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Proximal Impact of Transplant Tolerance-Promoting Antibody Therapies on Antigen-Specific T Cell Reactivity

by

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ABSTRACT

The development of a transient, tolerance-promoting therapy is a critical goal in transplantation. Antibody-perturbation of T cell activation signals is considered a promising candidate. However, the mechanisms of such therapies remain vague. The lack of cohesive and systematic knowledge in the requirements of generating long-term transplantation tolerance using short-term antibody treatments impedes the rational design of tolerance-promoting therapies. Therefore, using anti-LFA-1 and anti-CD154 as representatives of efficacious tolerance-inducing therapeutics, I interrogated their proximal impact on naïve antigen-specific T cells during initial antigen encounter.

Using both monoclonal TCR-transgenic and polyclonal T cells in an *in vivo* adoptive transfer model, I tracked T cell activation and differentiation in the presence of anti-LFA-1 and/or anti-CD154. The antibody therapies markedly reduced the number of T cells in the draining lymph nodes. However, those remaining in the nodes vigorously proliferated. This paradoxical decrease in cell number despite intact proliferative capability was not due to deletion, as the adoptively transferred T cells persisted past primary activation and responded productively to secondary antigen exposure. Surprisingly, while anti-LFA-1 and/or anti-CD154 partially inhibited effector cytokine production, they did not specifically induce differentiation of alternate, tolerogenic phenotypes. However, while the antibody therapies mediated neither complete suppression of T cell reactivity nor T cell tolerance, anti-LFA-1- and anti-CD154-mediated skin graft prolongation was maintained by a dominant regulatory mechanism that allowed

naïve graft-specific T cell activation and proliferation but inhibited their differentiation.

Taken together, anti-LFA-1 and anti-CD154 appeared to partially inhibit T cell reactivity without inducing early T cell tolerance. However, long-term graft survival and tolerance generated by these antibodies were maintained by an active regulatory mechanism protecting the graft from naïve T cells. I therefore hypothesize that, instead of immediately generating tolerance upon interaction with responding T cells, antibodies targeting T cell activation signals dampen initial T cell reactivity to allow early transplant survival in an immunologically quiescent microenvironment, which allows the transplant itself to then gradually tolerize graft-specific T cells and generate donor-specific tolerance in a time-dependent manner. In other words, the transplant, and not the therapeutic antibodies, is the key tolerogen for successful generation of tolerance.

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α	alpha
β	beta
γ	gamma
k	kilo (10^3)
m	milli (10^{-3})
μ	micro (10^{-6})
AICD	activation-induced cell death
Aire/AIRE	autoimmune regulator
APC	antigen-presenting cell(s)
APS1	Autoimmune Polyendocrinopathy Syndrome Type 1
B6	C57BL/6
B6-OVA	C57BL/6-Tg(ACTB-OVA)916Jen/J
BALB/c	BALB/cByJ
BAK	Bcl-2 antagonist/killer
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
ConA	concanavalin A
CTL(s)	cytotoxic T lymphocyte(s)
CTLA-4	cytotoxic T lymphocyte antigen-4
dLN(s)	draining lymph nodes
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DST	donor-specific transfusion
DTH	delay-type hypersensitivity
EMEM	Eagle's minimal essential medium
FACS	fluorescence-activated cell sorting/flow cytometry
FoxP3	forkhead box P3
ELISA	enzyme-linked immunosorbent assay
g	gram
x g	multiple of gravity
H-2	mouse major histocompatibility complex
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMPDH	Inosine monophosphate dehydrogenase
i.p.	intraperitoneal

iTreg	induced T regulatory cells
i.v.	intravenous
KO	knockout
L	litre
LFA-1	lymphocyte function-associated antigen-1
LN(s)	lymph node(s)
M	molar
MHC	major histocompatibility complex
mol	mole(s)
mTOR	mammalian target-of-rapamycin
NFAT/NFATc	nuclear factor of activated T cells
nTreg	natural T regulatory cells
OT-I	CD45.1 OT-I rag-/-
OT-II	CD45.1 OT-II rag-/-
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
pLN(s)	popliteal lymph node(s)
PMA	phorbol myristyl acetate
PML	progressive multifocal leukoencephalopathy
RAG	recombination activating gene
TCR	T cell receptor
T_H	T helper cells
TGF	transforming growth factor
TNF	tumour necrosis factor
Treg	T regulatory cells

CHAPTER ONE
INTRODUCTION

1.1 T CELLS AND IMMUNOSUPPRESSIVE TRANSPLANT THERAPIES

Solid organ and cellular transplantation is the standard treatment for end-stage organ failure. In the past five decades, advances in surgical techniques and therapeutics have steadily improved early transplant function. However, transplant rejection by the immune system remains the primary obstacle to long-term survival of the transplant. Transplant rejection is initiated by the recognition of graft allo-antigens, which leads to injury and destruction of a transplant through effector mechanisms involving both the innate and the adaptive immune responses. Immune cells that have been shown to contribute to graft injury include such diverse players such as macrophages, eosinophils, neutrophils, natural killer cells, natural killer T cells, B cells, and T cells (1–6). Amongst these, T cells are especially critical in facilitating transplant rejection, whether by directly damaging the graft or by shaping other graft-destructive immune responses. In fact, it has long been known that animals lacking T cells cannot reject transplants until reconstitution of T cells, thus highlighting T cells' essential role in the process (6–9). Because of this integral role of T cells in the immune response to a transplant, monitoring the fate of T cells during treatment with therapeutic agents aimed to prolong allograft survival will be the primary focus of this thesis.

1.1.1 T Cell-Mediated Transplant Rejection

Antigen-specific T cells are a primary component of the adaptive immune system. They are able to recognize specific peptide antigens ranging from pathogenic organisms to tumour-associated antigens and differentiate into appropriate effectors to control the precise threat to the host. Once activated against a specific threat, antigen-reactive T cell clones rapidly expand and differentiate to neutralize the insult. A population of the activated T cells eventually acquires long-living memory phenotype capable of rapid activation

and protection upon a secondary encounter with the same threat. To maximize the range of antigens able to be recognized by T cells, the T cell repertoire is generated in the thymus by a random rearrangement of gene segments coding for the T cell receptors (TCR), which results in a vast set of available TCR specificities. Generally, the precursor frequency of naive T cells specific for one nominal peptide antigen is usually less than 1/100,000, which necessitates a period of time lag after initial antigen encounter for naïve T cells to expand to the necessary magnitude in order to neutralize the immune insult efficiently (10). In contrast, the frequency of T cells able to react to a given non-self major histocompatibility complex (MHC) molecules is as high as 0.1% to 10% of an individual's total T cell repertoire, and can therefore achieve similar magnitude of expansion rapidly (11–13). A transplant can be seen both by the high-frequency T cells recognizing its allogeneic MHC molecules directly and by the low-frequency T cells recognizing donor antigens presented by self-MHC as nominal peptide antigen. Therefore, a transplant induces a dynamic and potent T cell response against itself.

The concept of alloreactivity is problematic in the context of the classical understanding of T cell development. During maturation, immature thymocytes undergo positive selection in the thymus so only those that recognize self-MHC survive (14). In fact, the concept of MHC restriction, or the requirement of nominal peptide antigens to be presented on self-MHC to activate T cells is one of the earliest observations made in the studies of T cell immunobiology (15, 16). An alloreactive T cell capable of recognizing non-self MHCs directly is therefore a curious phenomenon, more so that it exists in so high a frequency. Currently, it is thought that T cells are primed against transplant antigens through three distinct recognition pathways (17). The "direct" recognition pathway requires the recipient T cells to interact with intact allogeneic peptide-MHC complexes on donor-derived antigen-presenting cells (APCs), and thus involves these high-frequency, directly alloreactive T cells (17) (Fig. 1-1A). The "indirect" recognition pathway primes recipient T cells by presenting donor allo-antigens on recipient APC; the responding T cells recognize a combination of donor peptide

antigen with recipient MHC, and are therefore self-MHC-restricted (17) (Fig. 1-1B). More recently, an additional "semidirect" recognition pathway has been shown to occur when donor cell membrane fragments bearing donor antigen-MHC complexes are captured and transferred onto recipient APC surface (17, 18) (Fig. 1-1C). In other words, a recipient alloreactive T cell would recognize the combination of donor peptide antigen on donor MHC similar to the direct pathway, but this interaction is facilitated by recipient APCs instead. Since the finite number of donor passenger leukocytes within a transplant are eliminated relatively quickly after transplantation, the effect of the direct pathway on priming alloreactive T cells likely decreases with time and probably contributes mostly to acute graft rejection (19). On the other hand, indirect antigen recognition can occur as long as the transplant is present as a source of antigen. Therefore, this pathway plausibly becomes the primary mechanism of T cell priming against a transplant in the long term, and is a major component of chronic transplant rejection (17, 19).

1.1.1.1 CD8+ T Cells in Transplant Rejection

T cells are traditionally classified into two major categories based upon their co-receptors. T cells expressing the co-receptor CD8 are restricted to recognizing their cognate antigen presented on class I MHC molecules (MHC I), while those expressing CD4 co-receptor recognize antigens presented on class II MHC molecules (MHC II). CD4+ and CD8+ T cells can be further categorized into different subsets based upon the specific differentiation phenotypes and cytokine profiles they acquire upon activation. Most of these subsets have been shown to facilitate transplant rejection.

When naïve CD8+ T cells are activated by their cognate antigens (MHC I plus peptide), they differentiate into antigen-specific cytotoxic T lymphocytes (CTLs). The generation of graft-specific CTLs is a critical barrier to transplant survival. Numerous studies have conclusively shown the potency of CTLs in graft rejection (20–27). CD8+ effector T cells are consistently isolated from human patients with rejecting allografts (20). Moreover, adoptive transfer of CD8+ T

cells alone effectively induced rejection of MHC I-mismatched skin grafts or islet allografts in mice, and mice undergoing heart rejection episodes had expanded and activated CD8+ T cell infiltration in the graft (8, 22, 28). Furthermore, adoptive transfer of a single clone of antigen-specific CD8+ T cells into thymectomized and T cell-depleted recipients was sufficient to reject fully MHC-mismatched cardiac allografts in mice (23). Taken together, a variety of studies clearly indicated graft-reactive CD8+ T cells as a key facilitator of transplant rejection.

CTLs mediate graft destruction primarily through specific interaction with cells presenting their cognate peptide-MHC complexes (17). Once recognition is established, CTLs release granules containing cytotoxic molecules such as perforin and granzyme B, which then trigger target cell apoptosis cascade including the activation of caspase 3 and caspase 9 (17, 29, 30). Some results suggest a role of caspase-initiated apoptosis in transplant destruction. Caspase 3 staining in human cardiac transplant biopsies correlated with rejection, and inhibiting caspase 3 prevented acute cardiac allograft rejection in rats (29, 31). Furthermore, when graft-infiltrating T cells from rejecting human renal allografts were incubated with inhibitors of the granzyme exocytosis pathway, their ability to induce *in vitro* apoptosis or proximal tubular epithelial cells was significantly reduced, indicating a direct link between CTL granule release and transplant tissue damage (29). Alternatively, CTLs can also facilitate similar apoptosis pathways through the Fas-Fas ligand (FasL) pathway (17, 29), and Fas expression has been found on rejecting allografts in humans (29). More importantly, while these two pathways have been correlated to potent allograft damage and rejection, CTLs have been shown to reject transplants even in the absence of either mechanism (24). Diamond *et al.* used primed CD8+ T cells from perforin- or FasL-deficient mice to effectively reject allogeneic pancreatic islet transplants (24), suggesting the presence of multiple redundant mechanisms by which CTLs can cause allograft destruction. For instance, while the specific mechanisms are still unclear, CTLs can cause graft injury through a range of proinflammatory cytokines including interferon-gamma (IFN-γ) and tumour necrosis factor-alpha

(TNFα) similar to CD4+ T cells. (20). Later studies by Sleater *et al.* found that the bulk of CD8 T cell-mediated islet allograft rejection involved the alternative use of perforin and/or FasL (28). That is, perforin- or FasL-knockout CD8+ T cells did not readily reject FasL-deficient pancreatic islet allograft (28). Taken together, activated CD8+ T cells present a major barrier to transplant survival through its efficiency in direct tissue destruction and flexibility in employing multiple mechanisms in causing such destruction.

1.1.1.2 CD4+ T Cells in Transplant Rejection

MHC class II-restricted CD4+ T cell-mediated transplant rejection is another major component of cell-mediated transplant rejection. Clear evidence indicate that CD4+ T cells are capable of rejecting a transplant independently of any other immune cell types. For example, mice with transgenic monoclonal antigen-specific CD4+ T cells could rapidly reject skin grafts bearing the cognate antigen in the absence of CD8+ T cells, and other studies showed that selective depletion of CD8+ T cells *in vivo* did not prevent skin graft rejection (32–34). Moreover, adoptive transfer of CD4+ T cells isolated from a CD8-knockout mouse into an immune-deficient recipient was also sufficient to induce skin graft rejection (35). CD4+ T cells also accounted for primary acute cardiac allograft rejection independent of either CD8 T cells or B cells (36). CD4+ T cells were also demonstrated to be required for the rejection of pancreatic islet allo- and xeno-grafts (37–39). Furthermore, CD4+ T cells can collaborate with naïve CD8+ T cells and B cells to promote the generation of CTLs and/or antibody-producing B cells, respectively (32). Taken together, CD4+ T cells can mediate transplant rejection both as the direct mediator of transplant injury or as a helper in the functions of other immune cells.

Unlike CD8+ T cells, which generally ‘default’ into pro-inflammatory effector cells, CD4+ T cells have great plasticity in their differentiation and functional phenotype depending on the context under which they are activated. A naïve CD4+ T cell potentially can differentiate into several different types of effector T cells. Conversely, while different types CD4+ T effector cells have the

capability to cause graft injury, certain subsets of them can actually mediate protection of the transplant; these particular subsets of CD4+ T cells will be discussed in detail later in section 1.2.1.2.3. The two CD4+ T cell subsets that have been demonstrated most extensively in transplant rejection are T helper-1 (T_H-1) and T helper-2 (T_H-2) T cells. T_H-1-mediated graft rejection is more commonly referred to as delayed-type hypersensitivity (DTH) in the clinic, which describes the general inflammation at the graft site associated with the presence of T_H-1 effectors. T_H-1 cells characteristically produce pro-inflammatory cytokines IL-2, IFN-γ and TNF-α and mediate acute inflammation by facilitating the activation and infiltration of monocytes, macrophages and CTLs into the graft site and the subsequent generation of proteolytic enzymes, nitric oxide, and other factors in the local environment (17, 29, 34). T_H-1-mediated graft rejection has been demonstrated by adoptively transferring a single clone of T_H-1 alloreactive cell line recognizing donor MHC peptides indirectly (40). These monoclonal T_H-1 cells alone were sufficient to reject skin grafts in SCID recipients, and the grafts histologically showed macrophage infiltration consistent with a DTH-type response (40). T_H-1 T cells can also reject cardiac allografts primarily using the cytotoxic mediators perforin and FasL (41). Also, other studies achieved long-term graft acceptance by decreasing the magnitude of T_H-1 response (29, 42, 43). In humans, peripheral blood mononuclear cells (PBMCs) from patients with functioning allografts despite cessation of immunosuppressants showed particularly blunted DTH response (43). As such, T_H-1-associated allograft response is closely connected to transplant rejection and survival.

