J. Pettigrew. 2016b. "Diversity of the CD4 T Cell Alloresponse: The Short and the Long of It." *Cell Rep* 14 (5):1232-1245. doi: http://dx.doi.org/10.1016/j.celrep.2015.12.099.

Almond, P. Stephen, Arthur Matas, Kristen Gillingham, David L. Dunn, William D. Payne, John S. Najarian, Gores Paul, and Gruessner Rainer. 1993. "Risk factors for chronic rejection in renal allograft recipients." *Transplantation* 55 (4).

Amarante-Mendes, Gustavo P., Sandy Adjemian, Laura Migliari Branco, Larissa C. Zanetti, Ricardo Weinlich, and Karina R. Bortoluci. 2018. "Pattern Recognition Receptors and the Host Cell Death Molecular Machinery." *Frontiers in Immunology* 9:2379.

Anderson, Mark S., Emily S. Venanzi, Ludger Klein, Zhibin Chen, Stuart P. Berzins, Shannon J. Turley, Harald von Boehmer, Roderick Bronson, Andrée Dierich, Christophe Benoist, and Diane Mathis. 2002. "Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein." *Science* 298 (5597):1395. doi: 10.1126/science.1075958.

Anderton, S. M., and S. Fillatreau. 2008. "Activated B cells in autoimmune diseases: the case for a regulatory role." *Nat Clin Pract Rheumatol* 4 (12):657-66. doi: 10.1038/ncprheum0950.

Andrews, Lawrence P., Ariel E. Marciscano, Charles G. Drake, and Dario A. A. Vignali. 2017. "LAG3 (CD223) as a cancer immunotherapy target." *Immunological reviews* 276 (1):80-96. doi: 10.1111/imr.12519.

Antunes, Inês, and George Kassiotis. 2010. "Suppression of innate immune pathology by regulatory T cells during Influenza A virus infection of immunodeficient mice." *Journal of virology* 84 (24):12564-12575. doi: 10.1128/JVI.01559-10.

Arpaia, Nicholas, Jesse A. Green, Bruno Moltedo, Aaron Arvey, Saskia Hemmers, Shaopeng Yuan, Piper M. Treuting, and Alexander Y. Rudensky. 2015. "A Distinct Function of Regulatory T Cells in Tissue Protection." *Cell* 162 (5):1078-1089. doi: 10.1016/j.cell.2015.08.021.

Atarashi, K., T. Tanoue, K. Oshima, W. Suda, Y. Nagano, H. Nishikawa, S. Fukuda, T. Saito, S. Narushima, K. Hase, S. Kim, J. V. Fritz, P. Wilmes, S. Ueha, K. Matsushima, H. Ohno, B. Olle, S. Sakaguchi, T. Taniguchi, H. Morita, M. Hattori, and K. Honda. 2013. "Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota." *Nature* 500 (7461):232-6. doi: 10.1038/nature12331.

Atarashi, Koji, Takeshi Tanoue, Tatsuichiro Shima, Akemi Imaoka, Tomomi Kuwahara, Yoshika Momose, Genhong Cheng, Sho Yamasaki, Takashi Saito, Yusuke Ohba, Tadatsugu Taniguchi, Kiyoshi Takeda, Shohei Hori, Ivaylo I. Ivanov, Yoshinori Umesaki, Kikuji Itoh, and Kenya Honda. 2011. "Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species." *Science* 331 (6015):337. doi: 10.1126/science.1198469.

Barker, Clyde F., and James F. Markmann. 2013. "Historical overview of transplantation." *Cold Spring Harbor perspectives in medicine* 3 (4):a014977-a014977. doi: 10.1101/cshperspect.a014977.

Bautista, Jhoanne L., Chan-Wang J. Lio, Stephanie K. Lathrop, Katherine Forbush, Yuqiong Liang, Jingqin Luo, Alexander Y. Rudensky, and Chyi-Song Hsieh. 2009. "Intraclonal competition limits the fate determination of regulatory T cells in the thymus." *Nature Immunology* 10 (6):610-617. doi: 10.1038/ni.1739.

Belkaid, Yasmine, and Timothy W Hand. 2014. "Role of the Microbiota in Immunity and Inflammation." *Cell* 157 (1):121-141. doi: 10.1016/j.cell.2014.03.011.

Belladonna, Maria L., Ciriana Orabona, Ursula Grohmann, and Paolo Puccetti. 2009. "TGF-β and kynurenines as the key to infectious tolerance." *Trends in Molecular Medicine* 15 (2):41-49. doi: https://doi.org/10.1016/j.molmed.2008.11.006.

Bellon, M., and C. Nicot. 2017. "Telomere Dynamics in Immune Senescence and Exhaustion Triggered by Chronic Viral Infection." *Viruses* 9 (10). doi: 10.3390/v9100289.

Besançon, Alix, Marije Baas, Tania Goncalves, Fabrice Valette, Herman Waldmann, Lucienne Chatenoud, and Sylvaine You. 2017. "The Induction and Maintenance of Transplant Tolerance Engages Both Regulatory and Anergic CD4(+) T cells." *Frontiers in immunology* 8:218-218. doi: 10.3389/fimmu.2017.00218.

Bettelli, Estelle, Yijun Carrier, Wenda Gao, Thomas Korn, Terry B. Strom, Mohamed Oukka, Howard L. Weiner, and Vijay K. Kuchroo. 2006. "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells." *Nature* 441 (7090):235-238. doi: 10.1038/nature04753.

Bettini, Matthew L., Maria Bettini, and Dario A. A. Vignali. 2012. "T-cell receptor retrogenic mice: a rapid, flexible alternative to T-cell receptor transgenic mice." *Immunology* 136 (3):265-272. doi: 10.1111/j.1365-2567.2012.03574.x.

Beyer, Marc, Yasser Thabet, Roman-Ulrich Müller, Timothy Sadlon, Sabine Classen, Katharina Lahl, Samik Basu, Xuyu Zhou, Samantha L. Bailey-Bucktrout, Wolfgang Krebs, Eva A. Schönfeld, Jan Böttcher, Tatiana Golovina, Christian T. Mayer, Andrea Hofmann, Daniel Sommer, Svenja Debey-Pascher, Elmar Endl, Andreas Limmer, Keli L. Hippen, Bruce R. Blazar, Robert Balderas, Thomas Quast, Andreas Waha, Günter Mayer, Michael Famulok, Percy A. Knolle, Claudia Wickenhauser, Waldemar Kolanus, Bernhard Schermer, Jeffrey A. Bluestone, Simon C. Barry, Tim Sparwasser, James L. Riley, and Joachim L. Schultze. 2011. "Repression of the genome organizer SATB1 in regulatory T

cells is required for suppressive function and inhibition of effector differentiation." *Nature Immunology* 12 (9):898-907. doi: 10.1038/ni.2084.

Bhat, Mamatha, Kristin Mara, Ross Dierkhising, and Kymberly D. S. Watt. 2018. *Mayo Clinic Proceedings* 93 (9):1236-1246. doi: 10.1016/j.mayocp.2018.04.025.

Billingham, R. E., L. Brent, and P. B. Medawar. 1953. "Actively acquired tolerance of foreign cells." *Nature* 172 (4379):603-6. doi: 10.1038/172603a0.

Billingham, R. E., L. Brent, and P. B. Medawar. 1956. "Quantitative Studies on Tissue Transplantation Immunity. III. Actively Acquired Tolerance." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 239 (666):357-414. doi: 10.1098/rstb.1956.0006.

Billingham, Rupert Everett, W. K. Silvers, and D. B. Wilson. 1965. "A second study on the H-Y transplantation antigen in mice." *Proceedings of the Royal Society of London. Series B. Biological Sciences* 163 (990):61-89. doi: 10.1098/rspb.1965.0060.

Black, C. M., T. D. Armstrong, and E. M. Jaffee. 2014. "Apoptosis-regulated low-avidity cancer-specific CD8(+) T cells can be rescued to eliminate HER2/neu-expressing tumors by costimulatory agonists in tolerized mice." *Cancer Immunol Res* 2 (4):307-19. doi: 10.1158/2326-6066.cir-13-0145.

Boissonnas, Alexandre, Alix Scholer-Dahirel, Virginie Simon-Blancal, Luigia Pace, Fabien Valet, Adrien Kissenpfennig, Tim Sparwasser, Bernard Malissen, Luc Fetler, and Sebastian Amigorena. 2010. "Foxp3+ T Cells Induce Perforin-Dependent Dendritic Cell Death in Tumor-Draining Lymph Nodes." *Immunity* 32 (2):266-278. doi: https://doi.org/10.1016/j.immuni.2009.11.015.

Bos, Rinke, Kristi L. Marquardt, Jocelyn Cheung, and Linda A. Sherman. 2012. "Functional differences between low- and high-affinity CD8+ T cells in the tumor environment." *OncoImmunology* 1 (8):1239-1247. doi: 10.4161/onci.21285.

Boyman, Onur, Marek Kovar, Mark P. Rubinstein, Charles D. Surh, and Jonathan Sprent. 2006. "Selective Stimulation of T Cell Subsets with Antibody-Cytokine Immune Complexes." *Science* 311 (5769):1924. doi: 10.1126/science.1122927.

Brennan, T. V., V. Hoang, K. R. Garrod, F. C. Liu, T. Hayden, J. Kim, and S. M. Kang. 2008. "A new T-cell receptor transgenic model of the CD4+ direct pathway: level of priming determines acute versus chronic rejection." *Transplantation* 85 (2):247-55. doi: 10.1097/TP.0b013e31815e883e.

Brouard, S., A. Pallier, K. Renaudin, Y. Foucher, R. Danger, A. Devys, A. Cesbron, C. Guillot-Guegen, J. Ashton-Chess, S. Le Roux, J. Harb, G. Roussey, J. F. Subra, F. Villemain, C. Legendre, F. J. Bemelman, G. Orlando, A. Garnier, H. Jambon, H. Le Monies De Sagazan, L. Braun, C. Noel, E. Pillebout, M. C. Moal, C. Cantarell, A. Hoitsma, M. Ranbant, A. Testa, J. P. Soulillou, and M. Giral. 2012. "The natural history of clinical

operational tolerance after kidney transplantation through twenty-seven cases." *Am J Transplant* 12 (12):3296-307. doi: 10.1111/j.1600-6143.2012.04249.x.

Brown, C. C., H. Gudjonson, Y. Pritykin, D. Deep, V. P. Lavallee, A. Mendoza, R. Fromme, L. Mazutis, C. Ariyan, C. Leslie, D. Pe'er, and A. Y. Rudensky. 2019. "Transcriptional Basis of Mouse and Human Dendritic Cell Heterogeneity." *Cell* 179 (4):846-863.e24. doi: 10.1016/j.cell.2019.09.035.

Brunkow, Mary E., Eric W. Jeffery, Kathryn A. Hjerrild, Bryan Paeper, Lisa B. Clark, Sue-Ann Yasayko, J. Erby Wilkinson, David Galas, Steven F. Ziegler, and Fred Ramsdell. 2001. "Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse." *Nature Genetics* 27 (1):68-73. doi: 10.1038/83784.

Burnet, Frank Macfarlane. 1941. *The Production of Antibodies: A Review and a Theoretical Discussion*: Macmillan Limited.

Burns, Audrea M., and Anita S. Chong. 2011. "Alloantibodies Prevent the Induction of Transplantation Tolerance by Enhancing Alloreactive T Cell Priming." *The Journal of Immunology* 186 (1):214. doi: 10.4049/jimmunol.1001172.

Burzyn, Dalia, Wilson Kuswanto, Dmitriy Kolodin, Jennifer L Shadrach, Massimiliano Cerletti, Young Jang, Esen Sefik, Tze Guan Tan, Amy J Wagers, Christophe Benoist, and Diane Mathis. 2013. "A Special Population of Regulatory T Cells Potentiates Muscle Repair." *Cell* 155 (6):1282-1295. doi: 10.1016/j.cell.2013.10.054.

Busch, D. H., and E. G. Pamer. 1999. "T cell affinity maturation by selective expansion during infection." *J Exp Med* 189 (4):701-10.

Bushell, A., T. C. Pearson, P. J. Morris, and K. J. Wood. 1995. "Donor-recipient microchimerism is not required for tolerance induction following recipient pretreatment with donor-specific transfusion and anti-CD4 antibody. Evidence of a clear role for short-term antigen persistence." *Transplantation* 59 (10):1367-71. doi: 10.1097/00007890-199505270-00001.

Cai, Guifang, Robert A. Kastelein, and Christopher A. Hunter. 1999. "IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-γ when combined with IL-18." *European Journal of Immunology* 29 (9):2658-2665. doi: 10.1002/(SICI)1521-4141(199909)29:09<2658::AID-IMMU2658>3.0.CO;2-G.

Callahan, Benjamin J., Paul J. McMurdie, and Susan P. Holmes. 2017. "Exact sequence variants should replace operational taxonomic units in marker-gene data analysis." *The ISME Journal* 11 (12):2639-2643. doi: 10.1038/ismej.2017.119.

Cao, Xuefang, Sheng F. Cai, Todd A. Fehniger, Jiling Song, Lynne I. Collins, David R. Piwnica-Worms, and Timothy J. Ley. 2007. "Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance." *Immunity* 27 (4):635-646. doi: 10.1016/j.immuni.2007.08.014.

Caporaso, J. Gregory, Justin Kuczynski, Jesse Stombaugh, Kyle Bittinger, Frederic D. Bushman, Elizabeth K. Costello, Noah Fierer, Antonio Gonzalez Peña, Julia K. Goodrich, Jeffrey I. Gordon, Gavin A. Huttley, Scott T. Kelley, Dan Knights, Jeremy E. Koenig, Ruth E. Ley, Catherine A. Lozupone, Daniel McDonald, Brian D. Muegge, Meg Pirrung, Jens Reeder, Joel R. Sevinsky, Peter J. Turnbaugh, William A. Walters, Jeremy Widmann, Tanya Yatsunenko, Jesse Zaneveld, and Rob Knight. 2010. "QIIME allows analysis of high-throughput community sequencing data." *Nature Methods* 7 (5):335-336. doi: 10.1038/nmeth.f.303.

Caporaso, J. Gregory, Christian L. Lauber, William A. Walters, Donna Berg-Lyons, James Huntley, Noah Fierer, Sarah M. Owens, Jason Betley, Louise Fraser, Markus Bauer, Niall Gormley, Jack A. Gilbert, Geoff Smith, and Rob Knight. 2012. "Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms." *The ISME Journal* 6 (8):1621-1624. doi: 10.1038/ismej.2012.8.

Carter, Natalie A., Rita Vasconcellos, Elizabeth C. Rosser, Calogero Tulone, Alba Muñoz-Suano, Masahito Kamanaka, Michael R. Ehrenstein, Richard A. Flavell, and Claudia Mauri. 2011. "Mice Lacking Endogenous IL-10–Producing Regulatory B Cells Develop Exacerbated Disease and Present with an Increased Frequency of Th1/Th17 but a Decrease in Regulatory T Cells." *The Journal of Immunology* 186 (10):5569. doi: 10.4049/jimmunol.1100284.

Castro, Flávia, Ana Patrícia Cardoso, Raquel Madeira Gonçalves, Karine Serre, and Maria José Oliveira. 2018. "Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion." *Frontiers in immunology* 9:847-847. doi: 10.3389/fimmu.2018.00847.

Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. "Activation of human dendritic cells through CD40 cross-linking." *J Exp Med* 180 (4):1263-72. doi: 10.1084/jem.180.4.1263.

Cawthon, A. G., H. Lu, and M. A. Alexander-Miller. 2001. "Peptide requirement for CTL activation reflects the sensitivity to CD3 engagement: correlation with CD8alphabeta versus CD8alphaalpha expression." *J Immunol* 167 (5):2577-84. doi: 10.4049/jimmunol.167.5.2577.

Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. "Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation." *J Exp Med* 184 (2):747-52. doi: 10.1084/jem.184.2.747.

Chai, Jian-Guo, Kulachelvy Ratnasothy, R. Pat Bucy, Randolph J. Noelle, Robert Lechler, and Giovanna Lombardi. 2015. "Allospecific CD4+ T cells retain effector function and are actively regulated by Treg cells in the context of transplantation tolerance." *European Journal of Immunology* 45 (7):2017-2027. doi: 10.1002/eji.201545455.

Chapman, Jeremy R., Angela C. Webster, and Germaine Wong. 2013. "Cancer in the transplant recipient." *Cold Spring Harbor perspectives in medicine* 3 (7):a015677. doi: 10.1101/cshperspect.a015677.

Charbonnier, L. M., B. Vokaer, P. H. Lemaître, K. A. Field, O. Leo, and A. Le Moine. 2012. "CTLA4-Ig Restores Rejection of MHC Class-II Mismatched Allografts by Disabling IL-2-Expanded Regulatory T Cells." *American Journal of Transplantation* 12 (9):2313-2321. doi: 10.1111/j.1600-6143.2012.04184.x.

Chaudhry, Ashutosh, Robert M. Samstein, Piper Treuting, Yuqiong Liang, Marina C. Pils, Jan-Michael Heinrich, Robert S. Jack, F. Thomas Wunderlich, Jens C. Brüning, Werner Müller, and Alexander Y. Rudensky. 2011. "Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation." *Immunity* 34 (4):566-578. doi: 10.1016/j.immuni.2011.03.018.

Chen, Lieping, and Dallas B. Flies. 2013. "Molecular mechanisms of T cell co-stimulation and co-inhibition." *Nature reviews. Immunology* 13 (4):227-242. doi: 10.1038/nri3405.

Chen, Wanjun, Mohamed H. Sayegh, and Samia J. Khoury. 1998. "Mechanisms of Acquired Thymic Tolerance In Vivo: Intrathymic Injection of Antigen Induces Apoptosis of Thymocytes and Peripheral T Cell Anergy." *The Journal of Immunology* 160 (3):1504.

Chesneau, Mélanie, Laure Michel, Nicolas Degauque, and Sophie Brouard. 2013. "Regulatory B cells and tolerance in transplantation: from animal models to human." *Frontiers in immunology* 4:497-497. doi: 10.3389/fimmu.2013.00497.

Chhabra, A. Y., J. Leventhal, A. R. Merchak, and S. Ildstad. 2017. "HSCT-Based Approaches for Tolerance Induction in Renal Transplant." *Transplantation* 101 (11):2682-2690. doi: 10.1097/tp.0000000000001837.

Chou, Jennifer P., and Rita B. Effros. 2013. "T cell replicative senescence in human aging." *Current pharmaceutical design* 19 (9):1680-1698. doi: 10.2174/138161213805219711.

Chung, Yeonseok, Shinya Tanaka, Fuliang Chu, Roza I. Nurieva, Gustavo J. Martinez, Seema Rawal, Yi-Hong Wang, Hoyong Lim, Joseph M. Reynolds, Xiao-hui Zhou, Hui-min Fan, Zhong-ming Liu, Sattva S. Neelapu, and Chen Dong. 2011. "Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions." *Nature Medicine* 17 (8):983-988. doi: 10.1038/nm.2426.

Cipolletta, Daniela, Markus Feuerer, Amy Li, Nozomu Kamei, Jongsoon Lee, Steven E. Shoelson, Christophe Benoist, and Diane Mathis. 2012. "PPAR-γ is a major driver of the accumulation and phenotype of adipose tissue Treg cells." *Nature* 486 (7404):549-553. doi: 10.1038/nature11132.

Collison, Lauren W., Creg J. Workman, Timothy T. Kuo, Kelli Boyd, Yao Wang, Kate M. Vignali, Richard Cross, David Sehy, Richard S. Blumberg, and Dario A. A. Vignali. 2007. "The inhibitory cytokine IL-35 contributes to regulatory T-cell function." *Nature* 450 (7169):566-569. doi: 10.1038/nature06306.

Coomes, S. M., Y. Kannan, V. S. Pelly, L. J. Entwistle, R. Guidi, J. Perez-Lloret, N. Nikolov, W. Müller, and M. S. Wilson. 2017. "CD4+ Th2 cells are directly regulated by IL-10 during

allergic airway inflammation." *Mucosal Immunology* 10 (1):150-161. doi: 10.1038/mi.2016.47.

Corry, Robert J., Henry J. Winn, and Paul S. Russell. 1973. "PRIMARILY VASCULARIZED ALLOGRAFTS OF HEARTS IN MICE: THE ROLE OF H-2D, H-2K, AND NON-H-2 ANTIGENS IN REJECTION." *Transplantation* 16 (4).

Cossart, Amelia Rose, Christine Elizabeth Staatz, Scott Bryan Campbell, Nicole Maree Isbel, and William Neil Cottrell. 2019. "Investigating barriers to immunosuppressant medication adherence in renal transplant patients." *Nephrology* 24 (1):102-110. doi: 10.1111/nep.13214.

Couper, Kevin N., Daniel G. Blount, and Eleanor M. Riley. 2008. "IL-10: The Master Regulator of Immunity to Infection." *The Journal of Immunology* 180 (9):5771. doi: 10.4049/jimmunol.180.9.5771.

Cunha, Cristina, Agostinho Carvalho, Antonella Esposito, Francesco Bistoni, and Luigina Romani. 2012. "DAMP signaling in fungal infections and diseases." *Frontiers in immunology* 3:286-286. doi: 10.3389/fimmu.2012.00286.

De Vries, V. C., A. Wasiuk, K. A. Bennett, M. J. Benson, R. Elgueta, T. J. Waldschmidt, and R. J. Noelle. 2009. "Mast Cell Degranulation Breaks Peripheral Tolerance." *American Journal of Transplantation* 9 (10):2270-2280. doi: 10.1111/j.1600-6143.2009.02755.x.

Deaglio, Silvia, Karen M. Dwyer, Wenda Gao, David Friedman, Anny Usheva, Anna Erat, Jiang-Fan Chen, Keiichii Enjyoji, Joel Linden, Mohamed Oukka, Vijay K. Kuchroo, Terry B. Strom, and Simon C. Robson. 2007. "Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression." *Journal of Experimental Medicine* 204 (6):1257-1265. doi: 10.1084/jem.20062512.

Demetris, A. J., N. Murase, R. G. Lee, P. Randhawa, A. Zeevi, S. Pham, R. Duquesnoy, J. J. Fung, and T. E. Starzl. 1997. "Chronic rejection. A general overview of histopathology and pathophysiology with emphasis on liver, heart and intestinal allografts." *Annals of transplantation* 2 (2):27-44.

Doycheva, Iliana, Syed Amer, and Kymberly D. Watt. 2016. "De Novo Malignancies After Transplantation: Risk and Surveillance Strategies." *Medical Clinics of North America* 100 (3):551-567. doi: https://doi.org/10.1016/j.mcna.2016.01.006.

Dutoit, V., V. Rubio-Godoy, P. Y. Dietrich, A. L. Quiqueres, V. Schnuriger, D. Rimoldi, D. Liénard, D. Speiser, P. Guillaume, P. Batard, J. C. Cerottini, P. Romero, and D. Valmori. 2001. "Heterogeneous T-cell response to MAGE-A10(254-262): high avidity-specific cytolytic T lymphocytes show superior antitumor activity." *Cancer Res* 61 (15):5850-6.

Eck, Steven C., David Chang, Andrew D. Wells, and Laurence A. Turka. 1997. "Differential Down-Regulation Of CD28 By B7-1 And B7-2 Engagement1." *Transplantation* 64 (10).

Engelhardt, Karin R., and Bodo Grimbacher. 2014. "IL-10 in Humans: Lessons from the Gut, IL-10/IL-10 Receptor Deficiencies, and IL-10 Polymorphisms." In *Interleukin-10 in Health and Disease*, edited by Simon Fillatreau and Anne O'Garra, 1-18. Berlin, Heidelberg: Springer Berlin Heidelberg.

Engels, Eric A., Ruth M. Pfeiffer, Joseph F. Fraumeni, Jr., Bertram L. Kasiske, Ajay K. Israni, Jon J. Snyder, Robert A. Wolfe, Nathan P. Goodrich, A. Rana Bayakly, Christina A. Clarke, Glenn Copeland, Jack L. Finch, Mary Lou Fleissner, Marc T. Goodman, Amy Kahn, Lori Koch, Charles F. Lynch, Margaret M. Madeleine, Karen Pawlish, Chandrika Rao, Melanie A. Williams, David Castenson, Michael Curry, Ruth Parsons, Gregory Fant, and Monica Lin. 2011. "Spectrum of cancer risk among US solid organ transplant recipients." *JAMA* 306 (17):1891-1901. doi: 10.1001/jama.2011.1592.

Enouz, S., L. Carrie, D. Merkler, M. J. Bevan, and D. Zehn. 2012. "Autoreactive T cells bypass negative selection and respond to self-antigen stimulation during infection." *J Exp Med* 209 (10):1769-79. doi: 10.1084/jem.20120905.

Enouz, Sarah, Lucie Carrié, Doron Merkler, Michael J. Bevan, and Dietmar Zehn. 2012. "Autoreactive T cells bypass negative selection and respond to self-antigen stimulation during infection." *Journal of Experimental Medicine* 209 (10):1769-1779. doi: 10.1084/jem.20120905.

Eren, A. Murat, Gary G. Borisy, Susan M. Huse, and Jessica L. Mark Welch. 2014. "Oligotyping analysis of the human oral microbiome." *Proceedings of the National Academy of Sciences* 111 (28):E2875. doi: 10.1073/pnas.1409644111.

Eren, A. Murat, Özcan C. Esen, Christopher Quince, Joseph H. Vineis, Hilary G. Morrison, Mitchell L. Sogin, and Tom O. Delmont. 2015. "Anvi'o: an advanced analysis and visualization platform for 'omics data." *PeerJ* 3:e1319. doi: 10.7717/peerj.1319.

Eren, A. Murat, Loïs Maignien, Woo Jun Sul, Leslie G. Murphy, Sharon L. Grim, Hilary G. Morrison, and Mitchell L. Sogin. 2013. "Oligotyping: differentiating between closely related microbial taxa using 16S rRNA gene data." *Methods in Ecology and Evolution* 4 (12):1111-1119. doi: 10.1111/2041-210X.12114.

Eren, A. Murat, Hilary G. Morrison, Pamela J. Lescault, Julie Reveillaud, Joseph H. Vineis, and Mitchell L. Sogin. 2015. "Minimum entropy decomposition: Unsupervised oligotyping for sensitive partitioning of high-throughput marker gene sequences." *The ISME Journal* 9 (4):968-979. doi: 10.1038/ismej.2014.195.

Eren, A. Murat, Mitchell L. Sogin, and Loïs Maignien. 2016. "Editorial: New Insights into Microbial Ecology through Subtle Nucleotide Variation." *Frontiers in Microbiology* 7 (1318). doi: 10.3389/fmicb.2016.01318.

Ertelt, James M., Jared H. Rowe, Tanner M. Johanns, Joseph C. Lai, James B. McLachlan, and Sing Sing Way. 2009. "Selective Priming and Expansion of Antigen-Specific Foxp3[−]CD4⁺ T Cells during Listeria

monocytogenes Infection." *The Journal of Immunology* 182 (5):3032. doi: 10.4049/jimmunol.0803402.

Esensten, Jonathan H, Ynes A Helou, Gaurav Chopra, Arthur Weiss, and Jeffrey A Bluestone. 2016. "CD28 Costimulation: From Mechanism to Therapy." *Immunity* 44 (5):973-988. doi: https://doi.org/10.1016/j.immuni.2016.04.020.

Fahmy, Tarek M., Joan Glick Bieler, Michael Edidin, and Jonathan P. Schneck. 2001. "Increased TCR Avidity after T Cell Activation: A Mechanism for Sensing Low-Density Antigen." *Immunity* 14 (2):135-143. doi: https://doi.org/10.1016/S1074-7613(01)00096-6.

Fallarino, Francesca, Ursula Grohmann, Kwang Woo Hwang, Ciriana Orabona, Carmine Vacca, Roberta Bianchi, Maria Laura Belladonna, Maria Cristina Fioretti, Maria-Luisa Alegre, and Paolo Puccetti. 2003. "Modulation of tryptophan catabolism by regulatory T cells." *Nature Immunology* 4 (12):1206-1212. doi: 10.1038/ni1003.

Fallarino, Francesca, Ursula Grohmann, Sylvaine You, Barbara C. McGrath, Douglas R. Cavener, Carmine Vacca, Ciriana Orabona, Roberta Bianchi, Maria L. Belladonna, Claudia Volpi, Pere Santamaria, Maria C. Fioretti, and Paolo Puccetti. 2006. "The Combined Effects of Tryptophan Starvation and Tryptophan Catabolites Down-Regulate T Cell Receptor ζ-Chain and Induce a Regulatory Phenotype in Naive T Cells." *The Journal of Immunology* 176 (11):6752. doi: 10.4049/jimmunol.176.11.6752.

Farkash, E, A Naik, H Tedesco-Silva, B Nashan, O Witzke, M van den Hoogen, S Berger, DM Cibrik, S Mulgaonkar, DB Leeser, R Alloway, A Patel, J Pratchke, C Sommerer, A Wiseman, A van Zuilen, U Laessing, J Rush, and B Haraldsson. 2019. CNI-Free Therapy with Iscalimab (anti-CD40 mAb) Preserves Allograft Histology Compared to Standard of Care after Kidney Transplantation [abstract]. . Am J Transplant.

Farrar, Conrad A., Jerzy W. Kupiec-Weglinski, and Steven H. Sacks. 2013. "The Innate Immune System and Transplantation." *Cold Spring Harbor Perspectives in Medicine* 3 (10). doi: 10.1101/cshperspect.a015479.

Fekete, Andrea, Ondřej Viklický, Jaroslav A. Hubáček, Krisztina Rusai, Gábor Erdei, András Treszl, Štefan Vítko, Tivadar Tulassay, Uwe Heemann, György Reusz, and Attila J. Szabó. 2006. "Association between heat shock protein 70s and Toll-like receptor polymorphisms with long-term renal allograft survival." *Transplant International* 19 (3):190-196. doi: 10.1111/j.1432-2277.2006.00265.x.

Ferrer, Ivana R., Maylene E. Wagener, Minqing Song, Allan D. Kirk, Christian P. Larsen, and Mandy L. Ford. 2011. "Antigen-specific induced Foxp3+ regulatory T cells are generated following CD40/CD154 blockade." *Proceedings of the National Academy of Sciences of the United States of America* 108 (51):20701-20706. doi: 10.1073/pnas.1105500108.

Feuerer, Markus, Laura Herrero, Daniela Cipolletta, Afia Naaz, Jamie Wong, Ali Nayer, Jongsoon Lee, Allison B. Goldfine, Christophe Benoist, Steven Shoelson, and Diane Mathis. 2009. "Lean, but not obese, fat is enriched for a unique population of regulatory

T cells that affect metabolic parameters." *Nature Medicine* 15 (8):930-939. doi: 10.1038/nm.2002.

Fillatreau, Simon, Claire H. Sweenie, Mandy J. McGeachy, David Gray, and Stephen M. Anderton. 2002. "B cells regulate autoimmunity by provision of IL-10." *Nature Immunology* 3 (10):944-950. doi: 10.1038/ni833.

Fisher, James D., Stephen C. Balmert, Wensheng Zhang, Riccardo Schweizer, Jonas T. Schnider, Chiaki Komatsu, Liwei Dong, Vasil E. Erbas, Jignesh V. Unadkat, Ali Mübin Aral, Abhinav P. Acharya, Yalcin Kulahci, Heth R. Turnquist, Angus W. Thomson, Mario G. Solari, Vijay S. Gorantla, and Steven R. Little. 2019. "Treg-inducing microparticles promote donor-specific tolerance in experimental vascularized composite allotransplantation." *Proceedings of the National Academy of Sciences* 116 (51):25784. doi: 10.1073/pnas.1910701116.

Fisher, James D., Wensheng Zhang, Stephen C. Balmert, Ali M. Aral, Abhinav P. Acharya, Yalcin Kulahci, Jingjing Li, Heth R. Turnquist, Angus W. Thomson, Mario G. Solari, Vijay S. Gorantla, and Steven R. Little. 2020. "In situ recruitment of regulatory T cells promotes donor-specific tolerance in vascularized composite allotransplantation." *Science Advances* 6 (11):eaax8429. doi: 10.1126/sciadv.aax8429.

Fishman, J. A. 2017. "Infection in Organ Transplantation." *American Journal of Transplantation* 17 (4):856-879. doi: 10.1111/ajt.14208.

Fishman, Jay A. 2007. "Infection in Solid-Organ Transplant Recipients." *New England Journal of Medicine* 357 (25):2601-2614. doi: 10.1056/NEJMra064928.

Flippe, Léa, Séverine Bézie, Ignacio Anegon, and Carole Guillonneau. 2019. "Future prospects for CD8+ regulatory T cells in immune tolerance." *Immunological Reviews* 292 (1):209-224. doi: 10.1111/imr.12812.

Fontenot, Jason D., Marc A. Gavin, and Alexander Y. Rudensky. 2003. "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." *Nature Immunology* 4 (4):330-336. doi: 10.1038/ni904.

Fraser, J. D., B. A. Irving, G. R. Crabtree, and A. Weiss. 1991. "Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28." *Science* 251 (4991):313-6. doi: 10.1126/science.1846244.

Frimpong, Augustina, Kwadwo Asamoah Kusi, Dennis Adu-Gyasi, Jones Amponsah, Michael Fokuo Ofori, and Wilfred Ndifon. 2019. "Phenotypic Evidence of T Cell Exhaustion and Senescence During Symptomatic Plasmodium falciparum Malaria." *Frontiers in Immunology* 10:1345.

Galluzzi, Lorenzo, Ilio Vitale, Stuart A. Aaronson, John M. Abrams, Dieter Adam, Patrizia Agostinis, Emad S. Alnemri, Lucia Altucci, Ivano Amelio, David W. Andrews, Margherita Annicchiarico-Petruzzelli, Alexey V. Antonov, Eli Arama, Eric H. Baehrecke, Nickolai A. Barlev, Nicolas G. Bazan, Francesca Bernassola, Mathieu J. M.

Bertrand, Katiuscia Bianchi, Mikhail V. Blagosklonny, Klas Blomgren, Christoph Borner, Patricia Boya, Catherine Brenner, Michelangelo Campanella, Eleonora Candi, Didac Carmona-Gutierrez, Francesco Cecconi, Francis K. M. Chan, Navdeep S. Chandel, Emily H. Cheng, Jerry E. Chipuk, John A. Cidlowski, Aaron Ciechanover, Gerald M. Cohen, Marcus Conrad, Juan R. Cubillos-Ruiz, Peter E. Czabotar, Vincenzo D'Angiolella, Ted M. Dawson, Valina L. Dawson, Vincenzo De Laurenzi, Ruggero De Maria, Klaus-Michael Debatin, Ralph J. DeBerardinis, Mohanish Deshmukh, Nicola Di Daniele, Francesco Di Virgilio, Vishva M. Dixit, Scott J. Dixon, Colin S. Duckett, Brian D. Dynlacht, Wafik S. El-Deiry, John W. Elrod, Gian Maria Fimia, Simone Fulda, Ana J. García-Sáez, Abhishek D. Garg, Carmen Garrido, Evripidis Gavathiotis, Pierre Golstein, Eyal Gottlieb, Douglas R. Green, Lloyd A. Greene, Hinrich Gronemeyer, Atan Gross, Gyorgy Hajnoczky, J. Marie Hardwick, Isaac S. Harris, Michael O. Hengartner, Claudio Hetz, Hidenori Ichijo, Marja Jäättelä, Bertrand Joseph, Philipp J. Jost, Philippe P. Juin, William J. Kaiser, Michael Karin, Thomas Kaufmann, Oliver Kepp, Adi Kimchi, Richard N. Kitsis, Daniel J. Klionsky, Richard A. Knight, Sharad Kumar, Sam W. Lee, John J. Lemasters, Beth Levine, Andreas Linkermann, Stuart A. Lipton, Richard A. Lockshin, Carlos López-Otín, Scott W. Lowe, Tom Luedde, Enrico Lugli, Marion MacFarlane, Frank Madeo, Michal Malewicz, Walter Malorni, Gwenola Manic, Jean-Christophe Marine, Seamus J. Martin, Jean-Claude Martinou, Jan Paul Medema, Patrick Mehlen, Pascal Meier, Sonia Melino, Edward A. Miao, Jeffery D. Molkentin, Ute M. Moll, Cristina Muñoz-Pinedo, Shigekazu Nagata, Gabriel Nuñez, Andrew Oberst, Moshe Oren, Michael Overholtzer, Michele Pagano, Theocharis Panaretakis, Manolis Pasparakis, Josef M. Penninger, David M. Pereira, Shazib Pervaiz, Marcus E. Peter, Mauro Piacentini, Paolo Pinton, Jochen H. M. Prehn, Hamsa Puthalakath, Gabriel A. Rabinovich, Markus Rehm, Rosario Rizzuto, Cecilia M. P. Rodrigues, David C. Rubinsztein, Thomas Rudel, Kevin M. Ryan, Emre Sayan, Luca Scorrano, Feng Shao, Yufang Shi, John Silke, Hans-Uwe Simon, Antonella Sistigu, Brent R. Stockwell, Andreas Strasser, Gyorgy Szabadkai, Stephen W. G. Tait, Daolin Tang, Nektarios Tavernarakis, Andrew Thorburn, Yoshihide Tsujimoto, Boris Turk, Tom Vanden Berghe, Peter Vandenabeele, Matthew G. Vander Heiden, Andreas Villunger, Herbert W. Virgin, Karen H. Vousden, Domagoj Vucic, Erwin F. Wagner, Henning Walczak, David Wallach, Ying Wang, James A. Wells, Will Wood, Junying Yuan, Zahra Zakeri, Boris Zhivotovsky, Laurence Zitvogel, Gerry Melino, and Guido Kroemer. 2018. "Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018." *Cell Death & Differentiation* 25 (3):486-541. doi: 10.1038/s41418-017-0012-4.