While T_H-1 cells facilitate a pro-inflammatory response, T_H-2 cells promote humoural response with cytokines such as IL-4, IL-5 and IL-10 that assist in B cell maturation and eosinophil recruitment (29, 34). Traditionally, it was thought that since T_H-2 cytokines suppress T_H-1 differentiation and the downstream proinflammatory response, a T_H-2 response would be preferable in prolonging transplant survival. However, studies have subsequently demonstrated that a T_H-2 response alone is sufficient in mediating graft rejection (33, 44, 44). For instance, IFN-γ and IL-2 double-knockout mice unable to mount a T_H-1

response rapidly rejected cardiac allografts using a characteristic, "anti-inflammatory" T_H-2 cytokine profile (44). In fact, adoptive transfer of T_H-2-type T cell clones efficiently induced acute skin graft rejection by themselves (33). Therefore, despite producing cytokines not associated with acute inflammation and able to suppress pro-inflammatory T_H-1 cells, T_H-2 cells are highly effective in mediating transplant rejection. As such, whether a naïve T cell activates into a T_H-1 or a T_H-2 phenotype, it will acquire potent ability to cause graft injury.

Recently, more subsets of effector CD4+ T cells have been identified, such as the highly inflammatory T_H-17 cells, and their potential participation in transplant rejection are being investigated (45, 46). For the scope of this thesis, the CD4+ T cell effector paradigm will be confined to the pro-inflammatory T_H-1 and pro-humoural T_H-2 cells. However, the discovery of new effector phenotypes underlines the tremendous plasticity of CD4+ T cells and their potentially multi-faceted role in transplant immunity.

1.1.2 Immunosuppressive Therapies Preventing T Cell-Mediated Rejection

Since many T cell subsets have been demonstrated to independently or collaboratively promote acute transplant destruction, the design of transplant-protective therapies has revolved around control of T cell activities. Many different pharmaceutical agents, such as glucocorticoids, have been used and replaced as newer generations of immunosuppressants are continuously formulated and produced. In the following sections, a sample of the currently most used immunosuppressive agents in transplantation therapies will be discussed in detail (Table 1-1). These immunosuppressive therapies are generally designed to act in one of two approaches. First, small-molecule pharmaceutical agents have been used to interfere with cell cycle mechanisms such as DNA and RNA synthesis, thus arresting T cell activation and/or proliferation. Second, antibodies targeting key T cell surface molecules have been developed to specifically bind and deplete T cells. Current standard clinical post-transplant therapeutic regimens consist of a combination of these two types of immunosuppressive agents.

1.1.2.1 Small-Molecule Drugs

Most small-molecule immunosuppressive drugs are derived from microbial products. There have been many different classes of small-molecule immunosuppressants developed in the last fifty years. Three of them will be discussed here due to their wide-spread usage in almost all types of organ and cellular transplantations.

Calcineurin inhibitors bind to and form a complex with cytosolic immunophilin proteins that then inhibits calcineurin activity (47, 48). Calcineurin is a phosphatase downstream of the calcium channel, and it activates the transcription factor nuclear factor of activated T cell (NFATc) to translocate into the nucleus and initiate the transcription of interleukin-2 (IL-2), a cytokine crucial for promoting and perpetuating T cell expansion and survival during activation (48). Inhibition of calcineurin therefore prevents T cell activation and differentiation into effector function. Two calcineurin inhibitors, cyclosporine and tacrolimus, are currently widely used in solid organ transplantations. Cyclosporine was originally isolated from the fungus *Tolypocladium inflatum* and became one of the earliest immunosuppressive agents to be used in clinical transplantation (49, 50). Tacrolimus was discovered later from the bacteria *Streptomyces tsukubaensis* and exhibited greater molar potency than cyclosporine in inhibiting calcineurin activity (51). Both agents are used almost ubiquitously in combination with other immunosuppressants to prevent transplant rejection.

Target-of-Rapamycin inhibitors function similarly as calcineurin inhibitors to suppress T cell activity (47). Instead of calcineurin, these inhibitors form a complex with the cytosolic protein FKBP12 and subsequently block the activity of mammalian target of rapamycin (mTOR) (47). mTOR is a kinase regulating many downstream activities such as cell growth, proliferation, motility, survival, protein synthesis, and transcription in response to cytokines and growth factors (52–55). Inhibiting mTOR renders T cells unable to respond to cytokines such as IL-2 and generally inhibits T cell proliferation and activity (47, 55). The two mTOR inhibitors widely used in clinical transplantation today are sirolimus,

isolated from the bacteria *Streptomyces hygroscopicus*, and its derivative, everolimus (47, 56).

Inosine monophosphate dehydrogenase (IMPDH) inhibitors suppress the activity of IMPDH, a key enzyme driving purine synthesis (57). Interestingly, while T and B cell growth is particularly paralyzed without IMPDH, other cells are able to generate purine nucleotides by alternate pathways (47, 58). Therefore, inhibition of IMPDH became an attractive option for specifically suppressing lymphocyte responses without compromising universal cell activity. This is done clinically by the application of mycophenolate mofetil, a pro-drug that releases mycophenolic acid to inhibit IMPDH activity (47, 59–61).

1.1.2.2 Lymphocyte Depleting Antibodies

Unlike small-molecule drugs that inhibit T cell immune response by interfering with general cell growth pathways but not necessarily causing cell death, depleting antibodies cause the specific and universal destruction of T cells. Due to their potency in eliminating circulating T cells, depleting antibodies are routinely used as induction therapies, which are usually designed to result in immunosuppression of a much higher degree than maintenance therapies in order to prevent acute graft rejection (62). These antibodies work by binding to T cell-specific surface proteins and triggering T cell lysis or label T cells for clearance by other immune cells (62). The two most commonly used T cell depleting antibodies are antithymocyte globulins and alemtuzumab.

Antithymocyte globulins are polyclonal antibodies produced by immunizing horses or rabbits with human thymocytes (63). The resulting horse- or rabbit-anti-human-thymocyte antibodies bind a variety of T cell surface markers including CD2, CD3, CD4, CD8, CD11a, and CD18 and initiate complement-dependent lysis of bound T cells (64). Antithymocyte globulins induce such thorough and systemic depletion of both circulating T cells and resident T cells in the spleen and thymus, a short course of treatment with the globulins is sufficient to produce profound lymphopenia lasting beyond one year (47, 62, 64, 65).

Alemtuzumab is a humanized monoclonal antibody against CD52, which is found on almost all T and B cells (61, 62, 66, 67). Binding of alemtuzumab to CD52 induces antibody-dependent lysis of the cells (62). Similar to antithymocyte globulins, alemtuzumab depletes lymphocytes thoroughly and efficiently. While effective in preventing acute graft rejection, transient use of the antibody in an induction therapy results in lymphopenia that can take up to a year to reverse (62).

1.1.2.3 Problems Associated with Immunosuppressive Therapies

The immunosuppressive agents described so far are standard options for preventing transplant rejection. In fact, development of transplantation therapies since the first anti-rejection drug was used over fifty years ago has very much revolved around blunt suppression of immune responses. While incidences of acute rejection in every transplantation type have steadily decreased with each newer generation of immunosuppressants, long-term graft survival remains difficult to achieve and is wrought with complications. Unfortunately, many of these complications stem from the immunosuppressants themselves.

The primary side effect of immunosuppressants is their very mechanism of action. Immunosuppressive agents, by definition, indiscriminately inhibit the immune system. This leads to chronic immunodeficiency. While the transplant is thus protected from graft injury and rejection, the patient becomes vulnerable to pathogenic infections and malignancies (68–71). Since the patient has very little protective immunity upon treatment with immunosuppressive agents, even innocuous infections and otherwise treatable cancerous growths become life-threatening (71–73). As a result of preventing transplant rejection, immunosuppressants thus severely compromise a patient's immune defense against injurious assaults.

Besides from immunodeficiency due to the gross inhibition of immune responses, immunosuppressive agents are inherently toxic. The toxicity is amplified by their chronic and accumulative use necessary to maintain transplant survival. The two calcineurin inhibitors, cyclosporine and tacrolimus, induce a plethora of organ injuries including nephrotoxicity, neurotoxicity, and post-

transplantation diabetes mellitus (47). Side effects of sirolimus include hyperlipidemia, thrombocytopenia, delayed wound healing, and delayed graft function (47, 74). Sirolimus also exacerbates the toxicity of calcineurin inhibitors when used together, rendering therapeutic regimens containing both agents difficult to control (47). Mycophenolate mofetil is milder in its side effects but still causes blood-related dysregulations such as anemia, and a range of gastrointestinal complications (47, 75, 76). Byproducts of mycophenolate mofetil also appeared to induce release of inflammatory cytokines (77). Depleting antibodies induce severe cytokine-release syndrome during the process of lymphocyte lysis (47, 62). Furthermore, while depleting antibodies are usually used on a short-term basis, the resulting profound lymphopenia can lead to skewed reconstitution of the immune system during recovery (62). For instance, patients treated transiently with alemtuzumab for multiple sclerosis unexpectedly developed secondary autoimmunity driven by significantly increased level of serum interleukin-21 (78). Unfortunately, as these immunosuppressive agents are rarely effective independently and are routinely administered as a combined cocktail, the patients therefore suffer from the exacerbated adverse effects resulting from the amalgamation of multiple toxicities.

The chronic nature of immunosuppressive therapies and the subsequent immunodeficiency and organ toxicity negatively impact the prognosis for long-term graft and patient survival. Oftentimes these anti-rejection therapies paradoxically result in graft injuries. The elevated risks of infection, cancer, and adverse side effects also reduce the transplant patients' quality of life. As such, the goal of transplant therapy development is now aimed at inducing transplant tolerance rather than preventing rejection. **In contrast to immunosuppressants, the ideal tolerance-inducing transplant therapy should have low toxicity and require only a transient administration that will then result in transplant-specific tolerance. We define immune tolerance as an antigen-specific change in a competent immune system that will lead to no destructive immune response against this antigen**. In the context of transplantation, the immune

response will specifically accept the transplant while retaining all protective immunity against non-transplant pathogenic and mutagenic assaults.

1.2 T CELLS AND TOLERANCE-PROMOTING THERAPIES

The concept of immunological tolerance was first described in 1946 by Owen *et al.* when dizygotic cattle twins were observed to carry live erythrocytes of both their own and their siblings' genotypes (79). Since it was known at the time that an animal would normally mount an immune response to blood cells from another animal of the same species, this observation suggested a mechanism allowing non-responsiveness to a foreign antigen during fetal development. Burnet and Fenner subsequently developed the hypothesis that, if the immune system was exposed to an antigen prior to full development, it would not be able to respond to the antigen once it reaches maturity (80). This concept of actively acquired tolerance during the neonatal period was supported by several succeeding studies. Billingham, Brent, and Medawar injected strain A mouse fetuses with spleen cells from strain B, and these fetuses accepted strain B skin grafts when developed into adult mice (81). Concurrent with the demonstration from the Medawar group, Hasek and Habra performed embryonic parabiosis experiments connecting the circulatory systems of chicken embryos, which resulted in adults that accepted each other's skin grafts (82). Furthermore, later experiments fusing two heterozygous embryos created allophenic mice that did not reject grafted tissues from the parents (83). At this point, it was widely accepted that immunological tolerance required exposure to a foreign antigen during the fetal/neonatal development of an animal. As such, induction of transplantation tolerance in an adult would appear nigh on impossible.

However, an alternative hypothesis for acquired immune tolerance emerged when Lederberg proposed that it was the age of the lymphocyte, and not the animal, that determined whether antigen encounter would result in immunity or tolerance, since immature lymphocytes were likely to be more prone to being tolerogenic rather than immunogenic (84). He hypothesized that lymphocytes learn self-tolerance throughout an animal's lifetime by early exposure to self

antigens prior to cellular maturation (84). This concept allows for the possibility of conditioning the immune system to be tolerant to a transplant even after the recipients have reached adulthood. At around the same time period, large numbers of experiments began emerge that demonstrated induced antigen non-responsiveness in adult animals. It was found that if an adult animal was repeatedly injected intravenously with a non-immunogenic form of an antigen, such as soluble rather than aggregate proteins or proteins without adjuvants, the animal became non-responsive when subsequently exposed to the immunogenic forms of the antigen (85–89). Similarly, Rowley and Fitch observed that rat antibody response to xenogeneic sheep erythrocytes decreased steadily if the rat was injected with the erythrocytes consecutively for a prolonged period (90). Furthermore, adult female mice that normally rejected male skin grafts accepted male skins long-term when injected consecutively with disrupted male splenocytes prior to transplantation (91). Taken together, these experiments began to suggest acquired tolerance mechanisms that could be developed in an adult animal, thus rendering transplant tolerance a realistic possibility.

1.2.1 Self-Tolerance

The early observations of the existence of immunological tolerance were all determined by antibody responses to specific foreign antigens, as lymphocytes secreting antibodies were thought to be the sole mediators of adaptive immunity. However, after extensive studies into the function of the thymus, Miller and Mitchell demonstrated in 1967 that the thymus generates a subset of small circulating lymphocytes distinct from those originated from the bone marrow, thus identifying T cells as a unique component of the adaptive immune system (92–95). As T cells gained recognition as a potent mediator of adaptive immunity influencing many other components of the immune response, the role of T cells during the acquisition of immune tolerance became a key question. T cells are highly responsive to foreign antigens and initiate extensive destructive response against pathogens, malignancies and, unfortunately, non-self transplants. However, T cells are also selective in their response to their potential targets. In a

healthy host, the T cell repertoire is self-tolerant. This is especially remarkable since T cell receptors are generated randomly to maximize the range of antigen recognition, and they are specifically selected to retain a certain level of self-reactivity in order to recognize self-MHC molecules (MHC restriction). Nevertheless, these self-recognizing T cells do not induce immune damage against self-antigens. As demonstrations of induced immunological tolerance in adult animals increased, it became apparent that there existed multiple mechanisms preventing T cells from mounting a self-reactive response.

1.2.1.1 Central Tolerance

Parallel to Lederberg's hypothesis that immature lymphocytes can be conditioned to be tolerogenic, central tolerance, or negative selection, is a mechanism by which developing thymocytes showing high levels of auto-reactivity are eliminated in the thymus, thus rendering the overall mature T cell repertoire non-destructive to self (Fig. 1-2A). The clonal deletion of auto-reactive T cells was first described when animals tolerant to a particular antigen were demonstrated to have reduced frequency of mature T cells recognizing said antigen in the periphery (96). This concept was further illustrated by the use of a monoclonal antibody tracking the fate of a specific T cell family throughout the course of thymic maturation (97). The monoclonal antibody KJ23a recognized T cells with receptor subunit Vβ17a that interacted with the MHC class II I-E molecule (97). Using KJ23a as a detection tool, Kappler *et al.* demonstrated that T cells with Vβ17a receptor were absent in the peripheral T cell and single-positive thymocyte populations but present in the immature CD4+CD8+ double-positive thymocyte population, suggesting that clonal deletion of T cells reactive to self MHC class II occurred in the thymus prior to T cell maturation and emigration into the periphery (97). Clonal deletion has also been shown in mice with transgenic T cell receptors recognizing the male H-Y antigen presented on MHC class I, and CD8+ T cells with the transgenic T cell receptor were deleted in male but not female mice (98). These observations provided early evidence that

immature, self-reactive T cells underwent an elimination process within the thymus as a means of self-tolerance.