Ge, Yu-Zheng, Peng Yu, Rui-Peng Jia, Ran Wu, Ai-Xing Ding, Liang-Peng Li, Yan Zhao, Yu-Ming Feng, Zan-Long Gui, and Sheng Liao. 2014. "Association between transforming growth factor beta-1 +869T/C polymorphism and acute rejection of solid organ allograft: A meta-analysis and systematic review." *Transplant Immunology* 30 (2):76-83. doi: https://doi.org/10.1016/j.trim.2014.01.001.

Goldstein, D. R., B. M. Tesar, S. Akira, and F. G. Lakkis. 2003. "Critical role of the Toll-like receptor signal adaptor protein MyD88 in acute allograft rejection." *J Clin Invest* 111 (10):1571-8.

Gong, Tao, Lei Liu, Wei Jiang, and Rongbin Zhou. 2020. "DAMP-sensing receptors in sterile inflammation and inflammatory diseases." *Nature Reviews Immunology* 20 (2):95-112. doi: 10.1038/s41577-019-0215-7.

Gorbachev, Anton V., and Robert L. Fairchild. 2010. "CD4+CD25+ regulatory T cells utilize FasL as a mechanism to restrict DC priming functions in cutaneous immune responses." *European journal of immunology* 40 (7):2006-2015. doi: 10.1002/eji.200939387.

Gorczynski, Reginald M., Z. Chen, H. Zeng, and X. M. Fu. 1998. "A ROLE FOR PERSISTING ANTIGEN, ANTIGEN PRESENTATION, AND ICAM-1 IN INCREASED RENAL GRAFT SURVIVAL AFTER ORAL OR PORTAL VEIN DONOR-SPECIFIC IMMUNIZATION1." *Transplantation* 66 (3).

Green, Douglas R., Nathalie Droin, and Michael Pinkoski. 2003. "Activation-induced cell death in T cells." *Immunological Reviews* 193 (1):70-81. doi: 10.1034/j.1600-065X.2003.00051.x.

Green, J. M., P. J. Noel, A. I. Sperling, T. L. Walunas, G. S. Gray, J. A. Bluestone, and C. B. Thompson. 1994. "Absence of B7-dependent responses in CD28-deficient mice." *Immunity* 1 (6):501-8. doi: 10.1016/1074-7613(94)90092-2.

Gregori, Silvia, Kevin Goudy, and Maria Grazia Roncarolo. 2012. "The Cellular and Molecular Mechanisms of Immuno-Suppression by Human Type 1 Regulatory T Cells." *Frontiers in Immunology* 3:30.

Grohmann, Ursula, Ciriana Orabona, Francesca Fallarino, Carmine Vacca, Filippo Calcinaro, Alberto Falorni, Paola Candeloro, Maria L. Belladonna, Roberta Bianchi, Maria C. Fioretti, and Paolo Puccetti. 2002. "CTLA-4–Ig regulates tryptophan catabolism in vivo." *Nature Immunology* 3 (11):1097-1101. doi: 10.1038/ni846.

Grossman, William J., James W. Verbsky, Winfried Barchet, Marco Colonna, John P. Atkinson, and Timothy J. Ley. 2004. "Human T Regulatory Cells Can Use the Perforin Pathway to Cause Autologous Target Cell Death." *Immunity* 21 (4):589-601. doi: 10.1016/j.immuni.2004.09.002.

Guillonneau, Carole, Elodie Picarda, and Ignacio Anegon. 2010. "CD8+ regulatory T cells in solid organ transplantation." *Current Opinion in Organ Transplantation* 15 (6).

Gupta, Pawan K., Christine M. McIntosh, Anita S. Chong, and Maria-Luisa Alegre. 2019. "The pursuit of transplantation tolerance: new mechanistic insights." *Cellular & Molecular Immunology* 16 (4):324-333. doi: 10.1038/s41423-019-0203-7.

Gurley, K. E., R. P. Lowry, and R. D. Forbes. 1983. "Immune mechanisms in organ allograft rejection. II. T helper cells, delayed-type hypersensitivity, and rejection of renal allografts." *Transplantation* 36 (4):401-5.

Gutcher, Ilona, Moses K. Donkor, Qian Ma, Alexander Y. Rudensky, Richard A. Flavell, and Ming O. Li. 2011. "Autocrine transforming growth factor-β1 promotes in vivo Th17 cell differentiation." *Immunity* 34 (3):396-408. doi: 10.1016/j.immuni.2011.03.005.

Gyurkocza, Boglarka, and Brenda M. Sandmaier. 2014. "Conditioning regimens for hematopoietic cell transplantation: one size does not fit all." *Blood* 124 (3):344-353. doi: 10.1182/blood-2014-02-514778.

Hancock, Wayne W, Mohamed H Sayegh, Xiang-Guang Zheng, Robert Peach, Peter S Linsley, and Laurence A Turka. 1996. "Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection." *Proceedings of the National Academy of Sciences* 93 (24):13967. doi: 10.1073/pnas.93.24.13967.

Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. "CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones." *Nature* 356 (6370):607-9. doi: 10.1038/356607a0.

Haririan, A., J. Nogueira, D. Kukuruga, E. Schweitzer, J. Hess, C. Gurk-Turner, S. Jacobs, C. Drachenberg, S. Bartlett, and M. Cooper. 2009. "Positive cross-match living donor kidney transplantation: longer-term outcomes." *Am J Transplant* 9 (3):536-42. doi: 10.1111/j.1600-6143.2008.02524.x.

Hart, A., J. M. Smith, M. A. Skeans, S. K. Gustafson, D. E. Stewart, W. S. Cherikh, J. L. Wainright, A. Kucheryavaya, M. Woodbury, J. J. Snyder, B. L. Kasiske, and A. K. Israni. 2017. "OPTN/SRTR 2015 Annual Data Report: Kidney." *American Journal of Transplantation* 17 (S1):21-116. doi: 10.1111/ajt.14124.

Hata, Akiko, and Ye-Guang Chen. 2016. "TGF-β Signaling from Receptors to Smads." *Cold Spring Harbor Perspectives in Biology* 8 (9).

Haynes, L. D., E. Jankowska-Gan, A. Sheka, M. R. Keller, M. P. Hernandez-Fuentes, R. I. Lechler, V. Seyfert-Margolis, L. A. Turka, K. A. Newell, and W. J. Burlingham. 2012. "Donor-specific indirect pathway analysis reveals a B-cell-independent signature which reflects outcomes in kidney transplant recipients." *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 12 (3):640-648. doi: 10.1111/j.1600-6143.2011.03869.x.

Heidt, Sebastiaan, and Kathryn J. Wood. 2012. "BIOMARKERS OF OPERATIONAL TOLERANCE IN SOLID ORGAN TRANSPLANTATION." *Expert opinion on medical diagnostics* 6 (4):281-293. doi: 10.1517/17530059.2012.680019.

Helg, C., B. Chapuis, J. F. Bolle, P. Morel, D. Salomon, E. Roux, V. Antonioli, M. Jeannet, and M. Leski. 1994. "Renal transplantation without immunosuppression in a host with tolerance induced by allogeneic bone marrow transplantation." *Transplantation* 58 (12):1420-2.

Hesse, M. D., A. Y. Karulin, B. O. Boehm, P. V. Lehmann, and M. Tary-Lehmann. 2001. "A T cell clone's avidity is a function of its activation state." *J Immunol* 167 (3):1353-61. doi: 10.4049/jimmunol.167.3.1353.

Holst, Jeff, Andrea L. Szymczak-Workman, Kate M. Vignali, Amanda R. Burton, Creg J. Workman, and Dario A. A. Vignali. 2006. "Generation of T-cell receptor retrogenic mice." *Nature Protocols* 1 (1):406-417. doi: 10.1038/nprot.2006.61.

Honjo, K., X. Yan Xu, J. A. Kapp, and R. P. Bucy. 2004. "Evidence for cooperativity in the rejection of cardiac grafts mediated by CD4 TCR Tg T cells specific for a defined allopeptide." *Am J Transplant* 4 (11):1762-8. doi: 10.1046/j.1600-6143.2004.00596.x.

Honjo, Kazuhito, Xiao yan Xu, and R. Pat Bucy. 2000. "HETEROGENEITY OF T CELL CLONES SPECIFIC FOR A SINGLE INDIRECT ALLOANTIGENIC EPITOPE (I-Ab/H-2Kd54–68) THAT MEDIATE TRANSPLANT REJECTION1." *Transplantation* 70 (10).

Huang, Jun, Xun Zeng, Natalia Sigal, Peder J. Lund, Laura F. Su, Huang Huang, Yueh-hsiu Chien, and Mark M. Davis. 2016. "Detection, phenotyping, and quantification of antigen-specific T cells using a peptide-MHC dodecamer." *Proceedings of the National Academy of Sciences* 113 (13):E1890. doi: 10.1073/pnas.1602488113.

Husain, Shahid, Simona Kahane, Maureen G. Friedman, David L. Paterson, Sean Studer, Kenneth R. McCurry, Dana G. Wolf, Adriana Zeevi, Joseph Pilewski, and David Greenberg. 2007. "Simkania Negevensis in Bronchoalveolar Lavage of Lung Transplant Recipients: A Possible Association with Acute Rejection." *Transplantation* 83 (2).

Huse, Susan M., Les Dethlefsen, Julie A. Huber, David Mark Welch, David A. Relman, and Mitchell L. Sogin. 2008. "Exploring Microbial Diversity and Taxonomy Using SSU rRNA Hypervariable Tag Sequencing." *PLOS Genetics* 4 (11):e1000255. doi: 10.1371/journal.pgen.1000255.

Iida, Noriho, Amiran Dzutsev, C. Andrew Stewart, Loretta Smith, Nicolas Bouladoux, Rebecca A. Weingarten, Daniel A. Molina, Rosalba Salcedo, Timothy Back, Sarah Cramer, Ren-Ming Dai, Hiu Kiu, Marco Cardone, Shruti Naik, Anil K. Patri, Ena Wang, Francesco M. Marincola, Karen M. Frank, Yasmine Belkaid, Giorgio Trinchieri, and Romina S. Goldszmid. 2013. "Commensal Bacteria Control Cancer Response to Therapy by Modulating the Tumor Microenvironment." *Science* 342 (6161):967. doi: 10.1126/science.1240527.

Ioannidou, K., P. Baumgaertner, P. O. Gannon, M. F. Speiser, M. Allard, M. Hebeisen, N. Rufer, and D. E. Speiser. 2017. "Heterogeneity assessment of functional T cell avidity." *Sci Rep* 7:44320. doi: 10.1038/srep44320.

Ishido, Nobuhiro, Junji Matsuoka, Tsuyoshi Matsuno, Kazuhiko Nakagawa, and Noriaki Tanaka. 1999. "INDUCTION OF DONOR-SPECIFIC HYPORESPONSIVENESS AND PROLONGATION OF CARDIAC ALLOGRAFT SURVIVAL BY JEJUNAL ADMINISTRATION OF DONOR SPLENOCYTES." *Transplantation* 68 (9).

Ivanov, Ivaylo I., Koji Atarashi, Nicolas Manel, Eoin L. Brodie, Tatsuichiro Shima, Ulas Karaoz, Dongguang Wei, Katherine C. Goldfarb, Clark A. Santee, Susan V. Lynch, Takeshi Tanoue, Akemi Imaoka, Kikuji Itoh, Kiyoshi Takeda, Yoshinori Umesaki, Kenya Honda, and Dan R. Littman. 2009. "Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria." *Cell* 139 (3):485-498. doi: 10.1016/j.cell.2009.09.033.

Jacobsen, N., E. Taaning, J. Ladefoged, J. K. Kristensen, and F. K. Pedersen. 1994. "Tolerance to an HLA-B,DR disparate kidney allograft after bone-marrow transplantation from same donor." In *Lancet*, 800. England.

Jenkins, M. K., and R. H. Schwartz. 1987. "Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo." *Journal of Experimental Medicine* 165 (2):302-319. doi: 10.1084/jem.165.2.302.

Jiang, X., W. Sun, D. Guo, Z. Cui, L. Zhu, L. Lin, Y. Tang, X. Wang, and J. Liang. 2011. "Cardiac allograft acceptance induced by blockade of CD40-CD40L costimulation is dependent on CD4+CD25+ regulatory T cells." *Surgery* 149 (3):336-46. doi: 10.1016/j.surg.2010.08.012.

Jones, N. D., N. C. Fluck, A. L. Mellor, P. J. Morris, and K. J. Wood. 1998. "The induction of transplantation tolerance by intrathymic (i.t.) delivery of alloantigen: a critical relationship between i.t. deletion, thymic export of new T cells and the timing of transplantation." *International Immunology* 10 (11):1637-1646. doi: 10.1093/intimm/10.11.1637.

Josefowicz, Steven Z., Rachel E. Niec, Hye Young Kim, Piper Treuting, Takatoshi Chinen, Ye Zheng, Dale T. Umetsu, and Alexander Y. Rudensky. 2012. "Extrathymically generated regulatory T cells control mucosal TH2 inflammation." *Nature* 482 (7385):395-399. doi: 10.1038/nature10772.

Kalekar, Lokesh A., Jarish N. Cohen, Nicolas Prevel, Priscila Muñoz Sandoval, Anubhav N. Mathur, Joshua M. Moreau, Margaret M. Lowe, Audrey Nosbaum, Paul J. Wolters, Anna Haemel, Francesco Boin, and Michael D. Rosenblum. 2019. "Regulatory T cells in skin are uniquely poised to suppress profibrotic immune responses." *Science Immunology* 4 (39):eaaw2910. doi: 10.1126/sciimmunol.aaw2910.

Kalekar, Lokesh A., Shirdi E. Schmiel, Sarada L. Nandiwada, Wing Y. Lam, Laura O. Barsness, Na Zhang, Gretta L. Stritesky, Deepali Malhotra, Kristen E. Pauken, Jonathan L. Linehan, M. Gerard O'Sullivan, Brian T. Fife, Kristin A. Hogquist, Marc K. Jenkins, and Daniel L. Mueller. 2016. "CD4(+) T cell anergy prevents autoimmunity and generates regulatory T cell precursors." *Nature immunology* 17 (3):304-314. doi: 10.1038/ni.3331.

Kamada, Takahiro, Yosuke Togashi, Christopher Tay, Danbee Ha, Akinori Sasaki, Yoshiaki Nakamura, Eiichi Sato, Shota Fukuoka, Yasuko Tada, Atsushi Tanaka, Hiromasa Morikawa, Akihito Kawazoe, Takahiro Kinoshita, Kohei Shitara, Shimon Sakaguchi, and Hiroyoshi Nishikawa. 2019. "PD-1⁺ regulatory T cells amplified by PD-1 blockade promote hyperprogression of cancer." *Proceedings of the National Academy of Sciences* 116 (20):9999. doi: 10.1073/pnas.1822001116.

Kamei, H., S. Masuda, T. Nakamura, F. Oike, Y. Takada, and N. Hamajima. 2013. "Association of transporter associated with antigen processing (TAP) gene polymorphisms in donors with acute cellular rejection in living donor liver transplantation." *J Gastrointestin Liver Dis* 22 (2):167-71.

Kamei, Hideya, Satohiro Masuda, Masatoshi Ishigami, Taro Nakamura, Yasuhiro Fujimoto, Yasutsugu Takada, and Nobuyuki Hamajima. 2016. "Association of interleukin4 gene polymorphisms of recipients and donors with acute rejection following living donor liver transplantation." *Clinics and Research in Hepatology and Gastroenterology* 40 (2):179-185. doi: https://doi.org/10.1016/j.clinre.2015.06.019.

Karahan, Gonca E., Frans H. J. Claas, and Sebastiaan Heidt. 2017. "B Cell Immunity in Solid Organ Transplantation." *Frontiers in immunology* 7:686-686. doi: 10.3389/fimmu.2016.00686.

Kasiske, Bertram L. 2001. "EPIDEMIOLOGY OF CARDIOVASCULAR DISEASE AFTER RENAL TRANSPLANTATION." *Transplantation* 72 (6).

Kawai, T., D. H. Sachs, B. Sprangers, T. R. Spitzer, S. L. Saidman, E. Zorn, N. Tolkoff-Rubin, F. Preffer, K. Crisalli, B. Gao, W. Wong, H. Morris, S. A. LoCascio, P. Sayre, B. Shonts, W. W. Williams Jr, R. N. Smith, R. B. Colvin, M. Sykes, and A. B. Cosimi. 2014. "Long-Term Results in Recipients of Combined HLA-Mismatched Kidney and Bone Marrow Transplantation Without Maintenance Immunosuppression." *American Journal of Transplantation* 14 (7):1599-1611. doi: 10.1111/ajt.12731.

Kawai, Tatsuo, David Andrews, Robert B. Colvin, David H. Sachs, and A. Benedict Cosimi. 2000. "Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand." *Nature Medicine* 6:114. doi: 10.1038/72162.

Kawai, Tatsuo, A. Benedict Cosimi, Thomas R. Spitzer, Nina Tolkoff-Rubin, Manikkam Suthanthiran, Susan L. Saidman, Juanita Shaffer, Frederic I. Preffer, Ruchuang Ding, Vijay Sharma, Jay A. Fishman, Bimalangshu Dey, Dicken S. C. Ko, Martin Hertl, Nelson B. Goes, Waichi Wong, Winfred W. Williams, Robert B. Colvin, Megan Sykes, and David H. Sachs. 2008. "HLA-Mismatched Renal Transplantation without Maintenance Immunosuppression." *New England Journal of Medicine* 358 (4):353-361. doi: 10.1056/NEJMoa071074.

Kellersmann, R, and R Zhong. 1998. "Surgical technique for skin transplantation in mice. ." In *Organ transplantation in Rats and Mice: Microsurgical Techniques and Immunological Principles*, edited by W Timmermann, H-J Gassel, K Ulrichs, R Zhong and A Thiede, 151-4. Berlin, Heidelberg: Springer Berlin Heidelberg.

Kenyon, N. S., M. Chatzipetrou, M. Masetti, A. Ranuncoli, M. Oliveira, J. L. Wagner, A. D. Kirk, D. M. Harlan, L. C. Burkly, and C. Ricordi. 1999. "Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154." *Proc Natl Acad Sci U S A* 96 (14):8132-7.

Kenyon, N. S., L. A. Fernandez, R. Lehmann, M. Masetti, A. Ranuncoli, M. Chatzipetrou, G. Iaria, D. Han, J. L. Wagner, P. Ruiz, M. Berho, L. Inverardi, R. Alejandro, D. H. Mintz, A. D. Kirk, D. M. Harlan, L. C. Burkly, and C. Ricordi. 1999. "Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154." *Diabetes* 48 (7):1473-81.

Kerdiles, Yann M., Erica L. Stone, Daniel R. Beisner, Maureen A. McGargill, Irene L. Ch'en, Christian Stockmann, Carol D. Katayama, and Stephen M. Hedrick. 2010. "Foxo transcription factors control regulatory T cell development and function." *Immunity* 33 (6):890-904. doi: 10.1016/j.immuni.2010.12.002.

Khan, O., J. R. Giles, S. McDonald, S. Manne, S. F. Ngiow, K. P. Patel, M. T. Werner, A. C. Huang, K. A. Alexander, J. E. Wu, J. Attanasio, P. Yan, S. M. George, B. Bengsch, R. P. Staupe, G. Donahue, W. Xu, R. K. Amaravadi, X. Xu, G. C. Karakousis, T. C. Mitchell, L. M. Schuchter, J. Kaye, S. L. Berger, and E. J. Wherry. 2019. "TOX transcriptionally and epigenetically programs CD8(+) T cell exhaustion." *Nature* 571 (7764):211-218. doi: 10.1038/s41586-019-1325-x.

Khiew, Stella H. W., Dharmendra Jain, Jianjun Chen, Jinghui Yang, Dengping Yin, James S. Young, Alexander Dent, Roger Sciammas, Maria-Luisa Alegre, and Anita S. Chong. 2020. "Transplantation tolerance modifies donor-specific B cell fate to suppress de novo alloreactive B cells." *The Journal of Clinical Investigation* 130 (7). doi: 10.1172/JCI132814.

Kim, Jeong M., Jeffrey P. Rasmussen, and Alexander Y. Rudensky. 2007. "Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice." *Nature Immunology* 8 (2):191-197. doi: 10.1038/ni1428.

Kim, W. R., J. R. Lake, J. M. Smith, M. A. Skeans, D. P. Schladt, E. B. Edwards, A. M. Harper, J. L. Wainright, J. J. Snyder, A. K. Israni, and B. L. Kasiske. 2017. "OPTN/SRTR 2015 Annual Data Report: Liver." *American Journal of Transplantation* 17 (S1):174-251. doi: 10.1111/ajt.14126.

Kirk, Allan D., Linda C. Burkly, D. Scott Batty, Roxanne E. Baumgartner, Justin D. Berning, Kelvin Buchanan, John H. Fechner Jr, Rhonda L. Germond, Robert L. Kampen, Noelle B. Patterson, S. John Swanson, Douglas K. Tadaki, Christopher N. TenHoor, Leonard White, Stuart J. Knechtle, and David M. Harlan. 1999. "Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates." *Nature Medicine* 5:686. doi: 10.1038/9536.

Kishimoto, K., X. Yuan, H. Auchincloss, Jr., A. H. Sharpe, D. A. Mandelbrot, and M. H. Sayegh. 2004. "Mechanism of action of donor-specific transfusion in inducing tolerance: role of donor MHC molecules, donor co-stimulatory molecules, and indirect antigen presentation." *J Am Soc Nephrol* 15 (9):2423-8. doi: 10.1097/01.asn.0000137883.20961.2d.

Kitagawa, Y., N. Ohkura, Y. Kidani, A. Vandenbon, K. Hirota, R. Kawakami, K. Yasuda, D. Motooka, S. Nakamura, M. Kondo, I. Taniuchi, T. Kohwi-Shigematsu, and S. Sakaguchi.

2017. "Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment." *Nat Immunol* 18 (2):173-183. doi: 10.1038/ni.3646.

Koch, Meghan A., Glady's Tucker-Heard, Nikole R. Perdue, Justin R. Killebrew, Kevin B. Urdahl, and Daniel J. Campbell. 2009. "The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation." *Nature Immunology* 10 (6):595-602. doi: 10.1038/ni.1731.

Koenecke, Christian, Niklas Czeloth, Anja Bubke, Susanne Schmitz, Adrien Kissenpfennig, Bernard Malissen, Jochen Huehn, Arnold Ganser, Reinhold Förster, and Immo Prinz. 2009. "Alloantigen-specific de novo-induced Foxp3+ Treg revert in vivo and do not protect from experimental GVHD." *European Journal of Immunology* 39 (11):3091-3096. doi: 10.1002/eji.200939432.

Koenig, Jeremy E., Aymé Spor, Nicholas Scalfone, Ashwana D. Fricker, Jesse Stombaugh, Rob Knight, Largus T. Angenent, and Ruth E. Ley. 2011. "Succession of microbial consortia in the developing infant gut microbiome." *Proceedings of the National Academy of Sciences* 108 (Supplement 1):4578. doi: 10.1073/pnas.1000081107.

Koo, Eun Hee, Hye Ryoun Jang, Jung Eun Lee, Jae Berm Park, Sung-Joo Kim, Dae Joong Kim, Yoon-Goo Kim, Ha Young Oh, and Wooseong Huh. 2015. "The impact of early and late acute rejection on graft survival in renal transplantation." *Kidney Research and Clinical Practice* 34 (3):160-164. doi: https://doi.org/10.1016/j.krcp.2015.06.003.

Kovarik, Pavel, Virginia Castiglia, Masa Ivin, and Florian Ebner. 2016. "Type I Interferons in Bacterial Infections: A Balancing Act." *Frontiers in immunology* 7:652-652. doi: 10.3389/fimmu.2016.00652.

Kreslavsky, Taras, Hye-Jung Kim, Sergei B. Koralov, Dvora Ghitza, Thorsten Buch, Harvey Cantor, Klaus Rajewsky, and Harald von Boehmer. 2013. "Negative selection, not receptor editing, is a physiological response of autoreactive thymocytes." *Journal of Experimental Medicine* 210 (10):1911-1918. doi: 10.1084/jem.20130876.

Krieger, N. R., D. P. Yin, and C. G. Fathman. 1996. "CD4+ but not CD8+ cells are essential for allorejection." *Journal of Experimental Medicine* 184 (5):2013-2018. doi: 10.1084/jem.184.5.2013.

Krishnamoorthy, V., S. Kannanganat, M. Maienschein-Cline, S. L. Cook, J. Chen, N. Bahroos, E. Sievert, E. Corse, A. Chong, and R. Sciammas. 2017. "The IRF4 Gene Regulatory Module Functions as a Read-Write Integrator to Dynamically Coordinate T Helper Cell Fate." *Immunity* 47 (3):481-497.e7. doi: 10.1016/j.immuni.2017.09.001.

Krogsgaard, Michelle, Nelida Prado, Erin J. Adams, Xiao-lin He, Dar-Chone Chow, Darcy B. Wilson, K. Christopher Garcia, and Mark M. Davis. 2003. "Evidence that Structural Rearrangements and/or Flexibility during TCR Binding Can Contribute to T Cell Activation." *Molecular Cell* 12 (6):1367-1378. doi: 10.1016/S1097-2765(03)00474-X.

Kuball, J., B. Hauptrock, V. Malina, E. Antunes, R. H. Voss, M. Wolfl, R. Strong, M. Theobald, and P. D. Greenberg. 2009. "Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain." *J Exp Med* 206 (2):463-75. doi: 10.1084/jem.20082487.

Kurobe, Hirotsugu, Cunlan Liu, Tomoo Ueno, Fumi Saito, Izumi Ohigashi, Natalie Seach, Rieko Arakaki, Yoshio Hayashi, Tetsuya Kitagawa, Martin Lipp, Richard L. Boyd, and Yousuke Takahama. 2006. "CCR7-Dependent Cortex-to-Medulla Migration of Positively Selected Thymocytes Is Essential for Establishing Central Tolerance." *Immunity* 24 (2):165-177. doi: 10.1016/j.immuni.2005.12.011.

Kühn, Ralf, Jürgen Löhler, Donna Rennick, Klaus Rajewsky, and Werner Müller. 1993. "Interleukin-10-deficient mice develop chronic enterocolitis." *Cell* 75 (2):263-274. doi: https://doi.org/10.1016/0092-8674(93)80068-P.

Labrecque, N., L. S. Whitfield, R. Obst, C. Waltzinger, C. Benoist, and D. Mathis. 2001. "How much TCR does a T cell need?" *Immunity* 15 (1):71-82. doi: 10.1016/s1074-7613(01)00170-4.

Laidlaw, Brian J., Joseph E. Craft, and Susan M. Kaech. 2016. "The multifaceted role of CD4+ T cells in CD8+ T cell memory." *Nature Reviews Immunology* 16 (2):102-111. doi: 10.1038/nri.2015.10.

Lapierre, L. A., W. Fiers, and J. S. Pober. 1988. "Three distinct classes of regulatory cytokines control endothelial cell MHC antigen expression. Interactions with immune gamma interferon differentiate the effects of tumor necrosis factor and lymphotoxin from those of leukocyte alpha and fibroblast beta interferons." *The Journal of experimental medicine* 167 (3):794-804. doi: 10.1084/jem.167.3.794.

Larsen, C. P., E. T. Elwood, D. Z. Alexander, S. C. Ritchie, R. Hendrix, C. Tucker-Burden, H. R. Cho, A. Aruffo, D. Hollenbaugh, P. S. Linsley, K. J. Winn, and T. C. Pearson. 1996. "Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways." *Nature* 381 (6581):434-8. doi: 10.1038/381434a0.

Lei, Yuk Man, Luqiu Chen, Ying Wang, Andrew T. Stefka, Luciana L. Molinero, Betty Theriault, Keston Aquino-Michaels, Ayelet S. Sivan, Cathryn R. Nagler, Thomas F. Gajewski, Anita S. Chong, Caroline Bartman, and Maria-Luisa Alegre. 2016. "The composition of the microbiota modulates allograft rejection." *The Journal of Clinical Investigation* 126 (7):2736-2744. doi: 10.1172/JCI85295.

Leventhal, Joseph, Michael Abecassis, Joshua Miller, Lorenzo Gallon, Kadiyala Ravindra, David J. Tollerud, Bradley King, Mary Jane Elliott, Geoffrey Herzig, Roger Herzig, and Suzanne T. Ildstad. 2012. "Chimerism and Tolerance Without GVHD or Engraftment Syndrome in HLA-Mismatched Combined Kidney and Hematopoietic Stem Cell Transplantation." *Science Translational Medicine* 4 (124):124ra28. doi: 10.1126/scitranslmed.3003509.

Leventhal, Joseph, Michael Abecassis, Joshua Miller, Lorenzo Gallon, David Tollerud, Mary Jane Elliott, Larry D. Bozulic, Christopher Houston, Nedjema Sustento-Reodica, and Suzanne T. Ildstad. 2013. "Tolerance Induction in HLA Disparate Living Donor Kidney Transplantation by Donor Stem Cell Infusion: Durable Chimerism Predicts Outcome." *Transplantation* 95 (1).

Levitsky, Josh, and Sandy Feng. 2018. "Tolerance in clinical liver transplantation." *Human Immunology* 79 (5):283-287. doi: https://doi.org/10.1016/j.humimm.2017.10.007.

Li, Ming O., Yisong Y. Wan, and Richard A. Flavell. 2007. "T Cell-Produced Transforming Growth Factor-β1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation." *Immunity* 26 (5):579-591. doi: https://doi.org/10.1016/j.immuni.2007.03.014.

Li, Xingrui, Efterpi Kostareli, Janine Suffner, Natalio Garbi, and Günter J. Hämmerling. 2010. "Efficient Treg depletion induces T-cell infiltration and rejection of large tumors." *European Journal of Immunology* 40 (12):3325-3335. doi: 10.1002/eji.201041093.

Li, Yijin, Lianli Ma, Dengping Yin, JiKun Shen, and Anita S. Chong. 2008. "Long-Term Control of Alloreactive B Cell Responses by the Suppression of T Cell Help." *The Journal of Immunology* 180 (9):6077. doi: 10.4049/jimmunol.180.9.6077.

Lian, R. H., G. J. Kotwal, S. R. Wellhausen, L. A. Hunt, and D. E. Justus. 1996. "IFN-gamma-induced MHC class II gene expression is suppressed in endothelial cells by dextran sulfate." *The Journal of Immunology* 157 (2):864.

Lin, Hua, Jeffrey C. Rathmell, Gary S. Gray, Craig B. Thompson, Jeffrey M. Leiden, and Maria-Luisa Alegre. 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." *The Journal of Experimental Medicine* 188 (1):199.

Linsley, P. S., J. Bradshaw, M. Urnes, L. Grosmaire, and J. A. Ledbetter. 1993. "CD28 engagement by B7/BB-1 induces transient down-regulation of CD28 synthesis and prolonged unresponsiveness to CD28 signaling." *The Journal of Immunology* 150 (8):3161.

Linsley, P. S., P. M. Wallace, J. Johnson, M. G. Gibson, J. L. Greene, J. A. Ledbetter, C. Singh, and M. A. Tepper. 1992. "Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule." *Science* 257 (5071):792-5. doi: 10.1126/science.1496399.

Linterman, Michelle A., Wim Pierson, Sau K. Lee, Axel Kallies, Shimpei Kawamoto, Tim F. Rayner, Monika Srivastava, Devina P. Divekar, Laura Beaton, Jennifer J. Hogan, Sidonia Fagarasan, Adrian Liston, Kenneth G. C. Smith, and Carola G. Vinuesa. 2011. "Foxp3+ follicular regulatory T cells control the germinal center response." *Nature Medicine* 17 (8):975-982. doi: 10.1038/nm.2425.

Lissina, A., K. Ladell, A. Skowera, M. Clement, E. Edwards, R. Seggewiss, H. A. van den Berg, E. Gostick, K. Gallagher, E. Jones, J. J. Melenhorst, A. J. Godkin, M. Peakman, D. A.

Price, A. K. Sewell, and L. Wooldridge. 2009. "Protein kinase inhibitors substantially improve the physical detection of T-cells with peptide-MHC tetramers." *J Immunol Methods* 340 (1):11-24. doi: 10.1016/j.jim.2008.09.014.

Lowe, M., I. R. Badell, P. Thompson, B. Martin, F. Leopardi, E. Strobert, A. A. Price, H. S. Abdulkerim, R. Wang, N. N. Iwakoshi, A. B. Adams, A. D. Kirk, C. P. Larsen, and K. A. Reimann. 2012. "A novel monoclonal antibody to CD40 prolongs islet allograft survival." *Am J Transplant* 12 (8):2079-87. doi: 10.1111/j.1600-6143.2012.04054.x.

Lumeng, Carey N., Jennifer L. Bodzin, and Alan R. Saltiel. 2007. "Obesity induces a phenotypic switch in adipose tissue macrophage polarization." *The Journal of Clinical Investigation* 117 (1):175-184. doi: 10.1172/JCI29881.

MacDonald, Katherine G., Romy E. Hoeppli, Qing Huang, Jana Gillies, Dan S. Luciani, Paul C. Orban, Raewyn Broady, and Megan K. Levings. 2016. "Alloantigen-specific regulatory T cells generated with a chimeric antigen receptor." *The Journal of Clinical Investigation* 126 (4):1413-1424. doi: 10.1172/JCI82771.

Macián, Fernando, Francisco García-Cózar, Sin-Hyeog Im, Heidi F. Horton, Michael C. Byrne, and Anjana Rao. 2002. "Transcriptional Mechanisms Underlying Lymphocyte Tolerance." *Cell* 109 (6):719-731. doi: 10.1016/S0092-8674(02)00767-5.

Madariaga, Maria Lucia L., Daniel Kreisel, and Joren C. Madsen. 2015. "Organ-specific differences in achieving tolerance." *Current opinion in organ transplantation* 20 (4):392-399. doi: 10.1097/MOT.0000000000000206.

Maeda, J., M. Nishida, H. Takikawa, H. Yoshida, T. Azuma, M. Yoshida, and Y. Mizushina. 2010. "Inhibitory effects of sulfobacin B on DNA polymerase and inflammation." *Int J Mol Med* 26 (5):751-8. doi: 10.3892/ijmm_00000522.

Malchow, Sven, Daniel S. Leventhal, Saki Nishi, Benjamin I. Fischer, Lynn Shen, Gladell P. Paner, Ayelet S. Amit, Chulho Kang, Jenna E. Geddes, James P. Allison, Nicholas D. Socci, and Peter A. Savage. 2013. "Aire-Dependent Thymic Development of Tumor-Associated Regulatory T Cells." *Science* 339 (6124):1219. doi: 10.1126/science.1233913.

Malchow, Sven, Daniel S Leventhal, Victoria Lee, Saki Nishi, Nicholas D Socci, and Peter A Savage. 2016. "Aire Enforces Immune Tolerance by Directing Autoreactive T Cells into the Regulatory T Cell Lineage." *Immunity* 44 (5):1102-1113. doi: 10.1016/j.immuni.2016.02.009.

Mallone, R., S. A. Kochik, H. Reijonen, B. Carson, S. F. Ziegler, W. W. Kwok, and G. T. Nepom. 2005. "Functional avidity directs T-cell fate in autoreactive CD4+ T cells." *Blood* 106 (8):2798-805. doi: 10.1182/blood-2004-12-4848.

Mandegary, Ali, Sara Rahmanian-Koshkaki, Mohammad-Amir Mohammadifar, Leila Pourgholi, Mohammad Mehdipour, Abbas Etminan, Mohammad-Reza Ebadzadeh, Faramarz Fazeli, and Jalal Azmandian. 2015. "Investigation of association between donors' and recipients'

NADPH oxidase p22phox C242T polymorphism and acute rejection, delayed graft function and blood pressure in renal allograft recipients." *Transplant Immunology* 32 (1):46-50. doi: https://doi.org/10.1016/j.trim.2014.08.004.