While it became clear that autoreactive thymocytes were deleted in the thymus during development, the exact mechanism separating self-reactive thymocytes from non-self-reactive ones was unclear. It was shown that the medullary thymus epithelial cells expressed a surprisingly large number of peripheral tissue-specific antigens, which presumably permitted the thymus to test thymocyte autoreactivity (99). However, an explanation for the thymus' unique ability to "promiscuously" express peripheral tissue antigens did not surface until the autoimmune regulator (Aire) transcription factor was discovered. This began with the observations that humans with Autoimmune Polyendocrinopathy Syndrome Type 1 (APS1), a rare autosomal recessive disorder characterized by multi-organ autoimmunity, had mutations in only one single gene: *aire* (100, 101). Subsequently, mice engineered with Aire deficiency acquired spontaneous autoimmune destruction of multiple organs similar to the human disease (102). In searching for the link between the Aire transcription factor and the induction of multi-organ autoimmunity, Aire-deficient mice were found to have markedly reduced expressions of peripheral tissue-specific antigens in their thymic medulla, thus pointing the cause of disease to thymic negative selection (102, 103). Ensuing microarray analyses showed that Aire controls the transcription of thousands of genes in the medullary thymic epithelial cells, especially genes with highly specialized tissue origin such as insulin, fatty acid binding protein, and salivary protein (102, 103). Direct connection between Aire and negative selection was finally established in a series of experiments crossing TCR transgenic mice to mice expressing their cognate antigens peripherally in the pancreas or thyroid (104, 105). Transgenic T cells were largely deleted in the thymus in the resultant off-springs, and the small residual number of transgenic T cells in the periphery was functionally anergic (104, 105). However, if these normally healthy mice were rendered Aire-deficient, the transgenic T cells escaped thymic deletion and remained fully functional in responding to their cognate antigens in the periphery, resulting in the induction of diabetes or

thyroiditis (104, 105). Therefore, the promiscuous expression of peripheral tissue-specific antigens in the thymic medulla allows for the deletion of autoreactive T cells during thymic development, and central tolerance is thus an integral component in the acquisition of self-tolerance.

1.2.1.2 Peripheral Tolerance

While central tolerance is an effective mechanism of eliminating autoreactive T cells, it is not sufficient alone. Central tolerance is dependent on identifying autoreactive T cell before they exit the thymus. However, Aire does not drive the expression of every tissue-specific antigen, and autoreactive T cells can still be found in the periphery in healthy individuals, yet these individuals remain free of autoimmune symptoms (106). This suggests a secondary mechanism controlling T cell autoreactivity in the periphery. Furthermore, the possibility of TCR rearrangement in the periphery has been hypothesized when CD40 engagement was demonstrated to induce expression and nuclear translocation of recombination activating gene (RAG) 1 and 2 and alter TCR V-alpha expression in peripheral T cells (107). If fully developed peripheral T cells could modify their TCR specificity or recognition strength, a peripheral tolerance mechanism would be essential. Evidence of a peripheral tolerance mechanism have also been observed in many different animal models. The repeated intravenous injections of non-immunogenic forms of protein antigens described in section 1.2 resulting in antigen-specific non-responsiveness essentially demonstrated a thymus-independent mechanism dampening T cell reactivity in the periphery. Adoptive transfer of TCR transgenic T cells into recipients expressing their cognate antigens has also demonstrated to result in peripheral deletion of the "autoreactive" transgenic T cells (108). Also, if the thymus is sufficient in tolerizing T cells to all peripheral antigens, presence of peripheral tissues should be irrelevant in the acquisition of self-tolerance. However, several studies have demonstrated the break-down of tolerance to specific peripheral tissues by temporarily removing organs from fetal animals, which resulted in the immune destruction of the organs or organ-specific antigens when they were re-

introduced into the adult animal (109–111). Thus a peripheral mechanism functions alongside thymic negative selection in the maintenance of self-tolerance (Fig. 1-2B). Two arms of peripheral regulation of mature autoreactive T cells will be discussed here: recessive and dominant tolerance.

1.2.1.2.1 Recessive Tolerance: Anergy

Anergy is defined as a cell-autonomous state in which T cells remain viable and persist in the host but are intrinsically and functionally inactive (112). The state of hyporesponsiveness was first demonstrated using T cells interacting with chemically-altered APCs (113, 114). Instead of a neutral non-event, T cell interaction with impaired APCs inherently drove the T cells into a state of unresponsiveness and inability to produce IL-2 and proliferate (114). Interestingly, this loss of responsiveness is an actively induced state, as blockade of new protein synthesis during T cell interaction with altered APCs abrogated anergy (115). As such, the induction of anergy required an activation signal that was strong enough to initiate protein synthesis, but weak enough to result in non-productive activation (112). For instance, anergy can be a consequence of either a strong TCR signal in the absence of costimulation or a weak TCR signal with low-affinity ligands in the presence of costimulation (112, 115). In the context of peripheral self-tolerance, a scenario of activation with TCR signal lacking costimulation would be an autoreactive T cell interacting with self-antigen directly on tissue parenchymal cells that do not express costimulatory ligands. Alternatively, naïve autoreactive T cells can also interactive with APCs that are passively presenting self-antigens as a normal physiological process of continuous antigen uptake without up-regulation of costimulatory signals. Furthermore, some evidence suggested that anergic T cells might interact with other APCs and inhibit their antigen-presenting function, thus perpetuate the maintenance of anergy (116). Taken together, in a healthy individual without pathogen infection, inflammation, tissue distress and injury, or any other cell-intrinsic or cell-extrinsic "danger signals" that can initiate the up-regulation of costimulatory molecules

(117), self-antigens would be presented to autoreactive T cells with subpar signal strength, thus inducing anergy and preventing autoreactive T cell activity.

1.2.1.2.2 Recessive Tolerance: Cell Death/Deletion

In contrast to functionally inactivated autoreactive T cells remaining in the host, another mechanism of peripheral tolerance is the death or deletion of autoreactive T cells in the periphery. A peripheral deletion mechanism of T cell tolerance was observed when mice made tolerant to a bacterial superantigen markedly decreased frequency of peripheral superantigen-specific T cells (118). This deletion was ascertained to be a thymus-independent mechanism since it also occurred in thymectomized mice (118). Further demonstration of peripheral deletion of autoreactive T cells has been shown by the deletion of adoptive transferred mature T cells specific for a self-antigen in the recipients (108). Deletion of autoreactive T cells in the periphery occurs by apoptosis similar to thymic negative selection (106). T cell apoptosis could be triggered by death signaling through the Fas/FasL pathway, and Fas- or FasL-knockout mice display systemic autoimmunity resembling human systemic lupus erythematosus and significant lymphadenopathy (106, 119). Alternatively, apoptosis could also be triggered through the Bcl-2-regulated family of pro- and anti-apoptotic proteins (106, 120). In the context of peripheral self-tolerance, death pathways have been shown to be induced by repeated and chronic T cell stimulation by self-antigens (106). Similar to anergy, apoptosis as a consequence of T cell interaction with peripheral self-antigens protects and adult animal from T cell-mediated autoimmunity.

1.2.1.2.3 Dominant Tolerance: Regulatory T Cells

While recessive, cell-intrinsic tolerance mechanisms such as anergy and cell death appeared to explain the paradox of the presence of autoreactive T cells in the periphery of a healthy, non-autoimmune individual, there exists another form of peripheral T cell tolerance that employs the dominant regulation of autoreactive T cells by a second population of T cells (121). The evidence of such

regulatory T cells (Tregs) was presented through a series of observations spanning the past forty years. One of the earliest observation of T cell-mediated control of self-reactivity was the spontaneous induction multi-organ autoimmunity by thymectomizing mice between days three and five after birth (122). This suggested that there was a burst of T cells emigrating the thymus during this developmental period that could control self-reactive T cells leaving the thymus prior to thymectomy. This first inkling of a T cell-mediated tolerance mechanism was followed up by several adoptive transfer models indicating that autoimmunity could be controlled by specific CD4+ T cell populations. Transfer of T cells from diabetes-resistant strain sublines inferred disease protection in diabetes-prone BB rats or NOD mice (123). Subsequently, adoptive transfer of CD4+ T cells abrogated autoimmune thyroiditis and diabetes induced in adult rats by thymectomy followed by sub-lethal irradiation (124–126) More conclusively, it was shown that systemic autoimmunity triggered by day-three thymectomy was ameliorated by the transfer of a population of CD4+ T cells expressing IL-2 receptor alpha chain (CD25), thus giving Tregs a concrete phenotype as T cells co-expressing CD4 and CD25 (127). The transcription factor forkhead box P3 (FoxP3) was also identified as a marker of Tregs, and mice with non-functional *foxp3* gene developed severe systemic autoimmunity and lymphoproliferative syndrome (128, 129). These regulatory CD4+ T cells are therefore an integral component in the maintenance of peripheral self-tolerance.

There are two types of regulatory CD4+ T cells, natural (nTreg) and induced (iTreg) (130, 131). Natural Tregs commit to their regulatory lineage during thymic development, and the exact mechanism of which is unclear. It is postulated that while negative selection eliminates thymocytes showing strong affinity to self-antigens, the process also allows a pool of self-reactive thymocytes to survive and acquire a regulatory phenotype (130). Therefore, nTregs likely have specificities to self tissue-specific antigens, and are the key regulators in peripheral self-tolerance. In contrast, induced Tregs exit the thymus with naïve CD4+ T cell phenotype and can be activated to become either tolerogenic or immunogenic effector cells depending upon the context of their activation (130,

131). Since iTregs survived the negative selection process as a naïve T cell, they likely express TCRs that recognize non-self peptides, and are therefore heavily implicated in tolerance induction to oral antigens, gut commensal bacteria, and transplants (131). Despite the divergence in their genesis, both natural and induced Tregs appear to share similar mechanisms for regulating immune tolerance.

In vivo studies of tolerance induction by Tregs have yielded large number of potential mechanisms. Regulatory T cells could suppress effector T cell response through cell-cell contact either directly with the responding T cell or indirectly through a APC intermediate (132). When interacting directly with an effector T cell, Tregs may secrete suppressor cytokines such as IL-10 and TGF-β or cell surface molecules such as galectin-1 to arrest cell cycle progression (132–134). Alternatively, Tregs may compete with the effector T cells for IL-2 using their high surface expression of CD25, thus promoting cytokine-deprivation-induced apoptosis (135). Lastly, Tregs may also directly kill the effector T cells using the perforin/granzyme pathway similar to CD8+ cytotoxic T cells (136). When interacting with an APC, Tregs may utilize an array of surface signaling molecules to down-regulate the strength of costimulation by these APCs and thereby preventing them from activating effector T cells productively. Tregs constitutively express cytotoxic T-lymphocyte antigen 4 (CTLA-4), which binds to CD80/CD86 on dendritic cells and prevents their maturation and up-regulation of costimulatory molecules (132, 137, 138). Alternatively, CD223 on Treg binds to MHC class II on dendritic cells and sends an inhibitory signal to suppress maturation (139). Tregs may also use the ectoenzyme CD39 to catalytically inactivate extracellular ATP and thus suppress metabolic energy source required for dendritic cell maturation (140). Finally, Tregs may competitively occupy dendritic cells through long interactions induced via neuropilin (Nrp-1) and block naïve effector T cells from antigen exposure (132). Therefore, CD4+CD25+FoxP3+ regulatory T cells could maintain peripheral self-tolerance by dominantly suppressing the function of autoreactive T cells either directly or

indirectly by a myriad of mechanisms. Many of these mechanisms are also considered keys to inducing transplantation tolerance.

1.2.2 Application of Self-Tolerance Concepts in Transplantation

The understanding of self-tolerance evolved in parallel with experiments testing the induction of transplantation tolerance. The observations made in one arena helped advance the other. The major difference between self and transplant tolerance is that transplant tolerance requires tolerance induction to a non-self-antigen in the periphery of an immunologically mature recipient. This is problematic, as the foundation of immune tolerance hinges on the segregation of self from non-self. In contrast, transplantation tolerance requires an active restraint of an appropriately activated destructive immune response against a foreign antigen. Therefore, transplantation tolerance necessitates a deliberate deviation from a default healthy immune response. However, as the mechanisms initiating and maintaining self-tolerance are elucidated through time, potential ways of "tricking" the immune system into a tolerance response against the non-self transplant antigen also started to emerge.

Ray Owen's early observation of red blood cell chimerism in dizygotic cattle twins sparked the concept of using chimerism to tolerize allo-immunity (141). This was followed by extensive studies by the Medawar group showing that splenocytes or bone-marrow cells injected into a neonatal recipient with an immature immune system allowed the adult recipients to accept donor strain skin grafts (142). Subsequently, adult mice with a developed immune system were shown to accept donor skin grafts after being treated with total body irradiation then donor strain bone-marrow (141). The idea of using a chimeric immune system created by bone-marrow transplantation to generate donor-specific tolerance to an accompanying organ transplant was thus engendered. This method of tolerance induction has shown some success in animal models (143). However, it quickly became apparent that allogeneic bone-marrow transplantation itself carried lethal risks of graft-versus-host disease, and creating a chimeric immune system with a completely allogeneic bone-marrow transplant naturally was

unlikely (141). It became difficult to justify the risk-versus-benefit ratio of using bone marrow transplantation as a means of prolonging the survival of a secondary organ graft. Therefore, when the first immunosuppressive agent, azathioprine (6-mercaptopurine), was described and applied to clinical transplantation, studies in transplantation therapies quickly shifted focus from natural induction of graft tolerance using bone marrow chimerism to pharmaceutical control of rejection (141, 144). However, as discussed in detail in Section 1.1.2, while advances in immunosuppression allowed transplantation an immense leap forward as a feasible clinical therapy, these pharmaceutical agents carry with them a wide range of debilitating adverse side effects. Therefore, the search for a method to promote transplant-specific tolerance continued.

Attempts have been made to imitate thymic negative selection in order to induce clonal deletion and "central tolerance" of donor-specific T cells. Early observations described a loss of antibody immunity to bovine gamma globulin in rats after total body irradiation followed by injection of the globulin protein directly into the thymus (145). Subsequently, Posselt *et al.* showed that allogeneic rat pancreatic islets could survive indefinitely in the recipient thymus with one single injection of anti-lymphocyte serum to deplete circulating lymphocytes prior to transplantation (146). Interestingly, rats carrying such long-term surviving islet grafts in the thymus went on to accept islets from the same donor strain transplanted under the renal capsule without any therapies (146). Jones *et al.* further developed this observation into a transplant tolerance protocol in which TCR transgenic mice were treated with depleting anti-CD8 and anti-CD4 antibodies followed by intrathymic injection of splenocytes carrying the cognate antigen (147). Cardiac transplants expressing the cognate antigen were then accepted indefinitely by the recipients (147). Both experiments elegantly demonstrated the effect of thymic negative selection in conditioning developing thymocytes to prevent immunity against antigens within the thymus. However, the major caveat of this tolerance protocol is the requirement of intrathymic exposure to donor antigens minimum one month prior to transplantation, which renders it impractical in clinical transplantation scenarios unless a living donor is

involved. Therefore, while mimicking central tolerance proved to be an effective method to induce transplantation tolerance, it was unfortunately not applicable in human clinical transplantation.

Induction of the peripheral self-tolerance mechanism of anergy has also been studied as a potential method to promote transplant tolerance. This draws upon the large body of early data showing that antigen-specific non-responsiveness could be induced in adult animals by intravenous injection with non-immunogenic forms of protein, red blood cells, or disrupted splenocytes (85–91). Fabre *et al.* then demonstrated that multiple intravenous injections of donor blood resulted in long-term survival of donor-strain renal allografts (148). Dallman *et al.* followed up the observations by identifying the mechanism of allograft tolerance induced by donor blood transfusion as the induction of clonal anergy by disrupting the production of IL-2 and expression of IL-2 receptor alpha and beta chains on donor-specific T cells (149). However, this method of tolerance induction shares the same clinical impracticality as intrathymic antigen exposure in that the recipients need to be exposed to donor antigens at least one week prior to transplantation. As such, a method to induce transplantation tolerance without prior donor antigen exposure to accommodate the organ donation after cardiac or brain death is still to be found.