Marietta, Eric V., Joseph A. Murray, David H. Luckey, Patricio R. Jeraldo, Abhinav Lamba, Robin Patel, Harvinder S. Luthra, Ashutosh Mangalam, and Veena Taneja. 2016. "Suppression of Inflammatory Arthritis by Human Gut-Derived Prevotella histicola in Humanized Mice." *Arthritis & Rheumatology* 68 (12):2878-2888. doi: 10.1002/art.39785.

Markees, T. G., N. E. Phillips, E. J. Gordon, R. J. Noelle, L. D. Shultz, J. P. Mordes, D. L. Greiner, and A. A. Rossini. 1998. "Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires CD4(+) T cells, interferon-gamma, and CTLA4." *The Journal of Clinical Investigation* 101 (11):2446-2455. doi: 10.1172/JCI2703.

Markmann, James F., and Tatsuo Kawai. 2012. "The quest for transplantation tolerance: have we finally sipped from the cup?" *Science translational medicine* 4 (124):124fs5-124fs5. doi: 10.1126/scitranslmed.3003678.

Marodon, Gilles, Sylvain Fisson, Béatrice Levacher, Monique Fabre, Benoît L. Salomon, and David Klatzmann. 2006. "Induction of antigen-specific tolerance by intrathymic injection of lentiviral vectors." *Blood* 108 (9):2972-2978. doi: 10.1182/blood-2006-03-010900.

Marson, Alexander, Karsten Kretschmer, Garrett M. Frampton, Elizabeth S. Jacobsen, Julia K. Polansky, Kenzie D. MacIsaac, Stuart S. Levine, Ernest Fraenkel, Harald von Boehmer, and Richard A. Young. 2007. "Foxp3 occupancy and regulation of key target genes during T-cell stimulation." *Nature* 445 (7130):931-935. doi: 10.1038/nature05478.

Martinez, Ryan J., Na Zhang, Stephanie R. Thomas, Sarada L. Nandiwada, Marc K. Jenkins, Bryce A. Binstadt, and Daniel L. Mueller. 2012. "Arthritogenic self-reactive CD4+ T cells acquire an FR4hiCD73hi anergic state in the presence of Foxp3+ regulatory T cells." *Journal of immunology (Baltimore, Md. : 1950)* 188 (1):170-181. doi: 10.4049/jimmunol.1101311.

Maruhashi, Takumi, Il-mi Okazaki, Daisuke Sugiura, Suzuka Takahashi, Takeo K. Maeda, Kenji Shimizu, and Taku Okazaki. 2018. "LAG-3 inhibits the activation of CD4+ T cells that recognize stable pMHCII through its conformation-dependent recognition of pMHCII." *Nature Immunology* 19 (12):1415-1426. doi: 10.1038/s41590-018-0217-9.

Marín, Eros, Maria Cristina Cuturi, and Aurélie Moreau. 2018. "Tolerogenic Dendritic Cells in Solid Organ Transplantation: Where Do We Stand?" *Frontiers in immunology* 9:274-274. doi: 10.3389/fimmu.2018.00274.

Massagué, Joan. 2012. "TGFβ signalling in context." *Nature Reviews Molecular Cell Biology* 13 (10):616-630. doi: 10.1038/nrm3434.

Massart, Annick, Annaïck Pallier, Julio Pascual, Ondrej Viklicky, Klemens Budde, Goce Spasovski, Marian Klinger, Mehmet Sukru Sever, Søren Schwartz Sørensen, Karine Hadaya, Rainer Oberbauer, Christopher Dudley, Johan W. De Fijter, Alexander Yussim, Marc Hazzan, Thomas Wekerle, David Berglund, Consuelo De Biase, María José Pérez-Sáez, Anja Mühlfeld, Giuseppe Orlando, Katia Clemente, Quirino Lai, Francesco Pisani, Aljosa Kandus, Marije Baas, Frederike Bemelman, Jadranka Buturovic Ponikvar, Hakim Mazouz, Piero Stratta, Jean-François Subra, Florence Villemain, Andries Hoitsma, Laura Braun, Maria Carmen Cantarell, Hulya Colak, Aisling Courtney, Giovanni Maria Frasca, Matthew Howse, Maarten Naesens, Tomas Reischig, Daniel Serón, Nurhan Seyahi, Cem Tugmen, Angel Alonso Hernandez, Luboslav Beňa, Luigi Biancone, Vania Cuna, Carmen Díaz-Corte, Alexandre Dufay, André Gaasbeek, Arnaud Garnier, Philippe Gatault, Miguel Angel Gentil Govantes, François Glowacki, Oliver Gross, Bruno Hurault de Ligny, Uyen Huynh-Do, Bénédicte Janbon, Luis Antonio Jiménez del Cerro, Frieder Keller, Gaetano La Manna, Ricardo Lauzurica, Hervé Le Monies De Sagazan, Friedrich Thaiss, Christophe Legendre, Séverine Martin, Marie-Christine Moal, Christian Noël, Evangeline Pillebout, Gian Benedetto Piredda, Ana Ramírez Puga, Wladyslaw Sulowicz, Serhan Tuglular, Michaela Prokopova, Mélanie Chesneau, Alain Le Moine, Pierrick Guérif, Jean-Paul Soulillou, Marc Abramowicz, Magali Giral, Judith Racapé, Umberto Maggiore, Sophie Brouard, and Daniel Abramowicz. 2016. "The DESCARTES-Nantes survey of kidney transplant recipients displaying clinical operational tolerance identifies 35 new tolerant patients and 34 almost tolerant patients." *Nephrology Dialysis Transplantation* 31 (6):1002-1013. doi: 10.1093/ndt/gfv437.

Masteller, Emma L., Ellen Chuang, Alan C. Mullen, Steve L. Reiner, and Craig B. Thompson. 2000. "Structural Analysis of CTLA-4 Function In Vivo." *The Journal of Immunology* 164 (10):5319. doi: 10.4049/jimmunol.164.10.5319.

Matson, Vyara, Jessica Fessler, Riyue Bao, Tara Chongsuwat, Yuanyuan Zha, Maria-Luisa Alegre, Jason J. Luke, and Thomas F. Gajewski. 2018. "The commensal microbiome is associated with anti–PD-1 efficacy in metastatic melanoma patients." *Science* 359 (6371):104. doi: 10.1126/science.aao3290.

Mayer, Lloyd, and Ling Shao. 2004. "Therapeutic potential of oral tolerance." *Nature Reviews Immunology* 4 (6):407-419. doi: 10.1038/nri1370.

Mazariegos, George V., Jorge Reyes, Ignazio R. Marino, Anthony J. Demetris, Bridget Flynn, William Irish, John McMichael, John J. Fung, and Thomas E. Starzl. 1997. "Weaning of Immunosuppression in Liver Transplant Recipients." *Transplantation* 63 (2):243-249.

McCaughtry , Tom M., Troy A. Baldwin , Matthew S. Wilken , and Kristin A. Hogquist 2008. "Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla." *Journal of Experimental Medicine* 205 (11):2575-2584. doi: 10.1084/jem.20080866.

McDonald, Benjamin D., Jeffrey J. Bunker, Steven A. Erickson, Masatsugu Oh-Hora, and Albert Bendelac. 2015. "Crossreactive αβ T Cell Receptors Are the Predominant Targets of

Thymocyte Negative Selection." *Immunity* 43 (5):859-869. doi: 10.1016/j.immuni.2015.09.009.

McIntosh, Christine M., Luqiu Chen, Alon Shaiber, A. Murat Eren, and Maria-Luisa Alegre. 2018. "Gut microbes contribute to variation in solid organ transplant outcomes in mice." *Microbiome* 6 (1):96. doi: 10.1186/s40168-018-0474-8.

McMurdie, Paul J., and Susan Holmes. 2013. "phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data." *PLOS ONE* 8 (4):e61217. doi: 10.1371/journal.pone.0061217.

McNab, Finlay, Katrin Mayer-Barber, Alan Sher, Andreas Wack, and Anne O'Garra. 2015. "Type I interferons in infectious disease." *Nature Reviews Immunology* 15 (2):87-103. doi: 10.1038/nri3787.

Meng, Xiangbo, Xiwei Liu, Xingdong Guo, Shutan Jiang, Tingting Chen, Zhiqiang Hu, Haifeng Liu, Yibing Bai, Manman Xue, Ronggui Hu, Shao-cong Sun, Xiaolong Liu, Penghui Zhou, Xiaowu Huang, Lai Wei, Wei Yang, and Chenqi Xu. 2018. "FBXO38 mediates PD-1 ubiquitination and regulates anti-tumour immunity of T cells." *Nature* 564 (7734):130-135. doi: 10.1038/s41586-018-0756-0.

Miller, Leslie W. 2002. "Cardiovascular Toxicities of Immunosuppressive Agents." *American Journal of Transplantation* 2 (9):807-818. doi: 10.1034/j.1600-6143.2002.20902.x.

Miller, M. L., M. D. Daniels, T. Wang, J. Chen, J. Young, J. Xu, Y. Wang, D. Yin, V. Vu, A. N. Husain, M. L. Alegre, and A. S. Chong. 2015. "Spontaneous restoration of transplantation tolerance after acute rejection." *Nat Commun* 6:7566. doi: 10.1038/ncomms8566.

Miller, M. L., M. D. Daniels, T. Wang, Y. Wang, J. Xu, D. Yin, A. S. Chong, and M. L. Alegre. 2016a. "Tracking of TCR-Transgenic T Cells Reveals That Multiple Mechanisms Maintain Cardiac Transplant Tolerance in Mice." *Am J Transplant* 16 (10):2854-2864. doi: 10.1111/ajt.13814.

Miller, M. L., M. D. Daniels, T. Wang, Y. Wang, J. Xu, D. Yin, A. S. Chong, and M. L. Alegre. 2016b. "Tracking of TCR-Transgenic T Cells Reveals That Multiple Mechanisms Maintain Cardiac Transplant Tolerance in Mice." *American Journal of Transplantation* 16 (10):2854-2864. doi: 10.1111/ajt.13814.

Miller, M. L., C. M. McIntosh, J. B. Williams, Y. Wang, M. K. Hollinger, N. J. Isaad, J. J. Moon, T. F. Gajewski, A. S. Chong, and M. L. Alegre. 2018. "Distinct Graft-Specific TCR Avidity Profiles during Acute Rejection and Tolerance." *Cell Rep* 24 (8):2112-2126. doi: 10.1016/j.celrep.2018.07.067.

Miller, Michelle L., Christine M. McIntosh, Ying Wang, Luqiu Chen, Peter Wang, Yuk Man Lei, Melvin D. Daniels, Elyse Watkins, Carolina Mora Solano, Anita S. Chong, and Maria-Luisa Alegre. 2019. "Resilience of T cell-intrinsic dysfunction in transplantation

tolerance." *Proceedings of the National Academy of Sciences* 116 (47):23682. doi: 10.1073/pnas.1910298116.

Minguet, S., M. Swamy, B. Alarcón, I. F. Luescher, and W. W. Schamel. 2007. "Full activation of the T cell receptor requires both clustering and conformational changes at CD3." *Immunity* 26 (1):43-54. doi: 10.1016/j.immuni.2006.10.019.

Miyao, Takahisa, Stefan Floess, Ruka Setoguchi, Hervé Luche, Hans Joerg Fehling, Herman Waldmann, Jochen Huehn, and Shohei Hori. 2012. "Plasticity of Foxp3⁺ T Cells Reflects Promiscuous Foxp3 Expression in Conventional T Cells but Not Reprogramming of Regulatory T Cells." *Immunity* 36 (2):262-275. doi: 10.1016/j.immuni.2011.12.012.

Molinero, Luciana L., Dengping Yin, Yuk Man Lei, Luqiu Chen, Ying Wang, Anita S. Chong, and Maria-Luisa Alegre. 2016. "High-Fat Diet–Induced Obesity Enhances Allograft Rejection." *Transplantation* 100 (5).

Moon, James J., Pradyot Dash, Thomas H. Oguin, Jennifer L. McClaren, H. Hamlet Chu, Paul G. Thomas, and Marc K. Jenkins. 2011. "Quantitative impact of thymic selection on Foxp3⁺ and Foxp3[−] subsets of self-peptide/MHC class II-specific CD4⁺ T cells." *Proceedings of the National Academy of Sciences* 108 (35):14602. doi: 10.1073/pnas.1109806108.

Moran, Amy E., Keli L. Holzapfel, Yan Xing, Nicole R. Cunningham, Jonathan S. Maltzman, Jennifer Punt, and Kristin A. Hogquist. 2011. "T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse." *Journal of Experimental Medicine* 208 (6):1279-1289. doi: 10.1084/jem.20110308.

Moreau, Aurélie, Emilie Varey, Ignacio Anegon, and Maria-Cristina Cuturi. 2013. "Effector mechanisms of rejection." *Cold Spring Harbor perspectives in medicine* 3 (11):a015461. doi: 10.1101/cshperspect.a015461.

Mori, Daniel N., Daniel Kreisel, James N. Fullerton, Derek W. Gilroy, and Daniel R. Goldstein. 2014. "Inflammatory triggers of acute rejection of organ allografts." *Immunological Reviews* 258 (1):132-144. doi: 10.1111/imr.12146.

Morris, Heather, Susan DeWolf, Harlan Robins, Ben Sprangers, Samuel a LoCascio, Brittany a Shonts, Tatsuo Kawai, Waichi Wong, Suxiao Yang, Julien Zuber, Yufeng Shen, and Megan Sykes. 2015. "Tracking donor-reactive T cells: Evidence for clonal deletion in tolerant kidney transplant patients." *Sci Transl Med* 7 (272):272ra10-272ra10. doi: 10.1126/scitranslmed.3010760.

Mostoslavsky, Raul, and Frederick W. Alt. 2004. "Receptor revision in T cells: an open question?" *Trends in Immunology* 25 (6):276-279. doi: 10.1016/j.it.2004.04.001.

Naesens, M., D. R. Kuypers, and M. Sarwal. 2009. "Calcineurin inhibitor nephrotoxicity." *Clin J Am Soc Nephrol* 4 (2):481-508. doi: 10.2215/cjn.04800908.

Needham, David M., Rohan Sachdeva, and Jed A. Fuhrman. 2017. "Ecological dynamics and co-occurrence among marine phytoplankton, bacteria and myoviruses shows microdiversity matters." *The ISME Journal* 11 (7):1614-1629. doi: 10.1038/ismej.2017.29.

Nemazee, David. 2017. "Mechanisms of central tolerance for B cells." *Nature reviews. Immunology* 17 (5):281-294. doi: 10.1038/nri.2017.19.

Nikolich-Žugich, Janko, Mark K. Slifka, and Ilhem Messaoudi. 2004. "The many important facets of T-cell repertoire diversity." *Nature Reviews Immunology* 4 (2):123-132. doi: 10.1038/nri1292.

Nish, Simone A., Dominik Schenten, F. Thomas Wunderlich, Scott D. Pope, Yan Gao, Namiko Hoshi, Shuang Yu, Xiting Yan, Heung Kyu Lee, Lesley Pasman, Igor Brodsky, Brian Yordy, Hongyu Zhao, Jens Brüning, and Ruslan Medzhitov. 2014. "T cell-intrinsic role of IL-6 signaling in primary and memory responses." *eLife* 3:e01949. doi: 10.7554/eLife.01949.

Nitta, Takeshi, Sachiko Nitta, Yu Lei, Martin Lipp, and Yousuke Takahama. 2009. "CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens." *Proceedings of the National Academy of Sciences* 106 (40):17129. doi: 10.1073/pnas.0906956106.

Ntokou, Irma-Sofia A., Aliki G. Iniotaki, Elissavet N. Kontou, Maria N. Darema, Maria D. Apostolaki, Alkibiadis G. Kostakis, and John N. Boletis. 2011. "Long-term follow up for anti-HLA donor specific antibodies postrenal transplantation: high immunogenicity of HLA class II graft molecules." *Transplant International* 24 (11):1084-1093. doi: 10.1111/j.1432-2277.2011.01312.x.

Nyström, Sofia N., Dorothée Bourges, Sarah Garry, Ellen M. Ross, Ian R. van Driel, and Paul A. Gleeson. 2014. "Transient Treg-cell depletion in adult mice results in persistent self-reactive CD4+ T-cell responses." *European Journal of Immunology* 44 (12):3621-3631. doi: 10.1002/eji.201344432.

O'Connell, Ryan M., Supriya K. Saha, Sagar A. Vaidya, Kevin W. Bruhn, Gustavo A. Miranda, Brian Zarnegar, Andrea K. Perry, Bidong O. Nguyen, Timothy F. Lane, Tadatsugu Taniguchi, Jeff F. Miller, and Genhong Cheng. 2004. "Type I interferon production enhances susceptibility to Listeria monocytogenes infection." *The Journal of experimental medicine* 200 (4):437-445. doi: 10.1084/jem.20040712.

Ochando, Jordi, Patricia Conde, Alberto Utrero-Rico, and Estela Paz-Artal. 2019. "Tolerogenic Role of Myeloid Suppressor Cells in Organ Transplantation." *Frontiers in immunology* 10:374-374. doi: 10.3389/fimmu.2019.00374.

Ochoa-Repáraz, Javier, Daniel W. Mielcarz, Lauren E. Ditrio, Ashley R. Burroughs, Sakhina Begum-Haque, Suryasarathi Dasgupta, Dennis L. Kasper, and Lloyd H. Kasper. 2010. "Central Nervous System Demyelinating Disease Protection by the Human Commensal Bacteroides fragilis Depends on Polysaccharide A Expression." *The Journal of Immunology* 185 (7):4101. doi: 10.4049/jimmunol.1001443.

Ohnmacht, Caspar, Joo-Hong Park, Sascha Cording, James B. Wing, Koji Atarashi, Yuuki Obata, Valérie Gaboriau-Routhiau, Rute Marques, Sophie Dulauroy, Maria Fedoseeva, Meinrad Busslinger, Nadine Cerf-Bensussan, Ivo G. Boneca, David Voehringer, Koji Hase, Kenya Honda, Shimon Sakaguchi, and Gérard Eberl. 2015. "The microbiota regulates type 2 immunity through RORγt⁺ T cells." *Science* 349 (6251):989. doi: 10.1126/science.aac4263.

Okoye, Isobel S, Stephanie M Coomes, Victoria S Pelly, Stephanie Czieso, Venizelos Papayannopoulos, Tanya Tolmachova, Miguel C Seabra, and Mark S Wilson. 2014. "MicroRNA-Containing T-Regulatory-Cell-Derived Exosomes Suppress Pathogenic T Helper 1 Cells." *Immunity* 41 (1):89-103. doi: 10.1016/j.immuni.2014.05.019.

Olson, Brian, Jeremy Sullivan, and William Burlingham. 2013. "Interleukin 35: A Key Mediator of Suppression and the Propagation of Infectious Tolerance." *Frontiers in Immunology* 4:315.

Orabona, Ciriana, Ursula Grohmann, Maria Laura Belladonna, Francesca Fallarino, Carmine Vacca, Roberta Bianchi, Silvia Bozza, Claudia Volpi, Benoît L. Salomon, Maria Cristina Fioretti, Luigina Romani, and Paolo Puccetti. 2004. "CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86." *Nature Immunology* 5 (11):1134-1142. doi: 10.1038/ni1124.

Orlando, Giuseppe, Peiman Hematti, Robert J. Stratta, George W. Burke, 3rd, Pierpaolo Di Cocco, Francesco Pisani, Shay Soker, and Kathryn Wood. 2010. "Clinical operational tolerance after renal transplantation: current status and future challenges." *Annals of surgery* 252 (6):915-928. doi: 10.1097/SLA.0b013e3181f3efb0.

Owen, Ray D. 1945. "Immunogenetic consequences of vascular anastomoses between bovine twins." *Science* 102 (2651):400. doi: 10.1126/science.102.2651.400.

Ozga, Aleksandra J., Federica Moalli, Jun Abe, Jim Swoger, James Sharpe, Dietmar Zehn, Mario Kreutzfeldt, Doron Merkler, Jorge Ripoll, and Jens V. Stein. 2016. "pMHC affinity controls duration of CD8+ T cell–DC interactions and imprints timing of effector differentiation versus expansion." *Journal of Experimental Medicine* 213 (12):2811-2829. doi: 10.1084/jem.20160206.

Pace, Luigia, Andy Tempez, Catharina Arnold-Schrauf, Fabrice Lemaitre, Philippe Bousso, Luc Fetler, Tim Sparwasser, and Sebastian Amigorena. 2012. "Regulatory T Cells Increase the Avidity of Primary CD8⁺ T Cell Responses and Promote Memory." *Science* 338 (6106):532. doi: 10.1126/science.1227049.

Palmer, Chana, Elisabeth M. Bik, Daniel B. DiGiulio, David A. Relman, and Patrick O. Brown. 2007. "Development of the Human Infant Intestinal Microbiota." *PLOS Biology* 5 (7):e177. doi: 10.1371/journal.pbio.0050177.

Palmer, Scott M., Lauranell H. Burch, R. Duane Davis, Walter F. Herczyk, David N. Howell, Nancy L. Reinsmoen, and David A. Schwartz. 2003. "The Role of Innate Immunity in

Acute Allograft Rejection after Lung Transplantation." *American Journal of Respiratory and Critical Care Medicine* 168 (6):628-632. doi: 10.1164/rccm.200303-447OC.

Pandiyan, Pushpa, Lixin Zheng, Satoru Ishihara, Jennifer Reed, and Michael J. Lenardo. 2007. "CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation–mediated apoptosis of effector CD4+ T cells." *Nature Immunology* 8 (12):1353-1362. doi: 10.1038/ni1536.

Panduro, Marisella, Christophe Benoist, and Diane Mathis. 2016. "Tissue Tregs." *Annual Review of Immunology* 34 (1):609-633. doi: 10.1146/annurev-immunol-032712-095948.

Parker, D. C., D. L. Greiner, N. E. Phillips, M. C. Appel, A. W. Steele, F. H. Durie, R. J. Noelle, J. P. Mordes, and A. A. Rossini. 1995. "Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand." *Proc Natl Acad Sci U S A* 92 (21):9560-4.

Pearson, T. C., D. Z. Alexander, K. J. Winn, P. S. Linsley, R. P. Lowry, and C. P. Larsen. 1994. "Transplantation tolerance induced by CTLA4-Ig." *Transplantation* 57 (12):1701-6.

Pearson, T. C., J. Trambley, K. Odom, D. C. Anderson, S. Cowan, R. Bray, A. Lin, D. Hollenbaugh, A. Aruffo, A. W. Siadak, E. Strobert, R. Hennigar, and C. P. Larsen. 2002. "Anti-CD40 therapy extends renal allograft survival in rhesus macaques." *Transplantation* 74 (7):933-40. doi: 10.1097/01.tp.0000020751.87264.61.

Penaranda, Cristina, Qizhi Tang, and Jeffrey A. Bluestone. 2011. "Anti-CD3 Therapy Promotes Tolerance by Selectively Depleting Pathogenic Cells while Preserving Regulatory T Cells." *The Journal of Immunology* 187 (4):2015. doi: 10.4049/jimmunol.1100713.

Perico, N., M. Rossini, O. Imberti, and G. Remuzzi. 1991. "Thymus-mediated immune tolerance to renal allograft is donor but not tissue specific." *Journal of the American Society of Nephrology* 2 (6):1063.

Persaud, S. P., C. R. Parker, W. L. Lo, K. S. Weber, and P. M. Allen. 2014. "Intrinsic CD4+ T cell sensitivity and response to a pathogen are set and sustained by avidity for thymic and peripheral complexes of self peptide and MHC." *Nat Immunol* 15 (3):266-74. doi: 10.1038/ni.2822.

Peterson, Pärt, Kentaro Nagamine, Hamish Scott, Maarit Heino, Jun Kudoh, Nobuyoshi Shimizu, Stylianos E. Antonarakis, and Kai J. E. Krohn. 1998. "APECED: a monogenic autoimmune disease providing new clues to self-tolerance." *Immunology Today* 19 (9):384-386. doi: https://doi.org/10.1016/S0167-5699(98)01293-6.

Pilon, C. B., S. Petillon, S. Naserian, G. H. Martin, C. Badoual, P. Lang, D. Azoulay, E. Piaggio, P. Grimbert, and J. L. Cohen. 2014. "Administration of Low Doses of IL-2 Combined to Rapamycin Promotes Allogeneic Skin Graft Survival in Mice." *American Journal of Transplantation* 14 (12):2874-2882. doi: 10.1111/ajt.12944.

Pinelli, D. F., M. E. Wagener, D. Liu, A. Yamniuk, J. Tamura, S. Grant, C. P. Larsen, A. Suri, S. G. Nadler, and M. L. Ford. 2013. "An Anti-CD154 Domain Antibody Prolongs Graft

Survival and Induces Foxp3+ iTreg in the Absence and Presence of CTLA-4 Ig." *American Journal of Transplantation* 13 (11):3021-3030. doi: 10.1111/ajt.12417.

Plitas, George, and Alexander Y. Rudensky. 2016. "Regulatory T Cells: Differentiation and Function." *Cancer immunology research* 4 (9):721-725. doi: 10.1158/2326-6066.CIR-16-0193.

Pober, J. S., M. A. Gimbrone, Jr., R. S. Cotran, C. S. Reiss, S. J. Burakoff, W. Fiers, and K. A. Ault. 1983. "Ia expression by vascular endothelium is inducible by activated T cells and by human gamma interferon." *Journal of Experimental Medicine* 157 (4):1339-1353. doi: 10.1084/jem.157.4.1339.

Podestà, Manuel Alfredo, Giuseppe Remuzzi, and Federica Casiraghi. 2019. "Mesenchymal Stromal Cells for Transplant Tolerance." *Frontiers in immunology* 10:1287-1287. doi: 10.3389/fimmu.2019.01287.

Pohar, Jelka, Quentin Simon, and Simon Fillatreau. 2018. "Antigen-Specificity in the Thymic Development and Peripheral Activity of CD4(+)FOXP3(+) T Regulatory Cells." *Frontiers in immunology* 9:1701-1701. doi: 10.3389/fimmu.2018.01701.

Polukort, Stephanie H., Jeffrey Rovatti, Logan Carlson, Chelsea Thompson, Jennifer Ser-Dolansky, Shannon R. M. Kinney, Sallie S. Schneider, and Clinton B. Mathias. 2016. "IL-10 Enhances IgE-Mediated Mast Cell Responses and Is Essential for the Development of Experimental Food Allergy in IL-10–Deficient Mice." *The Journal of Immunology* 196 (12):4865. doi: 10.4049/jimmunol.1600066.

Posselt, A. M., C. F. Barker, J. E. Tomaszewski, J. F. Markmann, M. A. Choti, and A. Naji. 1990. "Induction of donor-specific unresponsiveness by intrathymic islet transplantation." *Science* 249 (4974):1293. doi: 10.1126/science.2119056.

Qin, S., S. P. Cobbold, H. Pope, J. Elliott, D. Kioussis, J. Davies, and H. Waldmann. 1993. ""Infectious" transplantation tolerance." *Science* 259 (5097):974-7. doi: 10.1126/science.8094901.

Quezada, Sergio A., Kathy Bennett, Bruce R. Blazar, Alexander Y. Rudensky, Shimon Sakaguchi, and Randolph J. Noelle. 2005. "Analysis of the Underlying Cellular Mechanisms of Anti-CD154-Induced Graft Tolerance: The Interplay of Clonal Anergy and Immune Regulation." *The Journal of Immunology* 175 (2):771. doi: 10.4049/jimmunol.175.2.771.

Quezada, Sergio A., Bruce Fuller, Lamis Z. Jarvinen, Mercedes Gonzalez, Bruce R. Blazar, Alexander Y. Rudensky, Terry B. Strom, and Randolph J. Noelle. 2003. "Mechanisms of donor-specific transfusion tolerance: preemptive induction of clonal T-cell exhaustion via indirect presentation." *Blood* 102 (5):1920.

Qureshi, Omar S., Yong Zheng, Kyoko Nakamura, Kesley Attridge, Claire Manzotti, Emily M. Schmidt, Jennifer Baker, Louisa E. Jeffery, Satdip Kaur, Zoe Briggs, Tie Z. Hou, Clare E. Futter, Graham Anderson, Lucy S. K. Walker, and David M. Sansom. 2011. "Trans-

Endocytosis of CD80 and CD86: A Molecular Basis for the Cell-Extrinsic Function of CTLA-4." *Science* 332 (6029):600. doi: 10.1126/science.1202947.

Rajbhandari, Prashant, Brandon J. Thomas, An-Chieh Feng, Cynthia Hong, Jiexin Wang, Laurent Vergnes, Tamer Sallam, Bo Wang, Jaspreet Sandhu, Marcus M. Seldin, Aldons J. Lusis, Loren G. Fong, Melanie Katz, Richard Lee, Stephen G. Young, Karen Reue, Stephen T. Smale, and Peter Tontonoz. 2018. "IL-10 Signaling Remodels Adipose Chromatin Architecture to Limit Thermogenesis and Energy Expenditure." *Cell* 172 (1):218-233.e17. doi: 10.1016/j.cell.2017.11.019.

Read, Simon, Rebecca Greenwald, Ana Izcue, Nicholas Robinson, Didier Mandelbrot, Loise Francisco, Arlene H. Sharpe, and Fiona Powrie. 2006. "Blockade of CTLA-4 on CD4⁺CD25⁺ Regulatory T Cells Abrogates Their Function In Vivo." *The Journal of Immunology* 177 (7):4376. doi: 10.4049/jimmunol.177.7.4376.

Reinke, P., J. Lippert, R. Ewert, E. Fietze, S. Ode-Hakim, H. D. Volk, and S. Prösch. 1994. "Late-acute renal allograft rejection and symptomless cytomegalovirus infection." *The Lancet* 344 (8939):1737-1738. doi: 10.1016/S0140-6736(94)92887-8.

Remuzzi, G., P. Ferrazzi, M. Bontempelli, M. Senni, M. Glauber, P. Ruggenenti, and N. Perico. 1995. "Preliminary results of intrathymic injection of donor cells to prevent acute rejection in human heart transplantation." *Journal of the American Society of Nephrology* 6 (4):1291.

Ren, X., F. Ye, Z. Jiang, Y. Chu, S. Xiong, and Y. Wang. 2007. "Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4+CD25+ regulatory T cells." *Cell Death & Differentiation* 14 (12):2076-2084. doi: 10.1038/sj.cdd.4402220.

Richer, M. J., J. C. Nolz, and J. T. Harty. 2013. "Pathogen-specific inflammatory milieux tune the antigen sensitivity of CD8(+) T cells by enhancing T cell receptor signaling." *Immunity* 38 (1):140-52. doi: 10.1016/j.immuni.2012.09.017.

Ridge, John Paul, Francesca Di Rosa, and Polly Matzinger. 1998. "A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell." *Nature* 393 (6684):474-478. doi: 10.1038/30989.

Riella, L. V., T. Liu, J. Yang, S. Chock, T. Shimizu, B. Mfarrej, I. Batal, X. Xiao, M. H. Sayegh, and A. Chandraker. 2012. "Deleterious effect of CTLA4-Ig on a Treg-dependent transplant model." *Am J Transplant* 12 (4):846-55. doi: 10.1111/j.1600-6143.2011.03929.x.

Robbins, Paul D., and Adrian E. Morelli. 2014. "Regulation of immune responses by extracellular vesicles." *Nature reviews. Immunology* 14 (3):195-208. doi: 10.1038/nri3622.

Robles-Carrillo, Liza, Todd Meyer, Meghan Hatfield, Hina Desai, Mónica Dávila, Florian Langer, Mildred Amaya, Ellen Garber, John L. Francis, Yen-Ming Hsu, and Ali Amirkhosravi. 2010. "Anti-CD40L Immune Complexes Potently Activate Platelets In

Vitro and Cause Thrombosis in FCGR2A Transgenic Mice." *The Journal of Immunology* 185 (3):1577.

Rose-John, Stefan, Kevin Winthrop, and Leonard Calabrese. 2017. "The role of IL-6 in host defence against infections: immunobiology and clinical implications." *Nature Reviews Rheumatology* 13 (7):399-409. doi: 10.1038/nrrheum.2017.83.

Rousset, F., E. Garcia, T. Defrance, C. Péronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore, and J. Banchereau. 1992. "Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes." *Proceedings of the National Academy of Sciences* 89 (5):1890. doi: 10.1073/pnas.89.5.1890.

Roussey-Kesler, G., M. Giral, A. Moreau, J. F. Subra, C. Legendre, C. Noël, E. Pillebout, S. Brouard, and J. P. Soulillou. 2006. "Clinical Operational Tolerance after Kidney Transplantation." *American Journal of Transplantation* 6 (4):736-746. doi: 10.1111/j.1600-6143.2006.01280.x.

Rowe, Jared H., James M. Ertelt, Lijun Xin, and Sing Sing Way. 2012. "Listeria monocytogenes Cytoplasmic Entry Induces Fetal Wastage by Disrupting Maternal Foxp3+ Regulatory T Cell-Sustained Fetal Tolerance." *PLOS Pathogens* 8 (8):e1002873. doi: 10.1371/journal.ppat.1002873.

Rubtsov, Yuri P., Rachel E. Niec, Steven Josefowicz, Li Li, Jaime Darce, Diane Mathis, Christophe Benoist, and Alexander Y. Rudensky. 2010. "Stability of the Regulatory T Cell Lineage in Vivo." *Science* 329 (5999):1667. doi: 10.1126/science.1191996.

Rubtsov, Yuri P., Jeffrey P. Rasmussen, Emil Y. Chi, Jason Fontenot, Luca Castelli, Xin Ye, Piper Treuting, Lisa Siewe, Axel Roers, William R. Henderson, Werner Muller, and Alexander Y. Rudensky. 2008. "Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces." *Immunity* 28 (4):546-558. doi: https://doi.org/10.1016/j.immuni.2008.02.017.

Saborio, D. V., N. C. Chowdhury, M. X. Jin, A. Chandraker, M. H. Sayegh, and S. F. Oluwole. 1999. "Regulatory T cells maintain peripheral tolerance to islet allografts induced by intrathymic injection of MHC class I allopeptides." *Cell Transplant* 8 (4):375-81. doi: 10.1177/096368979900800405.

Safa, Kassem, Shunsuke Ohori, Thiago J. Borges, Mayuko Uehara, Ibrahim Batal, Tetsunosuke Shimizu, Ciara N. Magee, Roger Belizaire, Reza Abdi, Chuan Wu, Anil Chandraker, and Leonardo V. Riella. 2015. "Salt Accelerates Allograft Rejection through Serum- and Glucocorticoid-Regulated Kinase-1–Dependent Inhibition of Regulatory T Cells." *Journal of the American Society of Nephrology* 26 (10):2341. doi: 10.1681/ASN.2014090914.

Safinia, Niloufar, Nathali Grageda, Cristiano Scottà, Sarah Thirkell, Laura J. Fry, Trishan Vaikunthanathan, Robert I. Lechler, and Giovanna Lombardi. 2018. "Cell Therapy in Organ Transplantation: Our Experience on the Clinical Translation of Regulatory T Cells." *Frontiers in immunology* 9:354-354. doi: 10.3389/fimmu.2018.00354.

Sage, Peter T., and Arlene H. Sharpe. 2016. "T follicular regulatory cells." *Immunological Reviews* 271 (1):246-259. doi: 10.1111/imr.12411.

Sagoo, Pervinder, Esperanza Perucha, Birgit Sawitzki, Stefan Tomiuk, David A. Stephens, Patrick Miqueu, Stephanie Chapman, Ligia Craciun, Ruhena Sergeant, Sophie Brouard, Flavia Rovis, Elvira Jimenez, Amany Ballow, Magali Giral, Irene Rebollo-Mesa, Alain Le Moine, Cecile Braudeau, Rachel Hilton, Bernhard Gerstmayer, Katarzyna Bourcier, Adnan Sharif, Magdalena Krajewska, Graham M. Lord, Ian Roberts, Michel Goldman, Kathryn J. Wood, Kenneth Newell, Vicki Seyfert-Margolis, Anthony N. Warrens, Uwe Janssen, Hans-Dieter Volk, Jean-Paul Soulillou, Maria P. Hernandez-Fuentes, and Robert I. Lechler. 2010. "Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans." *The Journal of clinical investigation* 120 (6):1848-1861. doi: 10.1172/JCI39922.

Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases." *The Journal of Immunology* 155 (3):1151.

Sanchez-Fueyo, Alberto. 2013. "Tolerance profiles and immunosuppression." *Liver Transplantation* 19 (S2):S44-S48. doi: 10.1002/lt.23749.