While examples described above providing proof-of-concept that inducing transplantation tolerance in an immunologically mature recipient is achievable are clearly important, a key rate-limiting issue is the method of delivery and its efficient application in the clinic. As outlined in section 1.1.2.3, the ideal tolerance-promoting agent should be able to induce long-term donor-specific tolerance with a transient administration. Also, since organ donation originates primarily from cardiac- or brain-dead donors, tolerizing protocols that require pre-conditioning with specific donor antigens are highly impractical if not impossible. Therefore, tolerance-promoting therapy should be donor-independent agents that could be administered to patients around the time of transplantation. These agents should then specifically alter the early events of T cell activation in order to derail them from initiating a destructive response against the donor antigen. As the

detailed signaling events modulating peripheral self-tolerance were identified, it became apparent that a key deciding factor deciding between T cell tolerance and immunity is the context in which a naïve T cell become activated. The strength of T cell interaction with APCs, which involves the sum total of signals through TCR, costimulatory and coinhibitory molecules, and adhesion molecules, varied cytokines, and other cellular and humoural components all can influence the downstream effector function of the T cell following antigen encounter. Monoclonal antibodies disrupting specific T cell surface costimulation or adhesion molecules were thus developed on this premise and showed great promise as clinically applicable transplant-tolerance-promoting therapies. In the following section, major signals in T cell activation and induction of transplant tolerance by targeting these signals will be discussed.

1.2.2.1 Signals in T Cell Activation

To productively engage with a cognate antigen, naïve T cells interact with APCs presenting the antigens and become activated. The consequence of activation is dependent on the strength and quality of signals between the T cell and the APC, and the T cell could potentially proceed into various differentiation pathways that will determine their functionality. As described previously, peripheral self-tolerance is particularly dependent on utilizing reduced activation signal strength to impart tolerance to autoreactive T cells. Generally, T cell activation requires the interplay between three types of signals, including TCR engagement with antigen presented on MHC, costimulation, and adhesion. The cytokine milieu in the immune microenvironment, such as the presence of inflammatory cytokines such as interferons, is also considered as an additional signal of T cell activation. However, since these cytokines are the result of initial T cell activation and are therefore an amplification of an established activation phenotype, and targeting T cell activation signals as a means of inducing transplantation tolerance generally focuses on perturbing T cell activation immediately during initial T cell recognition of the transplant, the following discussion will focus on the signals from TCR engagement, costimulation, and

adhesion. The role of each of these signals in T cell activation will be described below (Fig. 1-3).

1.2.2.1.3 TCR:Antigen-MHC Engagement

T cells recognize specific antigens presented on self-MHC through their clonotypic TCRs. This was originally shown by Zinkernagel and Doherty that cytotoxic T cells could only kill virus-infected target cells if the target cell and the T cell matched at the MHC loci (15, 16). The requirement of self-MHC recognition was further supported by the process of positive selection, or MHC restriction, during T cell thymic maturation. Developing, double-positive (CD4+CD8+) thymocytes with weak or no recognition of self-MHC will die by neglect, thus requiring thymocytes to be able to interact with self-MHCs in order to survive maturation (150). T cell activation in this context is therefore a highly specific event requiring the correct antigen presented by self-MHC. It is interesting then that an adult individual actually harbors a high frequency of mature T cells capable of recognizing non-self (allogeneic) MHC molecules (10, 13, 151). This process of allorecognition is the basis of T cell rejection of an allogeneic transplant. Since the presence of alloreactive T cell in the periphery is very high, a transplant can elicit a rapid activation and expansion of direct alloreactive T cells leading to acute allograft rejection.

However, alloreactive T cells directly activated by foreign MHC are not the only T cell recognition event in the context of a transplant. As described in Section 1.1.1, there exists also a population of self-MHC-restricted T cells that can recognize processed allo-antigens presented on self-MHC (40, 152). These T cells can therefore be indirectly activated by transplant donor antigens processed through self-APCs (153). As the frequency of indirect alloreactive T cells resembles T cell clones recognizing a peptide antigen, it is much lower than direct alloreactive T cells. The indirect T cells are thus implicated in a slower, chronic form of transplant rejection. Taken as a whole, a transplant recipient harbors T cells that could engage donor antigen both directly through recognizing donor

MHC, or indirectly via donor antigen presented on self-MHC, and both T cell populations could mediate transplant rejection.

1.2.2.1.2 Costimulation

While TCR interaction with transplant antigens initiates alloreactive T cell recognition, this interaction alone is insufficient to activate a destructive response by the T cells. Binding of costimulatory molecules expressed on naive T cell surface with their ligands on APCs is another essential signal in T cell activation. This secondary signal could determine whether a naïve T cell become fully activated and proceed into productive clonal expansion and functional differentiation (154). Several costimulatory molecules have been identified to play crucial roles in T cell activation.

The B7 superfamily consists of several costimulatory molecules with strong influence on T cell immunity. Pathways in this family of costimulatory molecules include CD28/CTLA-4-CD80/CD86, and ICOS-ICOSL. CD80/CD86-CD28/CTLA-4 is one of the earliest costimulatory pathways identified, and is one of the most intensively studied signals in the induction of transplant tolerance. CD28 is constitutively expressed on the surface of naïve CD4+ and CD8+ T cells (155). CD28's role in T cell activation was originally recognized when it was discovered that TCR engagement of antigen without IL-2 led to T cell anergy instead of proliferation, and CD28 augmented T cell IL-2 production when bound to its ligands, CD80 and CD86, on APCs (113, 156). CD28 costimulation lowers the activation threshold of naïve T cells, thus allowing T cells to be activated with fewer TCR-antigen engagements (157). CD28 engagement also up-regulates T cell IL-2 production and promotes naïve T cell survival, thus enabling them to maintain optimal clonal expansion and differentiation (156, 158, 159). In contrast, CTLA-4, which also binds to CD80 and CD86, delivers a negative signal blocking signal transduction from TCR and CD28 (160–162). CTLA-4 was identified due to structural homology to CD28 (163). Unlike the constitutive expression of CD28 found on T cells, CTLA-4 expression is not up-regulated until a few days after T cell activation (161). Once expressed, it binds CD80 and

CD86 with greater affinity than CD28 and delivers an inhibitory signal that inhibits T cell IL-2 production and prevents entry into cell cycle and proliferation (161, 162, 164–166). In other words, upon TCR-antigen engagement, CD28 ligation with CD80/CD86 promotes T cell activation and survival, and CTLA-4 induces T cell anergy.

A second class of costimulatory molecules is the tumor necrosis factor (TNF)-TNF receptor superfamily with pathways including CD40-CD154, OX40-40L, 4-1BB-4-1BBL, CD27-CD70, and CD30-CD30L (120). The first members of the TNF-TNFR family to be identified as costimulatory molecules are CD40 and its ligand CD154 (CD40L) (120). CD40 is constitutively expressed on APCs including B cells, dendritic cells, macrophages, and thymic epithelium (167). Pro-inflammatory cytokines such as TNF-α, IL-1-β, and interferon-γ can also induce CD40 expression on endothelial cells and fibroblasts (167, 168). CD154, or CD40-ligand, is primarily expressed on activated CD4+ T cells but can also be found on a subset of CD8+ T cells, NK cells, and eosinophils (167). The ligation of T cell CD154 with CD40 on APCs up-regulates expression of costimulatory molecules CD80, CD86, and adhesion molecule ICAM-1 on APCs and promotes their ability to trigger T cell proliferation and production of pro-inflammatory cytokines (169, 170). Furthermore, APCs with augmented costimulatory capacities after CD40-CD154 interaction can provide help in priming the cytotoxic CD8+ T cell response (171, 172). Therefore, CD40-CD154 can be considered as a reverse costimulation mechanism in which T cells provide the costimulatory signal to APCs. The enhanced antigen-presentation and costimulatory capacity of "activated" APCs then indirectly augment T cell activation. In other words, CD40-CD154 pathway creates a feedback loop that broadly impacts both T cells and APCs in determining the consequence of T cell activation. As such, this pathway is a key candidate for manipulating T cell response to an allograft.

1.2.2.1.3 Adhesion

While specific TCR-antigen-MHC interaction and costimulatory signals are crucial in naïve T cell activation, these events cannot occur if the circulating T cell cannot sustain cell-cell contact with APCs. Thus, a third crucial component of T cell activation is the adhesion between T cells and APCs. T cells interact with APCs in three steps. First, a migrating T cell initiates contact with an APC. If the APC does not present its cognate antigen, this interaction is brief, and the T cell continues its migration. However, if the APC presents the correct antigen-MHC complex, the signal from the TCR-antigen-MHC engagement stops T cell migration and begins polarizing other T cell surface receptors towards the point of contact (173). Second, the T cell forms a stable interaction plane with the APC called the immunological synapse (174, 175). At this stage, the T cell is arrested on the APC, allowing ligation of costimulatory molecules and their receptors, and T cell activation usually occurs at this stage (175). Finally, the immunological synapse collapses, and the T cell regains its high motility state, detaches from the APC, and begins proliferation (176). A stable immunological synapse is thus essential in productive T cell costimulation and activation. The formation of the immunological synapse also occurs in three phases. The combined signals of TCR recognition of cognate antigen-MHC complex and the ligation of T cell leukocyte function antigen-1 (LFA-1) with intercellular adhesion molecule-1 (ICAM-1) on the APC initiate synapse formation, resulting in temporary arrest in T cell migration (173). Early immunological synapse formation is followed by recruitment of a host of signaling molecules, kinases, and phosphatases that lead to protein and lipid scaffolding and the establishment of a stable signaling platform between the T cell and the APC (175). This allows the maturation of the synapse and the third phase of synapse formation to occur. T cell co-receptors, costimulatory receptors and ligands, cytokine receptors, and more adhesion molecules are recruited to the synapse, and the T cell is now receptive to costimulation and cytokines influencing its activation and lineage commitment (175, 177–180). As such, adhesion molecules are crucial in establishing the immunological synapse and facilitating activation and costimulation of naïve T cells.

The key surface molecules mediating immunological synapse formation is LFA-1 on T cells and its ligand, ICAM-1, on APCs. LFA-1 is a β-2 integrin heterodimer consisted of an unique CD11a α chain and a common CD18 β chain shared by other β-2 integrins (181). Baseline level LFA-1-ICAM-1 interaction allows migrating T cells to form brief cell-cell contact with APCs and facilitates their rapid and constant survey of presented antigens (182). A specific TCR-antigen-MHC engagement sends intercellular signals to strengthen LFA-1-mediated cell adhesion and induces clustering of LFA-1 toward the point of TCR engagement, thereby initiating the early stages of immunological synapse (183). LFA-1-ICAM-1 interaction also maintains the synapse during T cell costimulation to ensure prolonged cell-cell contact between the T cells and APCs (175). Importantly, binding of LFA-1 to ICAM-1 also acts as a potent costimulatory pathway. Naïve T cells cross-linked by anti-CD3 antibody alone *in vitro* cannot proliferate, but proliferation is rescued by adding an anti-ICAM-1 antibody (184). LFA-1-ICAM-1 interaction also synergizes with CD28-CD80/CD86 costimulation to enhance T cell IL-2 production via non-redundant signaling pathways (185–187). In fact, it was shown that high TCR-antigen density cannot compensate for the lack of LFA-1-ICAM-1 engagement, and naïve CD4+ T cells could not be activated without LFA-1-ICAM-1 interaction, supporting the concept that these two molecules are critical for more than T cell adhesion (188). More recently, LFA-1-ICAM-1 signaling was also shown to induce a genetic profile with up-regulated TGF-β inhibitory molecules, implying a role for LFA-1 signaling to render T cells refractory to the dampening of immune reactivity by TGF-β (189). With their diverse role in T cell activation, LFA-1 and ICAM-1 have garnered great interest as potential targets for manipulating T cell response as a means of generating transplantation tolerance.

1.2.2.2 Targeting T Cell Activation Signals for Transplantation Tolerance

As naïve T cells require adhesion and costimulation in addition to TCR-antigen-MHC recognition for productive activation and differentiation, and various adhesion and costimulatory molecules involve in the decision between a

positive (induction of T cell immunity) and a negative (lack of T cell immunity) response following T cell activation, these molecules appear to be excellent candidates for therapeutic manipulation of antigen-specific T cell response. This is achieved by generating either antagonistic or agonistic monoclonal antibodies and similar biologic agents that specifically bind to the T cell surface adhesion or costimulatory molecules. Unlike the immunosuppressive depleting antibodies described in section 1.1.2.2, however, these antibodies interfere with T cell adhesion or signaling pathways without killing or lysing the T cells. Furthermore, disrupting these adhesion and costimulation pathways have shown great efficacy in prolonging allograft survival and generating donor-specific transplantation tolerance in rodent and non-human primate models. However, like the development of any therapeutic agents, manipulating cell signaling pathways using non-depleting antibodies was not without its difficulties and complexities. Each signaling pathway in the immune system potentially impacts a multitude of diverse responses, and some are not apparent until the disruption of individual pathways. Furthermore, many pathways share redundancy to compensate for each other, a phenomenon also not easily observed until specific pathways have been blocked. As such, there are still many variables and unexpected consequences resulting from the use of adhesion- or costimulation-specific antibody therapies. Despite the need for further elucidation of mechanisms and proximal impacts on T cell responses, these antibodies remain a promising alternative to immunosuppressive therapies (Table 1-2).

1.2.2.2.1 Costimulation: CD80/CD86-CD28/CTLA-4 Pathway

One way to target the CD80/CD86-CD28/CTLA-4 pathway is using monoclonal antibodies to directly disrupt CD80 and CD86. Combined anti-CD80 and anti-CD86 was first demonstrated to inhibit allograft rejection in mouse pancreatic islet transplantation model, in which the antibodies were shown to suppress alloreactive T cell proliferation against islet or splenocytes stimulation *in vitro* and delay CD4+ T cell infiltration into the graft site (190). However, results were less consistent when the antibodies were used in non-human primates.

Combined anti-CD80 and anti-CD86 therapy also delayed renal allograft rejection in non-human primates, but graft survival was not indefinite, and most recipients eventually rejected their transplant while still being treated with the antibodies (191). Allograft survival fared better when the two antibodies were used as induction therapy in conjunction with calcineurin inhibitors or sirolimus, but donor-specific tolerance was not generated in the primate model (192, 193). In addition to the rather lackluster allograft prolongation results, anti-CD80 and anti-CD86 also appeared to be a strictly immunosuppressive therapy. Therefore, direct interference of the CD80 and CD86 molecules in such manner does not appear to be a suitable candidate for generating transplantation tolerance.

Focusing on the other side of the CD80/CD86-CD28 costimulatory signaling, an anti-CD28 monoclonal antibody has also been tested in several transplantation models. Short-term administration of the antibody induced long-term renal allograft survival with generation of regulatory-type immune cells in rats and prevented graft-versus-host disease in a mouse bone-marrow transplant model (194–196). Paradoxically, this antibody turns out to send an agonistic signal through CD28. As CD28 costimulation abrogates T cell anergy and promotes positive T cell activation, facilitating allograft survival with agonistic anti-CD28 was a puzzling phenomenon. Anasetti *et al.* proposed that CD28 costimulation enhanced T cell activation with intermediate-avidity antigens but induced apoptosis when encountering high-avidity antigens, and further demonstrated interferon-γ-dependent donor-specific CD8+ T cell depletion in a mouse bone-marrow transplant model (196–198). The same antibody also promoted T_H-2 phenotype during CD4+ T cell activation with the production of IL-4 and IL-10 *in vitro* and expanded protective CD4+CD25+ regulatory T cells in a rat experimental autoimmune encephalomyelitis model (199, 200). Interestingly, the agonistic anti-CD28 showed no adverse effects in rodent and non-human primate models. Unfortunately, as a tragic illustration of the complexity and variability of immune responses across different animal models and the caution and care required to translate immune therapies from bench to bedside, a clinical safety trial of humanized agonistic anti-CD28 resulted in

immediate and massive cytokine storm and severe multi-organ failure in all of the healthy volunteers (201). It was later shown that, unlike the tolerizing effects seen in animal models, human lymphocytes underwent profound proliferation and cytokine release when treated with the antibody *in vitro* (202). Subsequently, anti-CD28 was eliminated as a potential candidate for transplantation tolerance therapy after imparting the grim lesson that a lack of stringent and multi-centre pre-clinical testing of immunological therapies potentially leads to dire consequences.