Sanjabi, Shomyseh, Soyoung A. Oh, and Ming O. Li. 2017. "Regulation of the Immune Response by TGF-β: From Conception to Autoimmunity and Infection." *Cold Spring Harbor perspectives in biology* 9 (6):a022236. doi: 10.1101/cshperspect.a022236.

Sansom, D. M. 2000. "CD28, CTLA-4 and their ligands: who does what and to whom?" *Immunology* 101 (2):169-177. doi: 10.1046/j.1365-2567.2000.00121.x.

Savage, P. A., J. J. Boniface, and M. M. Davis. 1999a. "A kinetic basis for T cell receptor repertoire selection during an immune response." *Immunity* 10 (4):485-92. doi: 10.1016/s1074-7613(00)80048-5.

Savage, Peter A., J. Jay Boniface, and Mark M. Davis. 1999b. "A Kinetic Basis For T Cell Receptor Repertoire Selection during an Immune Response." *Immunity* 10 (4):485-492. doi: https://doi.org/10.1016/S1074-7613(00)80048-5.

Savage, Thomas M., Brittany A. Shonts, Aleksandar Obradovic, Susan Dewolf, Saiping Lau, Julien Zuber, Michael T. Simpson, Erik Berglund, Jianing Fu, Suxiao Yang, Siu-Hong Ho, Qizhi Tang, Laurence A. Turka, Yufeng Shen, and Megan Sykes. 2018. "Early expansion of donor-specific Tregs in tolerant kidney transplant recipients." *JCI Insight* 3 (22). doi: 10.1172/jci.insight.124086.

Sawant, Deepali V., and Dario A. A. Vignali. 2014. "Once a Treg, always a Treg?" *Immunological reviews* 259 (1):173-191. doi: 10.1111/imr.12173.

Sawant, Deepali V., Hiroshi Yano, Maria Chikina, Qianxia Zhang, Mengting Liao, Chang Liu, Derrick J. Callahan, Zhe Sun, Tao Sun, Tracy Tabib, Arjun Pennathur, David B. Corry,

James D. Luketich, Robert Lafyatis, Wei Chen, Amanda C. Poholek, Tullia C. Bruno, Creg J. Workman, and Dario A. A. Vignali. 2019. "Adaptive plasticity of IL-10+ and IL-35+ Treg cells cooperatively promotes tumor T cell exhaustion." *Nature Immunology* 20 (6):724-735. doi: 10.1038/s41590-019-0346-9.

Sayegh, Mohamed H., Neil A. Fine, John L. Smith, Helmut G. Rennke, Edgar L. Milford, and Nicholas L. Tilney. 1991. "Immunologic Tolerance to Renal Allografts after Bone Marrow Transplants from the Same Donors." *Annals of Internal Medicine* 114 (11):954-955. doi: 10.7326/0003-4819-114-11-954.

Scandling, J. D., S. Busque, J. A. Shizuru, R. Lowsky, R. Hoppe, S. Dejbakhsh-Jones, K. Jensen, A. Shori, J. A. Strober, P. Lavori, B. B. Turnbull, E. G. Engleman, and S. Strober. 2015. "Chimerism, Graft Survival, and Withdrawal of Immunosuppressive Drugs in HLA Matched and Mismatched Patients After Living Donor Kidney and Hematopoietic Cell Transplantation." *American Journal of Transplantation* 15 (3):695-704. doi: 10.1111/ajt.13091.

Scharschmidt, Tiffany C, Kimberly S Vasquez, Hong-An Truong, Sofia V Gearty, Mariela L Pauli, Audrey Nosbaum, Iris K Gratz, Michael Otto, James J Moon, Jan Liese, Abul K Abbas, Michael A Fischbach, and Michael D Rosenblum. 2015. "A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes." *Immunity* 43 (5):1011-1021. doi: 10.1016/j.immuni.2015.10.016.

Scheel, Jennifer Felicia, Katharina Schieber, Sandra Reber, Lisa Stoessel, Elisabeth Waldmann, Sabine Jank, Kai-Uwe Eckardt, Franziska Grundmann, Frank Vitinius, Martina de Zwaan, Anna Bertram, and Yesim Erim. 2018. "Psychosocial Variables Associated with Immunosuppressive Medication Non-Adherence after Renal Transplantation." *Frontiers in Psychiatry* 9:23.

Scheid, Margrit, Edward A. Boyse, Elizabeth A. Carswell, and Lloyd J. Old. 1972. "SEROLOGICALLY DEMONSTRABLE ALLOANTIGENS OF MOUSE EPIDERMAL CELLS." *Journal of Experimental Medicine* 135 (4):938-955. doi: 10.1084/jem.135.4.938.

Scher, Jose U., Andrew Sczesnak, Randy S. Longman, Nicola Segata, Carles Ubeda, Craig Bielski, Tim Rostron, Vincenzo Cerundolo, Eric G. Pamer, Steven B. Abramson, Curtis Huttenhower, and Dan R. Littman. 2013. "Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis." *eLife* 2:e01202. doi: 10.7554/eLife.01202.

Schiering, Chris, Thomas Krausgruber, Agnieszka Chomka, Anja Fröhlich, Krista Adelmann, Elizabeth A. Wohlfert, Johanna Pott, Thibault Griseri, Julia Bollrath, Ahmed N. Hegazy, Oliver J. Harrison, Benjamin M. J. Owens, Max Löhning, Yasmine Belkaid, Padraic G. Fallon, and Fiona Powrie. 2014. "The alarmin IL-33 promotes regulatory T-cell function in the intestine." *Nature* 513 (7519):564-568. doi: 10.1038/nature13577.

Schietinger, Andrea, Jeffrey J. Delrow, Ryan S. Basom, Joseph N. Blattman, and Philip D. Greenberg. 2012. "Rescued Tolerant CD8 T Cells Are Preprogrammed to Reestablish the Tolerant State." *Science* 335 (6069):723. doi: 10.1126/science.1214277.

Schmidt, Angelika, Nina Oberle, and Peter Krammer. 2012. "Molecular Mechanisms of Treg-Mediated T Cell Suppression." *Frontiers in Immunology* 3:51.

Schneider, Helga, and Christopher E. Rudd. 2014. "Diverse Mechanisms Regulate the Surface Expression of Immunotherapeutic Target CTLA-4." *Frontiers in Immunology* 5:619.

Schwartz, R.H., D.L. Mueller, M.K. Jenkins, and H. Quill. 1989. "T-cell Clonal Anergy." *Cold Spring Harbor Symposia on Quantitative Biology* 54:605-610. doi: 10.1101/sqb.1989.054.01.072.

Schwartz, Ronald H. 2003. "T Cell Anergy." *Annual Review of Immunology* 21 (1):305-334. doi: 10.1146/annurev.immunol.21.120601.141110.

Scott, A. C., F. Dundar, P. Zumbo, S. S. Chandran, C. A. Klebanoff, M. Shakiba, P. Trivedi, L. Menocal, H. Appleby, S. Camara, D. Zamarin, T. Walther, A. Snyder, M. R. Femia, E. A. Comen, H. Y. Wen, M. D. Hellmann, N. Anandasabapathy, Y. Liu, N. K. Altorki, P. Lauer, O. Levy, M. S. Glickman, J. Kaye, D. Betel, M. Philip, and A. Schietinger. 2019. "TOX is a critical regulator of tumour-specific T cell differentiation." *Nature* 571 (7764):270-274. doi: 10.1038/s41586-019-1324-y.

Scott, Nicholas A., Yuxing Zhao, Balasubramanian Krishnamurthy, Stuart I. Mannering, Thomas W. H. Kay, and Helen E. Thomas. 2018. "IFNγ-Induced MHC Class II Expression on Islet Endothelial Cells Is an Early Marker of Insulitis but Is Not Required for Diabetogenic CD4+ T Cell Migration." *Frontiers in Immunology* 9:2800.

Sefik, Esen, Naama Geva-Zatorsky, Sungwhan Oh, Liza Konnikova, David Zemmour, Abigail Manson McGuire, Dalia Burzyn, Adriana Ortiz-Lopez, Mercedes Lobera, Jianfei Yang, Shomir Ghosh, Ashlee Earl, Scott B. Snapper, Ray Jupp, Dennis Kasper, Diane Mathis, and Christophe Benoist. 2015. "Individual intestinal symbionts induce a distinct population of RORγ⁺ regulatory T cells." *Science* 349 (6251):993. doi: 10.1126/science.aaa9420.

Sellon, R. K., S. Tonkonogy, M. Schultz, L. A. Dieleman, W. Grenther, E. Balish, D. M. Rennick, and R. B. Sartor. 1998. "Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice." *Infection and immunity* 66 (11):5224-5231.

Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. "Differential T cell costimulatory requirements in CD28-deficient mice." *Science* 261 (5121):609-12. doi: 10.1126/science.7688139.

Shibata, Yoshimi, L. Ann Foster, Masashi Kurimoto, Haruki Okamura, Reiko M. Nakamura, Katsuhide Kawajiri, J. Paul Justice, Michael R. Van Scott, Quentin N. Myrvik, and W.

James Metzger. 1998. "Immunoregulatory Roles of IL-10 in Innate Immunity: IL-10 Inhibits Macrophage Production of IFN-γ-Inducing Factors but Enhances NK Cell Production of IFN-γ." *The Journal of Immunology* 161 (8):4283.

Singh, Kailash, Marcus Hjort, Lina Thorvaldson, and Stellan Sandler. 2015. "Concomitant analysis of Helios and Neuropilin-1 as a marker to detect thymic derived regulatory T cells in naïve mice." *Scientific Reports* 5 (1):7767. doi: 10.1038/srep07767.

Sivan, Ayelet, Leticia Corrales, Nathaniel Hubert, Jason B. Williams, Keston Aquino-Michaels, Zachary M. Earley, Franco W. Benyamin, Yuk Man Lei, Bana Jabri, Maria-Luisa Alegre, Eugene B. Chang, and Thomas F. Gajewski. 2015. "Commensal Bifidobacterium promotes antitumor immunity and facilitates anti–PD-L1 efficacy." *Science* 350 (6264):1084. doi: 10.1126/science.aac4255.

Smith, Karen, Kathy D. McCoy, and Andrew J. Macpherson. 2007. "Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota." *Seminars in Immunology* 19 (2):59-69. doi: https://doi.org/10.1016/j.smim.2006.10.002.

Spangler, Jamie B., Jakub Tomala, Vincent C. Luca, Kevin M. Jude, Shen Dong, Aaron M. Ring, Petra Votavova, Marion Pepper, Marek Kovar, and K. Christopher Garcia. 2015. "Antibodies to Interleukin-2 Elicit Selective T Cell Subset Potentiation through Distinct Conformational Mechanisms." *Immunity* 42 (5):815-825. doi: 10.1016/j.immuni.2015.04.015.

Sprangers, B., S. DeWolf, T. M. Savage, T. Morokata, A. Obradovic, S. A. LoCascio, B. Shonts, J. Zuber, S. P. Lau, R. Shah, H. Morris, V. Steshenko, E. Zorn, F. I. Preffer, S. Olek, D. M. Dombkowski, L. A. Turka, R. Colvin, R. Winchester, T. Kawai, and M. Sykes. 2017. "Origin of Enriched Regulatory T Cells in Patients Receiving Combined Kidney-Bone Marrow Transplantation to Induce Transplantation Tolerance." *Am J Transplant* 17 (8):2020-2032. doi: 10.1111/ajt.14251.

Srivastava, Shivani, Meghan A. Koch, Marion Pepper, and Daniel J. Campbell. 2014. "Type I interferons directly inhibit regulatory T cells to allow optimal antiviral T cell responses during acute LCMV infection." *The Journal of experimental medicine* 211 (5):961-974. doi: 10.1084/jem.20131556.

Stagg, John, Upulie Divisekera, Helene Duret, Tim Sparwasser, Michele W. L. Teng, Phillip K. Darcy, and Mark J. Smyth. 2011. "CD73-Deficient Mice Have Increased Antitumor Immunity and Are Resistant to Experimental Metastasis." *Cancer Research* 71 (8):2892. doi: 10.1158/0008-5472.CAN-10-4246.

Stefka, Andrew T., Taylor Feehley, Prabhanshu Tripathi, Ju Qiu, Kathy McCoy, Sarkis K. Mazmanian, Melissa Y. Tjota, Goo-Young Seo, Severine Cao, Betty R. Theriault, Dionysios A. Antonopoulos, Liang Zhou, Eugene B. Chang, Yang-Xin Fu, and Cathryn R. Nagler. 2014. "Commensal bacteria protect against food allergen sensitization." *Proceedings of the National Academy of Sciences of the United States of America* 111 (36):13145-13150. doi: 10.1073/pnas.1412008111.

Strauss, Laura, Christoph Bergmann, and Theresa L. Whiteside. 2009. "Human circulating CD4+CD25highFoxp3+ regulatory T cells kill autologous CD8+ but not CD4+ responder cells by Fas-mediated apoptosis." *Journal of immunology (Baltimore, Md. : 1950)* 182 (3):1469-1480. doi: 10.4049/jimmunol.182.3.1469.

Suchin, Elliot J., Peter B. Langmuir, Ed Palmer, Mohamed H. Sayegh, Andrew D. Wells, and Laurence A. Turka. 2001. "Quantifying the Frequency of Alloreactive T Cells In Vivo: New Answers to an Old Question." *The Journal of Immunology* 166 (2):973. doi: 10.4049/jimmunol.166.2.973.

Sun, Li-Ying, Yun-Sheng Yang, Wei Qu, Zhi-Jun Zhu, Lin Wei, Zhi-Sheng Ye, Jian-Rui Zhang, Xiao-Ye Sun, and Zhi-Gui Zeng. 2017. "Gut microbiota of liver transplantation recipients." *Scientific Reports* 7 (1):3762. doi: 10.1038/s41598-017-03476-4.

Svendsen, U. G., S. Aggestrup, C. Heilmann, N. Jacobsen, C. Koch, B. Larsen, A. Svejgaard, B. Thisted, and G. Petterson. 1995. "Transplantation of a lobe of lung from mother to child following previous transplantation with maternal bone marrow." *European Respiratory Journal* 8 (2):334.

Szurek, Edyta, Anna Cebula, Lukasz Wojciech, Maciej Pietrzak, Grzegorz Rempala, Pawel Kisielow, and Leszek Ignatowicz. 2015. "Differences in Expression Level of Helios and Neuropilin-1 Do Not Distinguish Thymus-Derived from Extrathymically-Induced CD4+Foxp3+ Regulatory T Cells." *PLOS ONE* 10 (10):e0141161. doi: 10.1371/journal.pone.0141161.

Szymczak-Workman, Andrea L., Creg J. Workman, and Dario A. A. Vignali. 2009. "Cutting Edge: Regulatory T Cells Do Not Require Stimulation through Their TCR to Suppress." *The Journal of Immunology* 182 (9):5188. doi: 10.4049/jimmunol.0803123.

Tahiliani, Vikas, Tarun E. Hutchinson, Georges Abboud, Michael Croft, and Shahram Salek-Ardakani. 2017. "OX40 Cooperates with ICOS To Amplify Follicular Th Cell Development and Germinal Center Reactions during Infection." *The Journal of Immunology* 198 (1):218. doi: 10.4049/jimmunol.1601356.

Takaba, Hiroyuki, Yasuyuki Morishita, Yoshihiko Tomofuji, Lynett Danks, Takeshi Nitta, Noriko Komatsu, Tatsuhiko Kodama, and Hiroshi Takayanagi. 2015. "Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance." *Cell* 163 (4):975-987. doi: 10.1016/j.cell.2015.10.013.

Tang, Qizhi, and Jeffrey A. Bluestone. 2013. "Regulatory T-cell therapy in transplantation: moving to the clinic." *Cold Spring Harbor perspectives in medicine* 3 (11):a015552. doi: 10.1101/cshperspect.a015552.

Tang, Qizhi, Kammi J. Henriksen, Elisa K. Boden, Aaron J. Tooley, Jianqin Ye, Sumit K. Subudhi, Xin X. Zheng, Terry B. Strom, and Jeffrey A. Bluestone. 2003. "Cutting Edge: CD28 Controls Peripheral Homeostasis of CD4⁺CD25⁺ Regulatory T Cells." *The Journal of Immunology* 171 (7):3348. doi: 10.4049/jimmunol.171.7.3348.

Tang, Qizhi, and Flavio Vincenti. 2017. "Transplant trials with Tregs: perils and promises." *The Journal of Clinical Investigation* 127 (7):2505-2512. doi: 10.1172/JCI90598.

Thommen, Daniela S., and Ton N. Schumacher. 2018. "T Cell Dysfunction in Cancer." *Cancer Cell* 33 (4):547-562. doi: 10.1016/j.ccell.2018.03.012.

Thompson-Snipes, L., V. Dhar, M. W. Bond, T. R. Mosmann, K. W. Moore, and D. M. Rennick. 1991. "Interleukin 10: a novel stimulatory factor for mast cells and their progenitors." *Journal of Experimental Medicine* 173 (2):507-510. doi: 10.1084/jem.173.2.507.

Thornley, Thomas B., Nancy E. Phillips, Britte C. Beaudette-Zlatanova, Thomas G. Markees, Kapil Bahl, Michael A. Brehm, Leonard D. Shultz, Evelyn A. Kurt-Jones, John P. Mordes, Raymond M. Welsh, Aldo A. Rossini, and Dale L. Greiner. 2007. "Type 1 IFN Mediates Cross-Talk between Innate and Adaptive Immunity That Abrogates Transplantation Tolerance." *The Journal of Immunology* 179 (10):6620. doi: 10.4049/jimmunol.179.10.6620.

Thude, Hansjörg, Maike Janssen, Martina Sterneck, Björn Nashan, and Martina Koch. 2016. "14-bp ins/del polymorphism and +3142C>G SNP of the HLA-G gene have a significant impact on acute rejection after liver transplantation." *Human Immunology* 77 (12):1159-1165. doi: https://doi.org/10.1016/j.humimm.2016.09.009.

Tivol, Elizabeth A., Frank Borriello, A. Nicola Schweitzer, William P. Lynch, Jeffrey A. Bluestone, and Arlene H. Sharpe. 1995. "Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4." *Immunity* 3 (5):541-547. doi: https://doi.org/10.1016/1074-7613(95)90125-6.

Tomita, Y., A. Khan, and M. Sykes. 1994. "Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow transplantation in mice conditioned with a nonmyeloablative regimen." *J Immunol* 153 (3):1087-98.

Tourret, J., B. P. Willing, S. Dion, J. MacPherson, E. Denamur, and B. B. Finlay. 2017. "Immunosuppressive Treatment Alters Secretion of Ileal Antimicrobial Peptides and Gut Microbiota, and Favors Subsequent Colonization by Uropathogenic Escherichia coli." *Transplantation* 101 (1):74-82. doi: 10.1097/tp.0000000000001492.