With the remarkable damage induced by the agonist anti-CD28 therapy, focus in the CD80/86-CD28/CTLA-4 pathways returned to blocking, rather than inducing, a costimulatory signal. Since monoclonal antibody targeting CD80 and CD86 resulted in only mediocre transplant survival, an alternative strategy using a CTLA-4 immunoglobulin fusion protein that ligated CD80 and CD86 with high avidity and prevented CD28 binding was created instead (203). CTLA-4-Ig promoted allograft survival and donor-specific tolerance in mouse and rat transplantation models (204–207). Hypotheses of CTLA-4-Ig's mechanism of action vary. It may act as a suppressant of donor-specific cell-mediated immunity (204). It may also preferentially up-regulate a non-inflammatory T_H-2 response to inhibit graft injury from the inflammatory T_H-1 response (207). Induction of early donor-specific T cell anergy by blocking IL-2 production or T cell deletion through apoptosis or activation-induced cell death have also been suggested (208, 209). Subsequent to the early promising results, a second-generation of CTLA-4-Ig, LEA29Y, was developed to further increase binding avidity to CD80 and CD86 (203). LEA29Y was efficacious in prolonging allograft survival in non-human primate models when combined with immunosuppressive agents and human clinical trials suggested LEA29Y as a safer replacement for calcineurin-inhibitors (203). Interestingly, increasing the binding avidity appeared to eliminate the ability CTLA-4-Ig to promote tolerance and rendered it immunosuppressive. A potential reason for this may be that CD80 and CD86 are ligands to both the costimulatory CD28 and coinhibitory CTLA-4. Extensive and thorough blocking of CD80 and CD86, while preventing CD28 costimulation,

also thus abrogating immune regulation through CTLA-4. Nevertheless, the efficacy of LEY29Y in human transplantation trials presents a landmark for taking the concept of altering T cell activation signals as an immune therapy into the clinic.

1.2.2.2.2 Costimulation: CD40-CD154 Pathway

Monoclonal antibody against CD154 has shown great efficacy in prolonging allograft survival and promoting tolerance in different animal models. Transient anti-CD154 treatment combined with transfusion of donor lymphocytes reliably prolong allograft survival and generated donor-specific tolerance in mouse models (210–212). While short-term treatment with anti-CD154 alone was not as efficacious at tolerance induction, it proved to be excellent at synergizing with other transient antibody therapies such as CTLA-4-Ig, anti-LFA-1, anti-CD8, and anti-CD45RB to induce strong donor-specific tolerance in mouse allograft and xenograft models (213–219). While transient treatment with anti-CD154 alone was not successful in prolonging allograft survival in non-human primates, when used continuously or combined with other agents, anti-CD154 becomes highly effective in preventing acute rejection and promoting indefinite graft survival (220–224).

Although anti-CD154 appears to be the most successful non-depleting antibody in promoting transplantation tolerance so far, its mechanism is widely debated. While there is strong evidence that maintenance of anti-CD154-mediated graft survival is mediated by immune cells with regulatory functions, the induction phase of tolerance remains vague, and the proximal impact of the antibody on antigen-specific T cells is controversial (216, 225). For instance, anti-CD154 may or may not promote T cell anergy or deletion (209, 212, 215, 226–228). Anti-CD154 may generate adaptive CD4+ T regulatory cells (229, 230). Anti-CD154 may inhibit inflammatory T cell response by directly triggering a dominant non-inflammatory T_H-2 response or indirectly suppressing dendritic cell inflammatory cytokine production during antigen presentation (231–233). Regrettably, clinical trials of humanized anti-CD154 were suspended when

patients unexpectedly developed thromboembolic complications (234). It was then identified that, unlike rodents, CD154 was expressed on human platelets and treatment with anti-CD154 disrupted thrombostasis (203). Therefore, while anti-CD154 was amongst the most efficacious transplant tolerance-promoting agent to date and much of its potentials is still to be elucidated, it unfortunately was not feasible to be used as a clinical therapy.

Despite the setback, targeting the CD40-CD154 pathway with anti-CD154 remains one of the most successful transplant tolerance-promoting strategies observed. Furthermore, anti-CD154's ability to positively synergize with various antibodies and immunosuppressive agents made it a strong candidate for clinical application. Therefore, attempts are currently underway to develop an antibody against CD40 to potentially recapitulate the effect of anti-CD154. An early prototype of an antagonistic anti-CD40 was administered in conjunction with anti-CD86 in a non-human primate kidney allograft model (235). While the combination prevented acute rejection to a degree, all the animals rejected their grafts either during the treatment or immediately after its cessation (235). Larsen *et al.* subsequently developed and tested different isoforms of anti-human- and anti-mouse-CD40 (236–238). As a potential complication, however, these newer generations of antibodies still induced some agonistic signals, which was worrying considering the disastrous clinical trial with the agonistic anti-CD28 (236, 237). Nevertheless, while not as efficacious as a mono-therapy as anti-CD154, anti-CD40 developed by the Larsen groups achieved significant prolongation of allograft survival in mice and both allograft and xenograft survival in non-human primates when combined with LEA29Y, basiliximab (anti-IL-2-receptor), and sirolimus (236–238). However, it is unclear whether anti-CD40 can generate donor-specific transplantation tolerance beyond facilitating graft survival as anti-CD154. Taken together, anti-CD40 is a potential candidate for a transplant tolerance therapy provided more studies are carried out to interrogate its exact mechanisms and address safety concerns.

1.2.2.2.3 Adhesion and Costimulation: ICAM-1-LFA-1 Pathway

Similarly to anti-CD154, targeting the ICAM-1-LFA-1 pathway with anti-LFA-1 alone resulted in varied efficacy in prolonging allograft survival depending on the model (239–242). When combined with another biologic agent, however, transient treatment with anti-LFA-1 becomes strongly effective in promoting graft survival and donor-specific transplant tolerance (218, 219, 239, 243–245). For instance, a short course of anti-LFA-1 combined with anti-CD154 induced dominant and transferable donor-specific tolerance in a mouse pancreatic allograft model (218). The combination also promoted indefinite survival and tolerance in mice receiving xenogeneic porcine islet transplants (219, 246). Combination treatments including anti-LFA-1 as an induction therapy also proved to be successful in prolonging allograft survival in non-human primates (247). In fact, anti-LFA-1 appeared to be as promising a tolerance-promoting therapeutic candidate as anti-CD154 had been.

However, similarly to the antibodies described previously, mechanism and proximal impact of anti-LFA-1 treatment is unclear. It is known that anti-LFA-1 does not induce T cell depletion and allowed eventual T cell infiltration into the graft without disrupting tolerance (203). Interestingly, in certain models, complete abrogation of the ICAM-1-LFA-1 pathway by combining anti-ICAM-1 and anti-LFA-1 actually disrupted tolerance induction, suggesting induction of tolerance by transient anti-LFA-1 therapies required a certain threshold of T cell anti-donor reactivity (248). In other words, perturbing the ICAM-1-LFA-1 pathway could be either tolerogenic or immunosuppressive depending on the degree of interference. This was perhaps the reason that humanized anti-LFA-1 (efalizumab) given chronically to autoimmune psoriasis patients in a clinical trial resulted in two fatal cases of progressive multifocal leukoencephalopathy (PML), an opportunistic infection usually observed in severely immunosuppressed individuals (249, 250). While efalizumab, as a short-term induction therapy, had by then demonstrated compelling efficacy in promoting accelerated graft function and survival with few side effects in human renal and pancreatic islet transplantation trials, the antibody was unfortunately recalled from the market due to the PML cases (251–253).

Nevertheless, like anti-CD154, anti-LFA-1 has proven to be one of the most effective biologic agents in facilitating transplant survival and tolerance.

1.3 Rationale and Objectives of the Thesis

When solid organ transplantation proved to be a technical possibility but resulted in overwhelming immune responses, tremendous efforts were dedicated to understanding the immune system in search of methods to manipulate the responses into accommodating a foreign graft. In the last sixty years, we have gained significant insights into the mechanisms of allograft rejection and the essential role T cells play in the response. However, we still have not found an effective control of transplant rejection other than systemically and chronically suppressing the entire immune system. While the advancement in newer generations of immunosuppressive therapies allowed increasingly positive control of early transplant rejection, long-term and persistent graft survival remains difficult to accomplish. Parallel to knowledge gained in transplantation immunology, we have also unraveled some aspects of T cell's role in maintaining immune tolerance to self-antigens. It thus appeared to be a logical progression to apply knowledge learned from the studies in self-tolerance to achieving transplantation tolerance. If specific, self-perpetuating tolerance can be generated toward an allograft, a transplant patient will no longer need to rely on chronic immunosuppression in order to maintain transplant survival.

Since then, we have attempted to apply nearly all the self-tolerance concepts in the context of transplantation. As a result, we have recognized that using highly specific antibodies to target T cell activation signals appeared to be the most clinically applicable method of transplant tolerance induction. However, while successful promotion of transplantation tolerance has now been repeatedly observed in animal models, the mechanisms of tolerance induction remain unknown. Our knowledge is limited to the induction agents used and their final consequences regarding transplant survival. While many mechanisms have been proposed, systematic studies of the necessary events turning a destructive immune response into a tolerant one are still lacking. It is unknown whether there is one

predominant mechanism shared by all tolerance-promoting therapies, or if multiple possible pathways exist. This lack of cohesive knowledge impedes us from being able to rationally design a transplant therapy that maximizes benefits while minimizes undesirable adverse effects. Some of this lack is illustrated by the repeated suspension of antibody therapies that were rushed into clinical trials without a thorough understanding of their detailed impact on the immune system.

Therefore, the primary objective of the studies detailed in my thesis is to elucidate the mechanisms by which antibodies targeting T cell activation signals induces transplantation tolerance and their precise proximal impact on antigen-specific T cells during this process. We selected two representative monoclonal antibodies targeting T cell activation, anti-CD154 and anti-LFA-1, for these studies. The reasoning for their selection is two-fold. First, while many potential mechanisms have been proposed to explain the proximal impact of anti-LFA-1 and anti-CD154 on antigen-specific T cells, there is little consensus between studies, and a systematic interrogation of the issue has not been satisfactorily performed. Second, our laboratory has extensively demonstrated the efficacy of anti-CD154 and anti-LFA-1 in facilitating transplant survival. Significantly, when used as a combination therapy, these two antibodies consistently resulted in indefinite graft survival and generated potent donor-specific and transferable tolerance. Besides from our laboratory, many other groups have also reproducibly demonstrated these two antibodies' propensity in promoting transplantation tolerance. Therefore, anti-LFA-1 and anti-CD154 are reliable candidates for studying T cell responses during transplant tolerance induction, as tolerance is a known consequence resulting from transient treatments with these antibodies.

For my studies, I utilized two congenic mouse models to track the fate of adoptively transferred antigen-specific T cells *in vivo* during activation and treatments with anti-LFA-1 and anti-CD154. The first was a TCR transgenic model that allowed the tracking of a defined monoclonal population of responsive T cells. This provided an overall representation of any major impacts of anti-LFA-1 and anti-CD154 on T cell activation. To compensate for issues with precursor frequency and monoclonal T cell responses that came with using a TCR

transgenic model, I also performed my experiments in parallel on a second polyclonal, alloreactive model. By combining these two T cell activation models, I hope to present a thorough and systematic representation of antigen-specific T cell fate during the induction phase of anti-LFA-1 and anti-CD154 treatments.

1.4 Thesis Hypotheses

I hypothesize two models of the proximal impact of anti-LFA-1 and/or anti-CD154 on antigen-specific T cells based upon our existing understanding of the induction and maintenance of self-tolerance.

1.4.1 First Model: Induction of Alternate T Cell Phenotypes

The first mechanism of anti-LFA-1 and/or anti-CD154-mediated transplantation tolerance I hypothesize **requires an immediate change in T cell activation phenotypes upon antibody treatments**. The rationale for this hypothesis draws upon our understanding of peripheral self-tolerance, in which naïve autoreactive T cells enter the periphery with potential to initiate destructive immunity toward self-antigens. However, due to certain events during early T cell interaction with its cognate antigen such as lack of costimulation, inappropriate affinity or avidity, or activation state of the APCs, the post-activation fate of the autoreactive T cells becomes clonal anergy, clonal deletion, or differentiation into tolerance-prone phenotype. As the generation of transplantation tolerance requires the presence of these therapeutic antibodies, it is logical that the antibodies exert direct influence on graft-reactive T cells to render them non-destructive or tolerogenic (Fig. 1-4A).

1.4.2 Second Model: Inhibition of Early T Cell Reactivity

The alternative model of anti-LFA-1 and/or anti-CD154-mediated transplantation tolerance **requires the down-regulation of T cell reactivity without deviation in T cell phenotype upon antibody treatments**. The rationale of this hypothesis still bases upon our knowledge of the mechanisms of peripheral self-tolerance. However, in this case, instead of the therapeutic antibodies being the agents to

directly tolerize T cells, their primary role is to dampen immediate T cell reactivity sufficiently to allow for early transplant survival and suppress extensive inflammation. In this scenario, the transplant, in an immunological environment with down-regulated stress and stimulatory signals, can then tolerize graft-reactive T cells during interactions subsequent to antibody treatment. In other words, the transplant, and not the antibodies, is the T cell tolerogen, and it achieves this by mimicking peripheral self-antigens in an immunologically quiescent state (Fig. 1-4B).

1.5 FIGURES

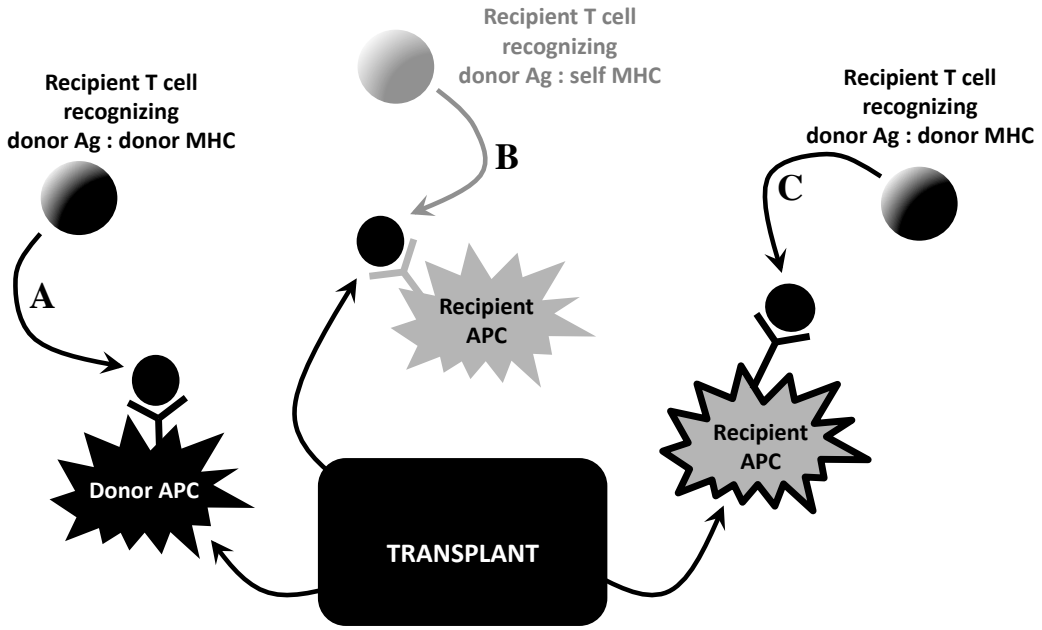
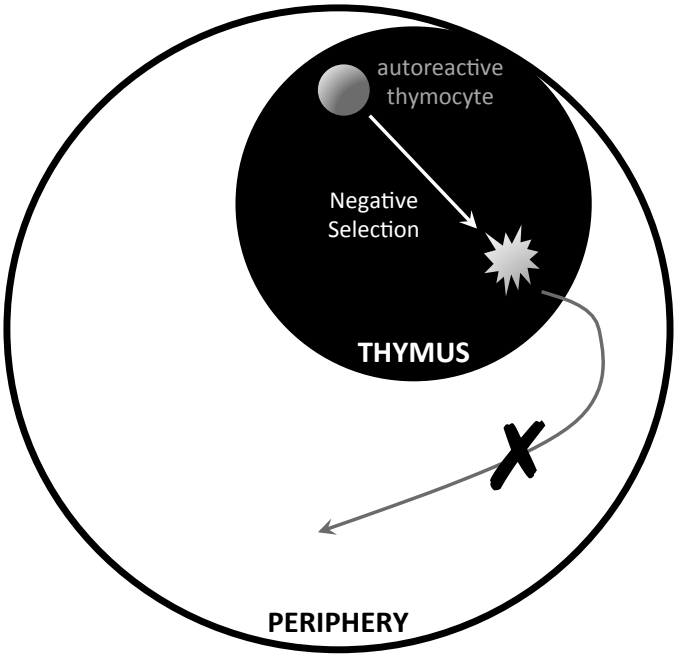


FIGURE 1-1. Pathways of T cell-mediated recognition and rejection of transplant. *A.* Direct recognition pathway. *B.* Indirect recognition pathway. *C.* Semidirect recognition pathway.

A



B

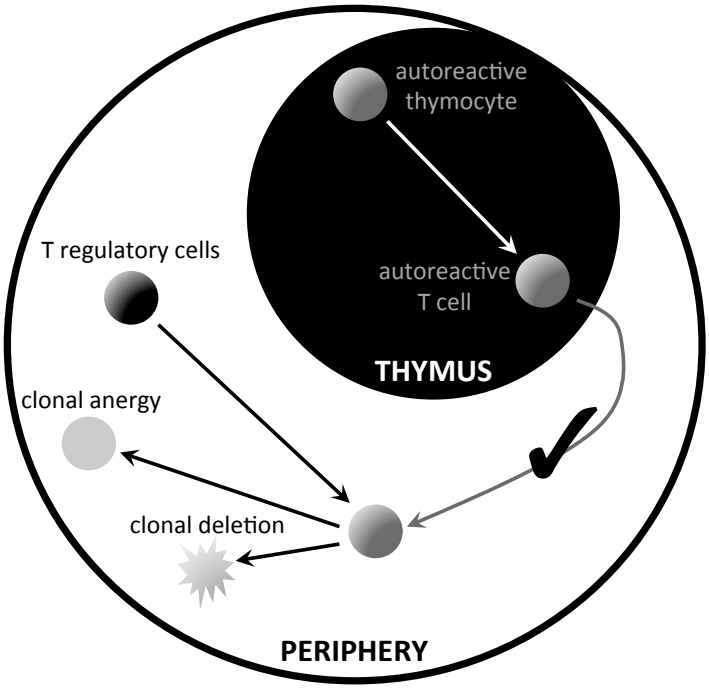


FIGURE 1-2. Mechanisms of self-tolerance. *A.* Central tolerance. *B.* Peripheral tolerance.

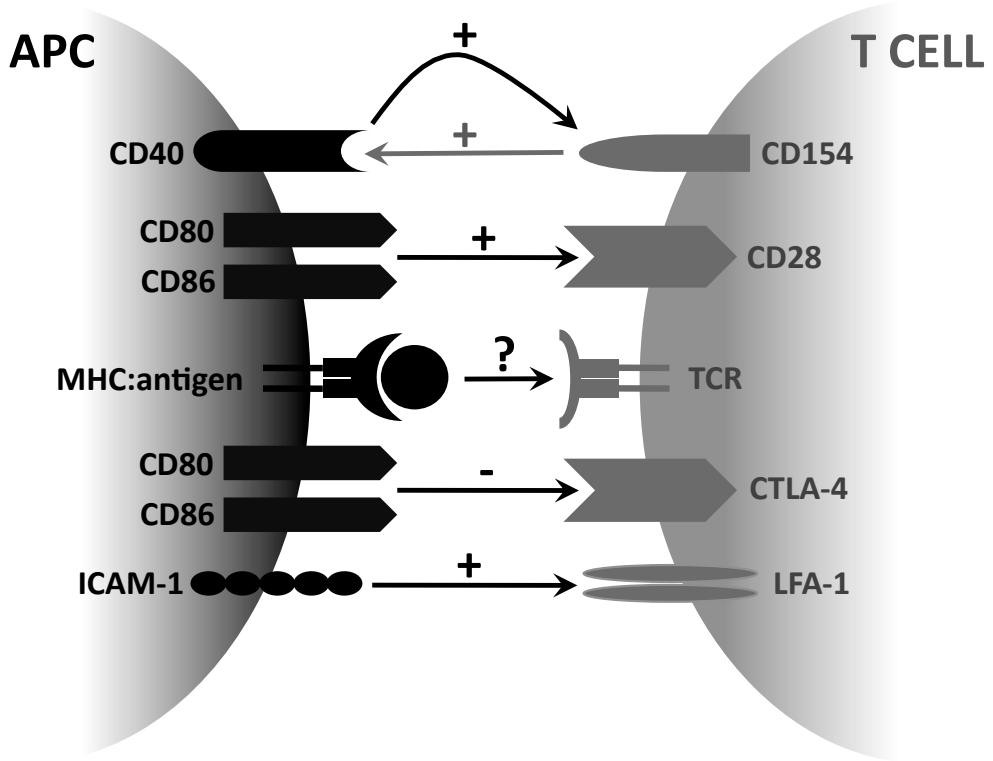
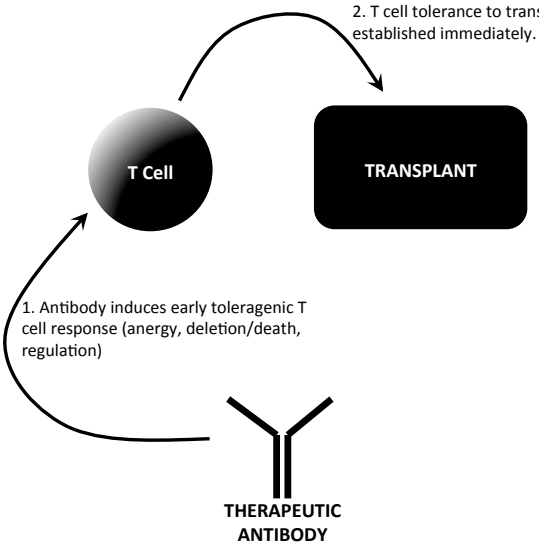


FIGURE 1-3. Signals affecting the outcome of T cell activation mentioned in this thesis. The effect of TCR-MHC-antigen recognition is indicated as unknown because whether this recognition leads to activation is dependent on the sum total of costimulatory versus coinhibitory signals received by the T cell.

A



B

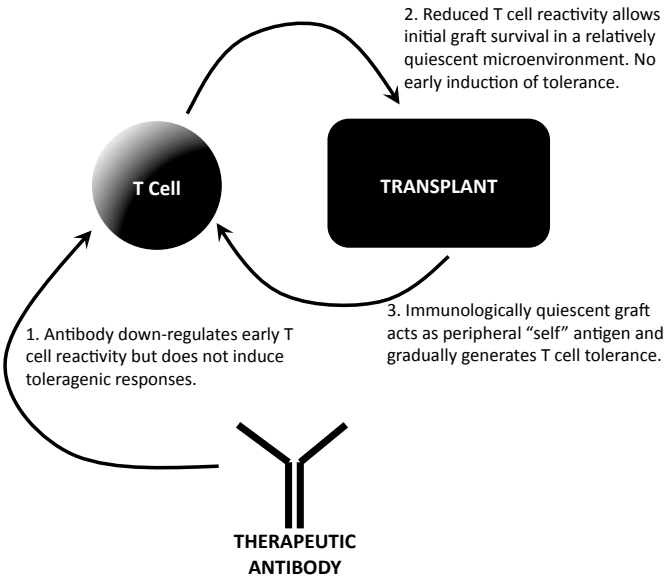


FIGURE 1-4. Hypothesized models of the proximal impact of anti-LFA-1 and/or anti-CD154 on graft-reactive T cells. *A.* Anti-LFA-1 and/or anti-CD154 directly tolerizes T cells by inducing alternate, non-destructive T cell phenotypes. *B.* Anti-LFA-1 and/or anti-CD154 do not directly tolerize T cells or induce alternate T cell phenotypes.

1.6 TABLES

Agent	Classification	Target	Mechanism on T Cells	Selected Adverse Effects
Cyclosporine	Calcineurin inhibitors	Cytosolic immunophilin: cyclophilin	Complexes with immunophilin and inhibits calcineurin activity. Blocks calcineurin-dependent IL-2 transcription. Inhibits T cell activation and proliferation.	Immunodeficiency. Nephrotoxicity. Neurotoxicity. Post-transplant diabetes mellitus.
Tacrolimus		Cytosolic immunophilin: FKBP12		
Sirolimus/ Rapamycin	Mammalian Target-of-Rapamycin (mTOR) inhibitors	Cytosolic immunophilin: FKBP12	Complexes with immunophilin and inhibits mTOR activity. Blocks T cell response to IL-2. Inhibits T cell activation and proliferation.	Immunodeficiency. Hyperlipidemia. Thrombocytopenia. Delayed wound healing Delayed graft function.
Everolimus				
Mycophenolate mofetil	Inosine monophosphate dehydrogenase (IMPDH) inhibitors	Catalytic GTP biosynthesis enzyme: IMPDH	Reversible inhibition of IMPDH. Blocks purine synthesis. Inhibits T cell growth.	Immunodeficiency. Gastrointestinal complications. Blood-related dysregulations such as anemia and hypoalbuminemia.
Antithymocyte globulin	Depletion antibodies	Multiple T cell surface markers including CD2, CD3, CD4, CD8, CD11a, CD18, CD45	Complement-dependent lysis of bound T cells	Immunodeficiency. Cytokine-release syndrome. Thrombocytopenia. Leukopenia. Serum sickness.
Alemtuzumab		CD52	Antibody-dependent lysis of bound T cells	Immunodeficiency. Cytokine-release syndrome. Neutropenia. Anemia. Autoimmune thrombocytopenia.

TABLE 1-1 Mechanisms and adverse effects of major immunosuppressive agents currently used in transplantation.

Biologic Agent	Target	T Cell Pathway	Efficacy
Anti-CD80/ Anti-CD86	CD80, CD86 on APCs	**Costimulation:** B7 Family	**Rodents and non-human primates:** Moderate graft prolongation if combined with other immunosuppressive agents. Do not appear to generate tolerance.
Anti-CD28	CD28 on T cells		**Rodents and non-human primates:** Good graft prolongation. Appeared to be toleragenic. **Humans:** agonist signaling caused cytokine storm
CTLA-4-Ig	CD80, CD86 on APCs	CD80/CD86 – CD28/CTLA-4	**Rodents and non-human primates:** Moderate graft prolongation but toleragenic using first-generation antibody. Second generation high-affinity antibody appeared immunosuppressive. **Humans:** Immunosuppressive. Good graft prolongation but increased incidents of acute rejection.
Anti-CD154	CD154 (CD40 ligand) on T cells	**Costimulation:** TNF Family CD40 – CD154	**Rodents and non-human primates:** Excellent graft prolongation and tolerance induction alone or in combined therapies. **Humans:** Thromboembolic complications
Anti-CD40	CD40 on APCs		**Rodents and non-human primates:** Moderate graft prolongation if combined with immunosuppressive agents. Ability to induce tolerance unknown.
Anti-LFA-1	LFA-1 on T cells, B cells macrophages, neutrophils	**Adhesion and Costimulation:** ICAM-1 – LFA-1	**Rodents and non-human primates:** Excellent graft prolongation and tolerance induction in combined therapies. **Humans:** Excellent graft acceptance and function when used as transient induction therapy in renal and pancreatic islet transplantation. Multifocal leukoencephalopathy complications when used as chronic immunosuppressant in psoriasis.

TABLE 1-2. Using monoclonal non-depleting antibodies and biologic agents targeting T cell activation pathways as transplantation therapies.

CHAPTER TWO
MATERIALS AND METHODS

2.1 MICE

All animals were maintained in accordance to the University of Alberta Animal Care and Use Committee and the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee guidelines.

2.1.1 Recipients

C57BL/6J (B6, H-2^b, CD45.2) mice aged 6-12 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME) or from in-house breeding colony with breeders purchased from the Jackson Laboratory

2.1.2 T Cell Donors

2.1.2.1 Monoclonal TCR Transgenic Model

The following mice were obtained from Taconic Farms (Hudson, NY) through the NIAID exchange program, NIH: B6.129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)1100Mjb (OT-I rag-/-, H-2^b; line 4175) and B6.129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)425Cbn (OT-II rag-/-, H-2^b; line 4234) (254, 255). The OT-I rag-/- and OT-II rag-/- mice were crossed with B6.SJL-*Ptprca Pep3b*/BoyJ (CD45.1 B6, H-2^b, CD45.1) mice purchased from the Jackson Laboratory until OT-I rag-/- and OT-II rag-/- mice were fixed with the congenic CD45.1 marker (CD45.1 OT-I rag-/-, CD45.1 OT-II rag-/-). In the thesis, the CD45.1 OT-I rag-/- will be referred to generically as OT-I and the CD45.1 OT-II rag-/- as OT-II. OT-I mice generate MHC-I-restricted CD8+ T cells with Vα2+Vβ5+ transgenic TCR specific for a chicken ovalbumin peptide ($OVA_{257-264}$, SIINFEKL) presented by H-$2K^b$ (256). OT-II mice generate MHC-II-restricted CD4+ T cells with Vα2+Vβ5+ TCR specific for a chicken ovalbumin peptide ($OVA_{323-339}$, ISQAVHAAHAEINEAGR) presented by H-2I-A^b (254).

2.1.2.2 Polyclonal Alloreactive Model

B6.SJL-*Ptprca Pep3b*/BoyJ (CD45.1 B6, H-2^b, CD45.1) mice were purchased from The Jackson Laboratory.

2.1.3 T Cell Antigen and Transplant Donors

2.1.3.1 Monoclonal TCR Transgenic Model

C57BL/6-Tg(ACTB-OVA)916Jen/J (B6-OVA, H-2^b) mice with membrane actin-driven expression of chicken ovalbumin protein were purchased from the Jackson Laboratory and maintained by in-house breeding (257).

2.1.3.2 Polyclonal Alloreactive Model

BALB/cByJ (BALB/c, H-2^d) mice were purchased from the Jackson Laboratory.

2.2 REAGENTS

2.2.1 General Preparations of Live Lymphocytes

Hank's balanced salt solution (HBSS) was made by diluting 10X HBSS (Sigma-Aldrich, St. Louis, MO) in purified water with 0.35 mg/L sodium bicarbonate (Sigma-Aldrich) and 10 mM N-2-hydroxyethylpiperazine-N"-2-Ethane Sulfonic Acid (HEPES; GIBCO, Invitrogen, Grand Island, NY). Eagle's essential minimal medium (EMEM) was made by adding 10% (v/v) fetal calf serum (Atlanta Biologicals, Lawrenceville, GA), 20 mM HEPES (GIBCO, Invitrogen), 1% (v/v) 100X GlutaMAX (GIBCO, Invitrogen), 1% (v/v) 100X non-essential amino acids (GIBCO, Invitrogen), 1% (v/v) penicillin/streptomycin/neomycin (GIBCO, Invitrogen), and 10^{-4} M 2-mercaptoethanol (Sigma-Aldrich) to minimal essential medium (MEM; GIBCO, Invitrogen). RBC lysing buffer was purchased from Sigma-Aldrich. Lympholyte cell separation media (Mammal) was purchased from Cedarlane (Burlington, NC). 0.4% Trypan Blue stain was purchased from GIBCO (Invitrogen).