Tsang, Julia Yuen-Shan, Yakup Tanriver, Shuiping Jiang, Shao-An Xue, Kulachelvy Ratnasothy, Daxin Chen, Hans J. Stauss, R. Pat Bucy, Giovanna Lombardi, and Robert Lechler. 2008. "Conferring indirect allospecificity on CD4+CD25+ Tregs by TCR gene transfer favors transplantation tolerance in mice." *The Journal of Clinical Investigation* 118 (11):3619-3628. doi: 10.1172/JCI33185.

Van Den Berg, Hugo A., and David A. Rand. 2007. "Quantitative theories of T-cell responsiveness." *Immunological Reviews* 216 (1):81-92. doi: 10.1111/j.1600-065X.2006.00491.x.

Van Parijs, Luk, Alexander Ibraghimov, and Abul K. Abbas. 1996. "The Roles of Costimulation and Fas in T Cell Apoptosis and Peripheral Tolerance." *Immunity* 4 (3):321-328. doi: 10.1016/S1074-7613(00)80440-9.

Vilchez, Regis A., James Dauber, Kenneth McCurry, Aldo Iacono, and Shimon Kusne. 2003. "Parainfluenza Virus Infection in Adult Lung Transplant Recipients: An Emergent Clinical Syndrome with Implications on Allograft Function." *American Journal of Transplantation* 3 (2):116-120. doi: 10.1034/j.1600-6143.2003.00024.x.

Vincenti, F., B. Charpentier, Y. Vanrenterghem, L. Rostaing, B. Bresnahan, P. Darji, P. Massari, G. A. Mondragon-Ramirez, M. Agarwal, G. Di Russo, C. S. Lin, P. Garg, and C. P. Larsen. 2010. "A Phase III Study of Belatacept-based Immunosuppression Regimens versus Cyclosporine in Renal Transplant Recipients (BENEFIT Study)." *American Journal of Transplantation* 10 (3):535-546. doi: 10.1111/j.1600-6143.2009.03005.x.

von Essen, M. R., M. Kongsbak, and C. Geisler. 2012. "Mechanisms behind functional avidity maturation in T cells." *Clin Dev Immunol* 2012:163453. doi: 10.1155/2012/163453.

Vrisekoop, Nienke, João P Monteiro, Judith N Mandl, and Ronald N Germain. 2014. "Revisiting Thymic Positive Selection and the Mature T Cell Repertoire for Antigen." *Immunity* 41 (2):181-190. doi: https://doi.org/10.1016/j.immuni.2014.07.007.

Vétizou, Marie, Jonathan M. Pitt, Romain Daillère, Patricia Lepage, Nadine Waldschmitt, Caroline Flament, Sylvie Rusakiewicz, Bertrand Routy, Maria P. Roberti, Connie P. M. Duong, Vichnou Poirier-Colame, Antoine Roux, Sonia Becharef, Silvia Formenti, Encouse Golden, Sascha Cording, Gerard Eberl, Andreas Schlitzer, Florent Ginhoux, Sridhar Mani, Takahiro Yamazaki, Nicolas Jacquelot, David P. Enot, Marion Bérard, Jérôme Nigou, Paule Opolon, Alexander Eggermont, Paul-Louis Woerther, Elisabeth Chachaty, Nathalie Chaput, Caroline Robert, Christina Mateus, Guido Kroemer, Didier Raoult, Ivo Gomperts Boneca, Franck Carbonnel, Mathias Chamaillard, and Laurence Zitvogel. 2015. "Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota." *Science* 350 (6264):1079. doi: 10.1126/science.aad1329.

Walker, Alesia, Barbara Pfitzner, Mourad Harir, Monika Schaubeck, Jelena Calasan, Silke S. Heinzmann, Dmitrij Turaev, Thomas Rattei, David Endesfelder, Wolfgang zu Castell, Dirk Haller, Michael Schmid, Anton Hartmann, and Philippe Schmitt-Kopplin. 2017. "Sulfonolipids as novel metabolite markers of Alistipes and Odoribacter affected by high-fat diets." *Scientific Reports* 7 (1):11047. doi: 10.1038/s41598-017-10369-z.

Walker, L. S., A. Gulbranson-Judge, S. Flynn, T. Brocker, C. Raykundalia, M. Goodall, R. Förster, M. Lipp, and P. Lane. 1999. "Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers." *The Journal of experimental medicine* 190 (8):1115-1122. doi: 10.1084/jem.190.8.1115.

Walunas, Theresa L., Deborah J. Lenschow, Christina Y. Bakker, Peter S. Linsley, Gordon J. Freeman, Jonathan M. Green, Craig B. Thompson, and Jeffrey A. Bluestone. 1994.

"CTLA-4 can function as a negative regulator of T cell activation." *Immunity* 1 (5):405-413. doi: https://doi.org/10.1016/1074-7613(94)90071-X.

Wang, Jun, Miguel F. Sanmamed, Ila Datar, Tina Tianjiao Su, Lan Ji, Jingwei Sun, Ling Chen, Yusheng Chen, Gefeng Zhu, Weiwei Yin, Linghua Zheng, Ting Zhou, Ti Badri, Sheng Yao, Shu Zhu, Agedi Boto, Mario Sznol, Ignacio Melero, Dario A. A. Vignali, Kurt Schalper, and Lieping Chen. 2019. "Fibrinogen-like Protein 1 Is a Major Immune Inhibitory Ligand of LAG-3." *Cell* 176 (1):334-347.e12. doi: 10.1016/j.cell.2018.11.010.

Wang, T., E. B. Ahmed, L. Chen, J. Xu, J. Tao, C. R. Wang, M. L. Alegre, and A. S. Chong. 2010a. "Infection with the intracellular bacterium, Listeria monocytogenes, overrides established tolerance in a mouse cardiac allograft model." *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 10 (7):1524-1533. doi: 10.1111/j.1600-6143.2010.03066.x.

Wang, T., E. B. Ahmed, L. Chen, J. Xu, J. Tao, C. R. Wang, M. L. Alegre, and A. S. Chong. 2010b. "Infection with the intracellular bacterium, Listeria monocytogenes, overrides established tolerance in a mouse cardiac allograft model." *Am J Transplant* 10 (7):1524-33. doi: 10.1111/j.1600-6143.2010.03066.x.

Wang, Tongmin, Luqiu Chen, Emily Ahmed, Lianli Ma, Dengping Yin, Ping Zhou, Jikun Shen, Honglin Xu, Chyung-Ru Wang, Maria-Luisa Alegre, and Anita S. Chong. 2008. "Prevention of Allograft Tolerance by Bacterial Infection with Listeria monocytogenes." *The Journal of Immunology* 180 (9):5991. doi: 10.4049/jimmunol.180.9.5991.

Wardemann, Hedda, Sergey Yurasov, Anne Schaefer, James W. Young, Eric Meffre, and Michel C. Nussenzweig. 2003. "Predominant Autoantibody Production by Early Human B Cell Precursors." *Science* 301 (5638):1374. doi: 10.1126/science.1086907.

Webster, Kylie E., Stacey Walters, Rachel E. Kohler, Tomas Mrkvan, Onur Boyman, Charles D. Surh, Shane T. Grey, and Jonathan Sprent. 2009. "In vivo expansion of T reg cells with IL-2–mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression." *Journal of Experimental Medicine* 206 (4):751-760. doi: 10.1084/jem.20082824.

Wells, Andrew D. 2009. "New Insights into the Molecular Basis of T Cell Anergy: Anergy Factors, Avoidance Sensors, and Epigenetic Imprinting." *The Journal of Immunology* 182 (12):7331. doi: 10.4049/jimmunol.0803917.

Wells, Andrew D., Xian Chang Li, Yongsheng Li, Matthew C. Walsh, Xin Xiao Zheng, Zihao Wu, Gabriel Nuñez, Aimin Tang, Mohamed Sayegh, Wayne W. Hancock, Terry B. Strom, and Laurence A. Turka. 1999. "Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance." *Nature Medicine* 5:1303. doi: 10.1038/15260.

Welsh, R. M., T. G. Markees, B. A. Woda, K. A. Daniels, M. A. Brehm, J. P. Mordes, D. L. Greiner, and A. A. Rossini. 2000. "Virus-induced abrogation of transplantation tolerance induced by donor-specific transfusion and anti-CD154 antibody." *J Virol* 74 (5):2210-8.

Wherry, E. John, and Makoto Kurachi. 2015. "Molecular and cellular insights into T cell exhaustion." *Nature reviews. Immunology* 15 (8):486-499. doi: 10.1038/nri3862.

Wing, Kajsa, Yasushi Onishi, Paz Prieto-Martin, Tomoyuki Yamaguchi, Makoto Miyara, Zoltan Fehervari, Takashi Nomura, and Shimon Sakaguchi. 2008. "CTLA-4 Control over Foxp3⁺ Regulatory T Cell Function." *Science* 322 (5899):271. doi: 10.1126/science.1160062.

Wohlfert, Elizabeth A., John R. Grainger, Nicolas Bouladoux, Joanne E. Konkel, Guillaume Oldenhove, Carolina Hager Ribeiro, Jason A. Hall, Ryoji Yagi, Shruti Naik, Ravikiran Bhairavabhotla, William E. Paul, Remy Bosselut, Gang Wei, Keji Zhao, Mohamed Oukka, Jinfang Zhu, and Yasmine Belkaid. 2011. "GATA3 controls Foxp3+ regulatory T cell fate during inflammation in mice." *The Journal of Clinical Investigation* 121 (11):4503-4515. doi: 10.1172/JCI57456.

Womer, K. L., C. C. Magee, N. Najafian, J. P. Vella, E. L. Milford, M. H. Sayegh, and C. B. Carpenter. 2008. "A pilot study on the immunological effects of oral administration of donor major histocompatibility complex class II peptides in renal transplant recipients." *Clin Transplant* 22 (6):754-9. doi: 10.1111/j.1399-0012.2008.00871.x.

Xing, Yan, and Kristin A. Hogquist. 2012. "T-cell tolerance: central and peripheral." *Cold Spring Harbor perspectives in biology* 4 (6):a006957. doi: 10.1101/cshperspect.a006957.

Yamada, Y., O. Nadazdin, S. Boskovic, S. Lee, E. Zorn, R. N. Smith, R. B. Colvin, J. C. Madsen, A. B. Cosimi, T. Kawai, and G. Benichou. 2015. "Repeated Injections of IL-2 Break Renal Allograft Tolerance Induced via Mixed Hematopoietic Chimerism in Monkeys." *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 15 (12):3055-3066. doi: 10.1111/ajt.13382.

Yang, Jaeseok, Matthew O. Brook, Manuela Carvalho-Gaspar, Jidong Zhang, Hilda E. Ramon, Mohamed H. Sayegh, Kathryn J. Wood, Laurence A. Turka, and Nick D. Jones.

Yang, Jaeseok, Matthew O. Brook, Manuela Carvalho-Gaspar, Jidong Zhang, Hilda E. Ramon, Mohamed H. Sayegh, Kathryn J. Wood, Laurence A. Turka, and Nick D. Jones. 2007. "Allograft rejection mediated by memory T cells is resistant to regulation." *Proceedings of the National Academy of Sciences* 104 (50):19954. doi: 10.1073/pnas.0704397104.

Yang, Liming, Barb DuTemple, Reginald M. Gorczynski, Gary Levy, and Li Zhang. 1999. "EVIDENCE FOR EPITOPE SPREADING AND ACTIVE SUPPRESSION IN SKIN GRAFT TOLERANCE AFTER DONOR-SPECIFIC TRANSFUSION1." *Transplantation* 67 (11).

You, S, and L Chatenoud. 2018. "The Concerted Action of Multiple Mechanisms to Induce and Sustain Transplant Tolerance." *OBM Transplantation* 2 (4). doi: 10.21926/obm.transplant.1804025.

Young, James S., Dengping Yin, Augustin Georges Louis Vannier, Maria-Luisa Alegre, and Anita S. Chong. 2018. "Equal Expansion of Endogenous Transplant-Specific Regulatory T Cell and Recruitment Into the Allograft During Rejection and Tolerance." *Frontiers in Immunology* 9 (1385). doi: 10.3389/fimmu.2018.01385.

Yu, Xuesong, Chibing Huang, Bo Song, Ya Xiao, Mingqi Fang, Jiayu Feng, and Pingxian Wang. 2013. "CD4+CD25+ regulatory T cells-derived exosomes prolonged kidney allograft survival in a rat model." *Cellular Immunology* 285 (1):62-68. doi: https://doi.org/10.1016/j.cellimm.2013.06.010.

Yurkovetskiy, Leonid, Michael Burrows, Aly A Khan, Laura Graham, Pavel Volchkov, Lev Becker, Dionysios Antonopoulos, Yoshinori Umesaki, and Alexander V Chervonsky. 2013. "Gender Bias in Autoimmunity Is Influenced by Microbiota." *Immunity* 39 (2):400-412. doi: 10.1016/j.immuni.2013.08.013.

Zavazava, Nicholas, Fred Fändrich, Xiaofeng Zhu, Anja Freese, Dirk Behrens, and Kyoung-Ae Yoo-Ott. 2000. "Oral feeding of an immunodominant MHC donor-derived synthetic class I peptide prolongs graft survival of heterotopic cardiac allografts in a high-responder rat strain combination." *Journal of Leukocyte Biology* 67 (6):793-800. doi: 10.1002/jlb.67.6.793.

Zeh, H. J., 3rd, D. Perry-Lalley, M. E. Dudley, S. A. Rosenberg, and J. C. Yang. 1999. "High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy." *J Immunol* 162 (2):989-94.

Zehn, D., and M. J. Bevan. 2006. "T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity." *Immunity* 25 (2):261-70. doi: 10.1016/j.immuni.2006.06.009.

Zhang, Qianxia, Maria Chikina, Andrea L. Szymczak-Workman, William Horne, Jay K. Kolls, Kate M. Vignali, Daniel Normolle, Maria Bettini, Creg J. Workman, and Dario A. A. Vignali. 2017. "LAG3 limits regulatory T cell proliferation and function in autoimmune diabetes." *Science immunology* 2 (9):eaah4569. doi: 10.1126/sciimmunol.aah4569.

Zhang, Qunfang, Weihui Lu, Chun-Ling Liang, Yuchao Chen, Huazhen Liu, Feifei Qiu, and Zhenhua Dai. 2018. "Chimeric Antigen Receptor (CAR) Treg: A Promising Approach to Inducing Immunological Tolerance." *Frontiers in immunology* 9:2359-2359. doi: 10.3389/fimmu.2018.02359.

Zhang, Z., L. Liu, H. Tang, W. Jiao, S. Zeng, Y. Xu, Q. Zhang, Z. Sun, A. Mukherjee, X. Zhang, and X. Hu. 2018. "Immunosuppressive effect of the gut microbiome altered by high-dose tacrolimus in mice." *Am J Transplant* 18 (7):1646-1656. doi: 10.1111/ajt.14661.

Zhang, Zheng, Linfu Zhu, Douglas Quan, Bertha Garcia, Necdet Ozcay, John Duff, Calvin Stiller, Andrew Lazarovits, David Grant, and Robert Zhong. 1996. "PATTERN OF LIVER, KIDNEY, HEART, AND INTESTINE ALLOGRAFT REJECTION IN DIFFERENT MOUSE STRAIN COMBINATIONS1." *Transplantation* 62 (9).

Zheng, Xin Xiao, Thomas G. Markees, Wayne W. Hancock, Yongsheng Li, Dale L. Greiner, Xian Chan Li, John P. Mordes, Mohammed H. Sayegh, Aldo A. Rossini, and Terry B. Strom. 1999. "CTLA4 Signals Are Required to Optimally Induce Allograft Tolerance with Combined Donor-Specific Transfusion and Anti-CD154 Monoclonal Antibody Treatment." *The Journal of Immunology* 162 (8):4983.

Zheng, Ye, Steven Josefowicz, Ashutosh Chaudhry, Xiao P. Peng, Katherine Forbush, and Alexander Y. Rudensky. 2010. "Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate." *Nature* 463 (7282):808-812. doi: 10.1038/nature08750.

Zhong, S., K. Malecek, L. A. Johnson, Z. Yu, E. Vega-Saenz de Miera, F. Darvishian, K. McGary, K. Huang, J. Boyer, E. Corse, Y. Shao, S. A. Rosenberg, N. P. Restifo, I. Osman, and M. Krogsgaard. 2013. "T-cell receptor affinity and avidity defines antitumor response and autoimmunity in T-cell immunotherapy." *Proc Natl Acad Sci U S A* 110 (17):6973-8. doi: 10.1073/pnas.1221609110.

Zhou, Liang, Jared E. Lopes, Mark M. W. Chong, Ivaylo I. Ivanov, Roy Min, Gabriel D. Victora, Yuelei Shen, Jianguang Du, Yuri P. Rubtsov, Alexander Y. Rudensky, Steven F. Ziegler, and Dan R. Littman. 2008. "TGF-β-induced Foxp3 inhibits TH17 cell differentiation by antagonizing RORγt function." *Nature* 453 (7192):236-240. doi: 10.1038/nature06878.

Zhou, Xuyu, Samantha L. Bailey-Bucktrout, Lukas T. Jeker, Cristina Penaranda, Marc Martínez-Llordella, Meredith Ashby, Maki Nakayama, Wendy Rosenthal, and Jeffrey A. Bluestone. 2009. "Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo." *Nature Immunology* 10 (9):1000-1007. doi: 10.1038/ni.1774.

Zhu, Yuwen, Sheng Yao, and Lieping Chen. 2011. "Cell surface signaling molecules in the control of immune responses: a tide model." *Immunity* 34 (4):466-478. doi: 10.1016/j.immuni.2011.04.008.

Zhu, Z., S. M. Cuss, V. Singh, D. Gurusamy, J. L. Shoe, R. Leighty, V. Bronte, and A. A. Hurwitz. 2015. "CD4+ T Cell Help Selectively Enhances High-Avidity Tumor Antigen-Specific CD8+ T Cells." *J Immunol* 195 (7):3482-9. doi: 10.4049/jimmunol.1401571.

Zou, Yizhou, Peter Stastny, Caner Süsal, Bernd Döhler, and Gerhard Opelz. 2007. "Antibodies against MICA Antigens and Kidney-Transplant Rejection." *New England Journal of Medicine* 357 (13):1293-1300. doi: 10.1056/NEJMoa067160.