2.2.2 Proliferation Dye Labeling

Washing solution was made by adding 10 μg/mL DNase (Sigma-Aldrich) and 20 mM HEPES to MEM (GIBCO, Invitrogen). Labeling solution was made by

adding 0.1% (v/v) fetal calf serum (Atlanta Biologicals) to filter-sterilized Dulbecco's phosphate-buffered saline (DPBS; GIBCO, Invitrogen). Quenching solution was made by adding 5% (v/v) fetal calf serum (Atlanta Biologicals) to filter-sterilized phosphate-buffered saline. Solid carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen Molecular Probes (Eugene, OR) and re-constituted into 5 mM stock solutions by dissolving in anhydrous dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO). Solid eFluor 670 was purchased from eBioscience (San Diego, CA) and re-constituted into 5 mM stock solutions by dissolving in anhydrous dimethyl sulfoxide (Sigma-Aldrich).

2.2.3 Flow Cytometry: Surface Staining

FACS washing buffer was DPBS (GIBCO, Invitrogen) with 0.01% (w/v) sodium azide (Sigma-Aldrich) and 1% (v/v) fetal calf serum (Atlanta Biologicals). FACS staining buffer is FACS washing buffer with 10 μg/mL anti-Fc receptor antibody (2.4G2; cell line purchased from American Type Culture Collection).

Antibodies CD45.1 (A20), CD4 (RM4-5), CD8α (53-6.7), CD44 (IM7), CD62L (MEL-14) and CD25 (7D4 or PC61.5). Antibodies were purchased from BioLegend (San Diego, CA), eBioscience and BD Biosciences (San Jose, CA).

2.2.4 Flow Cytometry: Intracellular Staining

Permeabilization washing buffer is 0.1% (w/v) saponin (Sigma-Aldrich) in FACS washing buffer. Fixing/Permeabilization buffer is HBSS (Sigma-Aldrich) with 4% (w/v) paraformaldehyde (Sigma-Aldrich), 0.1% (w/v) saponin (Sigma-Aldrich), and 10 mM HEPES (GIBCO, Invitrogen).

interferon-γ (XMG1.2, BD Biosciences), granzyme-B (GB11, BD Biosciences), interleukin-4 (11B11, BD Biosciences), interleukin-10 (JES5-16E3), and/or tumor necrosis factor-α (MP6-XT22), or isotype control antibodies (BD Biosciences), FoxP3 (FJK-16s, eBioscience)

polyclonal rabbit anti-mouse cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology, Danvers, MA), then followed by Alexa Fluor 647 goat anti-rabbit IgG (H+L) (Invitrogen Molecular Probes) and PE anti-Bcl-2

(BCL/10C4, BioLegend) or its isotype antibody PE mouse IgG1, κ (MOPC-21, BioLegend).

2.2.5 *in vitro* Re-Stimulation for Intracellular Cytokine Staining

Solid OT-I peptide ($OVA_{257-264}$, SIINFEKL) were purchased from GenScript (Piscataway, NJ) and re-constituted into 1 mg/mL stock solution by dissolving 1 mg of OT-I peptide in 50 μL anhydrous dimethyl sulfoxide then raise the concentration to 1 mg/mL by adding 950 μL of PBS.

Solid OT-II peptide ($OVA_{323-339}$, ISQAVHAAHAEINEAGR) were purchased from GenScript (Piscataway, NJ) and re-constituted into 1 mg/mL stock solution by dissolving 1 mg of OT-II peptide in 1 mL of PBS. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich and diluted in dimethyl sulfoxide (Sigma-Aldrich) to make 400 μg/mL stock solution. Ionomycin was purchased from Sigma-Aldrich and diluted in dimethyl sulfoxide (Sigma-Aldrich) to make 500 μg/mL stock solution. Brefeldin A were purchased from Sigma-Aldrich and re-constituted to 10 mg/mL stock solution in molecular grade 100% ethanol (Sigma-Aldrich).

2.2.6 Pancreatic Islet Transplantation

Collagenase Type V was purchased from Sigma-Aldrich. Medium A was made by adding 1% HEPES, 10 μg/mL DNase, and 1.5 g/L bovine serum albumin (Fisher Scientific, Hampton, NH). Medium B was made by adding 1% HEPES and 10% (v/v) bovine calf serum (Sigma-Aldrich) to HBSS. Histopaque was purchased from Sigma-Aldrich. Isoflurane (Fluriso) was purchased from Vet One (Boise, ID)

2.3 MONOCLONAL ANTIBODY THERAPIES

2.3.1 Anti-LFA-1 Therapy

Animals received 200 μg of anti-LFA-1 (KBA, rag IgG2a; cell line generously provided by Dr. A. Ihara at University of Tokyo) by intraperitoneal injections on days 0, 1, 7, 14 with day 0 being the day of antigen challenge or transplantation.

2.3.2 Anti-CD154 Therapy

Animal received 250 μg of anti-CD154 (MR-1, hamster IgG; purchased from Bio X Cell, West Lebanon, NH) by intraperitoneal injections on days -1, 2, 7, 9 with day 0 being the day of antigen challenge or transplantation.

2.3.3 Combined Anti-LFA-1 and Anti-CD154 Therapy

Animals received 200 μg of anti-LFA-1 on days 0, 1, 7, 14 plus 250 μg of anti-CD154 on days -1, 2, 7, 9 with day 0 being the day of antigen challenge or transplantation.

2.3.4 Ascites Preparation

Anti-LFA-1 was prepared from the KBA hybridoma cells using ascites. Out-bred ICR-SCID mice were purchased from Jackson Laboratory. The mice were injected intraperitoneally with 0.5 mL Pristane (2,6,10,14-Tetramethylpentadecane; Sigma-Aldrich) 14 to 21 days prior to injection of hybridoma cells. Pristane-primed mice were injected with $10x10^6$ KBA hybridoma cells intraperitoneally and monitored daily. When mice appeared bloated, they were euthanized and small incisions were made over their ventral peritoneum. Abdominal fluid was collected using a sterile plugged Pasteur pipet. The fluid was centrifuged at 478 x g for 10 minutes at 4-degree Celsius to remove the top Pristane layer and the red blood cell pellets. The middle ascites layer was centrifuged and purified again, then quantitated by ELISA. Unused ascites were stored in -80-degree Celsius freezer.

2.4 LABELING LYMPHOCYTES WITH PROLIFERATION DYES

Lymphocytes to be tracked for adoptive transfer experiments were harvested from donor spleens and lymph nodes (axial, brachial, inguinal, mesenteric, cervical).

The lymph nodes were dissociated by glass slides. The spleens were repeatedly cut with surgical scissors and ground through 70 micron cell strainers with a syringe plunger then depleted of red blood cells by incubating with red blood cell lysis buffer at 1 mL/spleen for 2 minutes then washed once by adding 10 mL HBSS to the lysis buffer-splenocyte mix. Lymphocytes from lymph nodes and spleens were then combined and counted. Lymphocytes were then washed once with washing solution. Single-cell suspensions were made in labeling solution at 10^6 cells/mL and incubated with either 5 μM eFluor 670 or 1 μM CFSE for 5 minutes at room temperature. The staining process was then quenched to adding equal-volume of quenching solution as the volume the cells were stained in then centrifuged. The cells were washed once in washing solution prior to resuspending in EMEM. The labeled cells were then counted again to account for any cell loss during the labeling process.

2.5 FLOW CYTOMETRY

All flow cytometric data were acquired on a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo analysis software version 9.5.2 (TreeStar, Ashland, OR).

2.5.1 Staining for Cell Surface Markers

Cells were harvested from individual experiments and processed as described in sections 2.7, 2.8, and 2.10. Single-cell suspensions of cells to be stained were transferred into 96-well V-bottom plates (Costar, Corning, Corning, NY) at 10^6 cells/well and centrifuged at 544 x g for 4 minutes. The cells were washed again by adding 200 μL FACS washing buffer per well prior to centrifuging. Fluorescence-conjugated antibodies against cell surface markers were diluted in FACS staining buffer to make a master mix. Generally, surface antibodies were diluted at a ratio of 1:500. The cells were incubated with 50 μL of master mix per well for 30 minutes in the dark at 4-degree Celsius. The cells were washed twice with then resuspended in FACS washing buffer prior to transfer into 5-mL

polystyrene round-bottom tubes (BD Falcon, BD Biosciences, San Jose, California) for flow cytometry acquisition.

2.5.2 Staining for Intracellular Cytokines

Cells were harvested from individual experiments and processed as described in sections 2.7, 2.8 and 2.10. Single-cell suspensions were re-stimulated *in vitro* for cytokine production as described in section 2.6. Cells were then transferred into 96-well V-bottom plates and centrifuged at 544 x g for 4 minutes followed by two washes with FACS washing buffer. Cells were stained with surface marker-specific antibodies as outlined in section 2.5.1. After the last wash with FACS washing buffer at the end of surface staining, cells were resuspended in fixation/permeabilization buffer at 100 μL/well for 15 minutes at room temperature. The cells were subsequently washed twice with 200 μL permeabilization buffer at 850 x g for 4 minutes. Fluorescence-conjugated antibodies specific to intracellular cytokines were diluted in permeabilization buffer to make a master mix. Intracellular cytokine antibodies were generally diluted at a ratio of 1:500. Cells were incubated with master mix at 50 μL/well for a minimum of 45 minutes in the dark at 4-degree Celsius. Cells were then washed twice with permeabilization buffer prior to resuspending in FACS washing buffer. Cells were transferred into flow cytometry tubes for acquisition.

2.5.3 Staining for the Transcription Factor FoxP3

Cells were harvested from individual experiments and processed as described in sections 2.7, 2.8 and 2.10. Single-cell suspension of harvested cells were then transferred into 96-well V-bottom plates and centrifuged at 544 x g for 4 minutes followed by two washes with FACS washing buffer. Cells were stained with surface marker-specific antibodies as outlined in section 2.5.1. After the last wash with FACS washing buffer at the end of surface staining, cells were resuspended in fixation/permeabilization buffer at 100 μL/well for 15 minutes at room temperature. The cells were subsequently washed twice with 200 μL permeabilization buffer at 850 x g for 4 minutes. Fluorescence-conjugated

antibodies specific to FoxP3 transcription factor were diluted in permeabilization buffer at a ratio of 1:200. Cells were incubated with diluted FoxP3 antibody at 50 μL/well for a minimum of 60 minutes in the dark at 4-degree Celsius. Cells were then washed twice with permeabilization buffer prior to resuspending in FACS washing buffer. Cells were transferred into flow cytometry tubes for acquisition.

2.5.4 Staining for Apoptosis Regulator Proteins

Cells were harvested from individual experiments and processed as described in sections 2.7, 2.8 and 2.10. Single-cell suspension of harvested cells were then transferred into 96-well V-bottom plates and centrifuged at 544 x g for 4 minutes followed by two washes with FACS washing buffer. Cells were stained with surface marker-specific antibodies as outlined in section 2.5.1. After the last wash with FACS washing buffer at the end of surface staining, cells were resuspended in fixation/permeabilization buffer at 100 μL/well for 15 minutes at room temperature. The cells were subsequently washed twice with 200 μL permeabilization buffer at 850 x g for 4 minutes. Polyclonal rabbit anti-mouse cleaved caspase-3 (Asp175) antibody was diluted in permeabilization buffer at a ratio of 1:200. Cells were incubated with diluted caspase 3 antibody at 50 μL/well for a minimum of 30 minutes in the dark at 4-degree Celsius then washed twice with 200 μL permeabilization buffer. Alexa Fluor 647 goat anti-rabbit IgG (H+L) antibody was diluted in permeabilization buffer at a ratio of 1:200 along with anti-Bcl-2 antibody or its isotype control at 1:50 to make master mixes. Cells were incubated with the master mix at 50 μL/well for a minimum of 30 minutes at 4-degrees Celsius in the dark. Cells were then washed twice with permeabilization buffer prior to resuspending in FACS washing buffer. Cells were transferred into flow cytometry tubes for acquisition.

Cultured thymocytes were used as positive apoptosis control. Thymi were harvested and dissociated by grinding through a 70-micron cell strainer with a 5-mL syringe plunger. Thymocytes were counted, resuspended in EMEM, and transferred into 12-well sterile cell culture plates at $5x10^6$ cells per well and cultured from 1 to 3 days prior to staining with caspase 3 antibody.

2.6 *in vitro* RE-STIMULATION FOR INTRACELLULAR CYTOKINE STAINING

2.6.1 Monoclonal TCR Transgenic Model

Lymphocytes were harvested and dissociated from lymphoid organs specified for each experiment type. Single-cell suspensions of lymphocytes were incubated in 96-well flat-bottom cell culture plates (Costar, Corning, Corning, NY) at 10^6 cells/well in EMEM containing 1 μg/mL brefeldin A with 0.1 μM OT-I peptide and/or 1.0 μM OT-II peptide depending on the transgenic T cells to be assessed for cytokines. The cells were incubated for 4 to 5 hours at 37-degree Celsius with 10% CO^2. The cells were then transferred into 96-well V-bottom plates and resuspended in FACS washing buffer for flow cytometry processing.

2.6.2 Polyclonal Alloreactive Model

Lymphocytes were harvested and dissociated from lymphoid organs specified for each experiment type. Single-cell suspensions of lymphocytes were incubated in 96-well flat-bottom cell culture-treated plates at 10^6 cells/well in Eagle's essential minimal medium containing 1 μg/mL brefeldin A with 40 ng/mL PMA and 500 ng/mL ionomycin. The cells were incubated for 4 to 5 hours at 37-degree Celsius with 10% CO^2. The cells were then transferred into 96-well V-bottom plates and resuspended in FACS washing buffer for flow cytometry processing.

2.7 LOCAL FOOTPAD ANTIGEN CHALLENGE

2.7.1 Monoclonal TCR Transgenic Model

2.7.1.1 Adoptive Transfer

Lymph nodes and spleens were harvested from naive CD45.1 OT-I rag-/- or CD45.1 OT-II rag-/- cell donors. Lymphocytes were dissociated from the lymphoid organs and labeled with proliferation dye as described in section 2.4. After labeling and cell counting, lymphocytes were resuspended in HBSS at

$5x10^6$ cells/0.1 mL for CD45.1 OT-I rag-/- and $10x10^6$ cells/0.1 mL for CD45.1 OT-II rag-/- in preparation for adoptive transfer. Recipients were briefly anesthetized by isoflurane inhalation and the lymphocytes were adoptively transferred into recipients via intravenous retro-orbital injection at a volume of 0.1 mL/recipient. Therefore, CD45.1 OT-I rag-/- were transferred at $5x10^6$ cells/recipient and CD45.1 OT-II rag-/- at $10x10^6$ cells/recipient.

To examine engraftment of the transferred lymphocytes in the recipients, 200 μL blood was collected from each recipient via submandibular bleeds 2 to 4 days post adoptive transfer. The blood was layered above 1 mL of lympholite (mammal) and centrifuged at 850 x g for 20 minutes at room temperature to separate the components. The lymphocyte layer was then collected and washed with 3 mL FACS washing buffer by centrifuging at 544 x g for 4 minutes prior to transfer into 96-well V-bottom plates for flow cytometry processing as described in section 2.5.1. CD45.1 OT-I rag-/- lymphocytes were identified by co-expression of CD45.1 and CD8, and CD45.1 OT-II rag-/- lymphocytes were identified by co-expression of CD45.1 and CD4.

2.7.1.2 Antigen Challenge and Therapy Treatment

2-4 days post adoptive transfer and after engraftment was confirmed, spleens were harvested from B6-OVA cell donor mice. The spleens were cut into small pieces and ground through 70-micron cell strainers with a 5-mL syringe plunger to dissociate the splenocytes. The splenocytes were counted then resuspended in HBSS at $1x10^6$ cells/25 μL in preparation for footpad injection. The recipients with previously adoptively transferred lymphocytes were transiently anesthetized by isoflurane inhalation and 25 μL of B6-OVA splenocytes were subcutaneously injected into each footpad of the hind paws. Therefore, each recipient received $1x10^6$ B6-OVA splenocytes per footpad in both footpads.

Therapy-treated recipients were given their respective monoclonal antibodies as outlined in section 2.3.

2.7.1.3 Harvest and Analysis

At 3 days post footpad challenge for CD45.1 OT-I rag-/- lymphocytes and 4 days post footpad challenge for CD45.1 OT-II rag-/- lymphocytes, draining popliteal

lymph nodes were harvested from recipients along with distal mesenteric lymph nodes, spleens, and blood. The blood was processed as described in section 2.7.1.1. The spleens were dissociated via described in section 2.7.1.2 and the lymph nodes via frosted glass slides. The splenocytes were RBC-depleted as described in section 2.4. Cells were then resuspended in EMEM and counted. At this point, the cells would be processed for flow cytometry as described in section 2.5 depending on the phenotypes being examined.

2.7.2 Polyclonal Alloreactive Model

2.7.2.1 Adoptive Transfer

Lymph nodes and spleens were harvested from naive CD45.1 B6 cell donors. Lymphocytes were dissociated from the lymphoid organs and labeled with proliferation dye as described in section 2.4. After labeling and cell counting, lymphocytes were resuspended in HBSS at $30x10^6$ cells/0.1 mL in preparation for adoptive transfer. Recipients were briefly anesthetized by isoflurane inhalation and the lymphocytes were adoptively transferred into recipients via intravenous retro-orbital injection at a volume of 0.1 mL/recipient. Therefore, each recipient received $30x10^6$ CD45.1 B6 lymphocytes.

The engraftments of the transferred lymphocytes were confirmed as described in section 2.7.1.1. CD45.1 B6 lymphocytes were identified as CD8+ and CD4+ cells also expressing CD45.1.

2.7.2.2 Antigen Challenge and Therapy Treatment

2-4 days post adoptive transfer and after engraftment was confirmed, spleens were harvested from BALB/c cell donor mice. Processing of splenocytes and subsequent footpad injections were as described in section 2.7.1.2.

Therapy-treated recipients were given their respective monoclonal antibodies as outlined in section 2.3.

2.7.2.3 Harvest and Analysis

At 5 days post footpad challenge, draining popliteal lymph nodes were harvested from recipients along with distal mesenteric lymph nodes, spleens, and blood. The cells were processed for analysis as described in section 2.7.1.3.

2.8 SECONDARY ANTIGEN CHALLENGE

2.8.1 *in vivo* Systemic Primary Antigen Stimulation

2.8.1.1 Monoclonal TCR Transgenic Model

CD45.1 OT-I rag-/- or CD45.1 OT-II rag-/- lymphocytes were harvested, processed, and adoptively transferred into recipients as described in section 2.7.1.1 with the exception that the lymphocytes were not labeled with proliferation dyes. 2-4 days post adoptive transfer, engraftments of transferred cells were confirmed as outlined in section 2.7.1.1. At this time, spleens were harvested from B6-OVA donor mice and processed for splenocytes as described in section 2.7.1.2. The splenocytes were resuspended in HBSS at $5x10^6$ cells/0.1 mL, and each recipient received 0.1 mL of the suspension by intraperitoneal injection. Therefore, each recipient was systemically challenged with $5x10^6$ B6-OVA splenocytes at the time of primary antigen stimulation. Therapy-treated recipients received their respective monoclonal antibodies as outlined in section 2.3.

2.8.1.2 Polyclonal Alloreactive Model

CD45.1 B6 lymphocytes were harvested, processed, and adoptively transferred into recipients as described in section 2.7.2.1 with the exception that the lymphocytes were not labeled with proliferation dyes. 2-4 days post adoptive transfer, engraftments of transferred cells were confirmed as outlined in section 2.7.2.1. At this time, spleens were harvested from BALB/c donor mice and processed for splenocytes as described in section 2.7.2.2. The splenocytes were resuspended in HBSS at $5x10^6$ cells/0.1 mL, and each recipient received 0.1 mL of the suspension by intraperitoneal injection. Therefore, each recipient was systemically challenged with $5x10^6$ BALB/c splenocytes at the time of primary antigen stimulation. Therapy-treated recipients received their respective monoclonal antibodies as outlined in section 2.3.

2.8.2 *in vitro* Secondary Antigen Challenge

2.8.2.1 Monoclonal TCR Transgenic Model

At day ≥ 21 post primary activation, the spleens from the recipients were harvested and the splenocytes dissociated as described in section 2.7.1.1. Spleens from B6-OVA mice were also harvested as secondary antigen source. The splenocytes were depleted of red blood cells as described in section 2.4, counted, and resuspended in EMEM. $1x10^6$ cells from each recipient were transferred into 96-well flat-bottom cell culture-treated plates and peptide-stimulated for 5 hours for cytokine generation as described in section 2.6.1. The cells were subsequently processed for intracellular cytokine staining and flow cytometry as described in section 2.5.2.

The remaining recipient splenocytes were labeled with proliferation dyes as described in section 2.4. The recipient cells were resuspended in EMEM at $10x10^6$ cells/mL, and the B6-OVA splenocytes at $30x10^6$ cells/mL. 1 mL ($10x10^6$ cells) of the recipient splenocytes were added into 25-cm^2 cell culture flasks (Corning, Corning, NY) standing upright with or without 1 mL ($30x10^6$ cells) of the B6-OVA splenocytes. The flasks were topped off to a final volume of 20 mL with EMEM. The cells were incubated at 37-degree Celsius and 10% CO_2 for three days. At which point, the cells were harvested from the culture flasks and processed for flow cytometric analysis as described in section 2.5.1.

2.8.2.2 Polyclonal Alloreactive Model

At day ≥ 21 post primary activation, the spleens from the recipients were harvested and the splenocytes dissociated as described in section 2.7.1.1. Spleens from BALB/c mice were also harvested as secondary antigen source. The splenocytes were depleted of red blood cells as described in section 2.4, counted, and resuspended in EMEM. $1x10^6$ cells from each recipient were transferred into 96-well flat-bottom cell culture-treated plates and stimulated for 5 hours with PMA/ionomycin for cytokine generation as described in section 2.6.2. The cells were subsequently processed for intracellular cytokine staining and flow cytometry as described in section 2.5.2.

The remaining recipient splenocytes were labeled with proliferation dyes as described in section 2.4. The recipient cells were resuspended in EMEM at

$10x10^6$ cells/mL, and the BALB/c splenocytes at $30x10^6$ cells/mL. 1 mL ($10x10^6$ cells) of the recipient splenocytes were added into 25-cm^2 cell culture flasks standing upright with or without 1 mL ($30x10^6$ cells) of the BALB/c splenocytes. The flasks were topped off to a final volume of 20 mL with EMEM. The cells were incubated at 37-degree Celsius and 10% CO_2 for three days. At which point, the cells were harvested from the culture flasks and processed for flow cytometric analysis as described in section 2.5.1.

2.9 TRANSPLANTATION SURGERIES

2.9.1 Pancreatic Islets

2.9.1.1 Pancreatic Islet Isolation

Donor mice were anesthetized via intraperitoneal Avertin injection, secured to the surgical board with abdomens facing upwards, and drenched with 70% denatured ethanol. Incisions were made into the skin and viscera to expose the peritoneum. Donors were exsanguinated at this point by incisions to the abdominal aorta/vena cava. Liver lobes were reflected to expose the gall bladder and common bile duct. The bile duct was clamped off at the junction with the small intestines, and the pancreas was injected with 4 mL of 2.5 mg/mL collagenase solution through the bile duct and removed. The pancreas was placed in a 50-mL tube and incubated in a 37-degree Celsius water bath until the islets had separated from the exocrine tissue. The incubation time was approximately 8-12 minutes and mouse strain-dependent. The incubation was terminated by shaking and disrupting the tube containing the digesting pancreas followed by adding and mixing 45 mL of medium A to the tube. The tube was placed on ice for 5 minutes to allow tissue settling and 20 mL of supernatant was vacuum-aspirated. This process was repeated twice, then the tissue was filtered into a new 50-mL tube through a wire filter screen to remove undigested pancreas and lymph nodes. The tube was topped off with medium B and centrifuged briefly at 850 x g to form a soft pellet. The supernatant was discarded, and the pellet was resuspended in 5 mL medium B. 5 mL of histopaque was layered beneath the pellet mix and centrifuged at 850

x g for 20 minutes, at which point the islets were collected at the interface. The islet interface layer was transferred to a 15 mL centrifuge tube, added with medium B, and centrifuged at 850 x g for 35 seconds. The supernatant was discarded and the tube was filled with medium B and placed on ice for 5 minutes to allow islets to settle. Most of the fluid was vacuum-aspirated, and the settling process was repeated until the supernatant appeared free of acinar tissue. The fluid containing the islets were transferred into a 100-mm petri dish on a dissecting microscope, and the islets were hand-picked and counted with a modified Pasteur pipet.

2.9.1.2 Chemical Induction of Diabetes with Streptozotocin

Pancreatic islet recipients were rendered diabetic using streptozotocin. Mice were given a single injection of streptozotocin intravenously minimum three days prior to islet transplantation. Streptozotocin was given at dosage of 160 to 220 mg/kg body weight depending on the mouse strain. Monitoring of diabetes onset in treated mice started two days post streptozotocin injection. Diabetes was defined as two consecutive blood glucose readings of more than 20 mM after streptozotocin treatment. Mice confirmed as diabetic were selected as recipients of pancreatic islets.

2.9.1.3 Pancreatic Islet Implantation

The islated islets were transferred into a PE-50 tubing and pelleted. Recipients were anesthetized via isoflurane inhalation and an incision was made at their left dorsal-lateral lumbar region. The surgical site was sterilized by wiping down with 70% ethanol pads followed by povidone-iodine pads. A 1 cm incision was made through the skin and peritoneal wall, and the left kidney was exposed through the incision. A 26-gauge needle was used to made a small hole in the kidney capsule. The end of the PE-50 tubing containing the islet pellet was inserted beneath the capsule, and the pellet was transferred into the capsule space using a micrometer. The peritoneal wall and the skin were closed with autoclips.

2.9.1.4 Monitoring Recipients

The recipients' blood glucose readings were monitored daily for the first week or until achieving euglycemia (<15 mM). At this point, the recipients were

monitored three times a week until rejection of the pancreatic islets or until graft survival exceeded 100 days. Graft rejection was determined as two consecutive blood glucose readings above euglycemic level, and the recipients were euthanized at this point. Recipients bearing grafts exceeding 100 days of survival were given nephrectomy to remove the islet graft-bearing kidney. Survival nephrectomy was used to confirm the dependence of recipient glucose control on the presence of the transplanted islets.

2.9.1.5 Survival Nephrectomy

Recipients were anesthetized via isoflurane inhalation and their left dorsal-lateral lumbar region was shaven. The surgical site was sterilized by wiping down with 70% ethanol pads followed by povidone-iodine pads. A 2 cm incision through the skin and peritoneal wall was made at the site of the prior islet transplantation, and the graft-bearing kidney was exposed. The adipose tissue surrounding the kidney and the renal vessels was dissected away, and the renal artery, vein, and ureter were ligated with nonabsorbable 5-0 silk sutures. The kidney was removed by excising the renal artery above the ligature. The peritoneal wall and the skin were then closed with autoclips. Nephrectomized recipients were monitored daily after the procedure for the recurrence of diabetes as determined by two consecutive blood glucose readings above euglycemic level (15 mM). Diabetic recipients were euthanized at this point.

2.9.2 Skin

2.9.2.1 Full-Thickness Trunk Skin Preparation

Skin donors (B6-OVA or BALB/c) were euthanized and shaven with electric clippers. The donors were drenched with 70% denatured ethanol. The donor skins were cut circumferentially around the shoulder joints and the hip joints; the two cuts were then joined by a longitudinal cut. The skins were peel off and rinsed in sterile PBS. The fat and subcutaneous tissues were scraped off of the skins, which were then cut into 1 cm^2 squares and kept in PBS.

2.9.2.2 Skin Grafting

The recipient mice (B6) were anesthetized with isoflurane inhalation and shaved from shoulder joint to approximately hip joint on the dorsal side of their trunks. The recipients were wiped down with ethanol gauze pads at the shaven site, and a square 1 cm^2 graft bed was cut on the dorsal side of the thorax but extended no further than the spine. The donor skins were placed on the graft beds with excess edges trimmed and affixed with small amounts of veterinary surgical glue (VetBond; 3M, St. Paul, MN). The grafts were covered with Vaseline-infused gauze (Tyco Healthcare Group, Mansfield, MA), and cohesive flexible bandages (Fisherbrand, Fisher HealthCare, Houston, TX) were applied circumferentially around the recipients.

2.9.2.3 Therapy Treatments and Monitoring of Recipients

Therapy-treated recipients were given their respective monoclonal antibodies as outlined in section 2.3. All recipients were monitored daily. The bandages were removed after minimum of 7 days. The grafts were observed daily after bandage removal. The day of rejection was determined as the day the entire graft had become necrotic.

2.10 NAÏVE T CELL RE-CHALLENGE OF LONG-TERM B6-OVA SKIN GRAFTS

2.10.1 Re-Challenge: Naïve T Cells Alone

Lymph nodes and spleens were harvested from naive CD45.1 OT-I rag-/- or CD45.1 OT-II rag-/- cell donor mice. Lymphocytes were dissociated and labeled with proliferation dye as described in section 2.4. 10^4 CD45.1 OT-I rag-/- or $2x10^4$ CD45.1 OT-II rag-/- labeled lymphocytes were adoptively transferred by intravenous retro-orbital injection into 1) combined anti-LFA-1 and anti-CD154 therapy-treated recipients bearing healthy B6-OVA skin grafts for a minimum of 60 days, 2) naïve recipients with fresh B6-OVA graft, and 3) naïve recipients with fresh B6 grafts. Recipients with CD45.1 OT-I rag-/- lymphocytes were harvested 5 days post transfer, and those with CD45.1 OT-II rag-/- lymphocytes 6 days post transfer. Skin draining lymph nodes (axial, brachial, inguinal) were harvested as

the primary site of reaction along with mesenteric lymph nodes and spleens. The lymphoid organs were dissociated and processed for cytokine re-stimulation (section 2.6.1) and flow cytometric analysis (section 2.5).

2.10.2 Re-Challenge: Naïve T Cells with Peripheral Donor Antigen Stimulation

Lymph nodes and spleens were harvested from naive CD45.1 OT-I rag-/- or CD45.1 OT-II rag-/- cell donor mice. Lymphocytes were dissociated and labeled with proliferation dye as described in section 2.4. 10^4 CD45.1 OT-I rag-/- or $2x10^4$ CD45.1 OT-II rag-/- labeled lymphocytes were adoptively transferred by intravenous retro-orbital injection into 1) combined anti-LFA-1 and anti-CD154 therapy-treated recipients bearing healthy graft for a minimum of 60 days, 2) recipients transplanted with and subsequently rejected B6-OVA graft minimally 60 days prior, and 3) recipients bearing healthy syngeneic B6 graft for minimally 60 days. All recipients were subsequently challenged in both footpads with $1x10^6$ live B6-OVA splenocytes one day post adoptive transfer. Recipients with CD45.1 OT-I rag-/- lymphocytes were harvested 4 days post transfer, and those with CD45.1 OT-II rag-/- lymphocytes 5 days post transfer. Skin draining lymph nodes (axial, brachial, inguinal) and popliteal lymph nodes were harvested as the primary sites of reaction along with mesenteric lymph nodes and spleens. The lymphoid organs were dissociated and processed for cytokine re-stimulation (section 2.6.1) and flow cytometric analysis (section 2.5).

2.11 STATICTICAL ANALYSIS

2.11.1 Transplant Survival Curves

Statistical significance of transplant survival curves were analyzed using Log-rank (Mantel-Cox) Test. Significance is defined as a minimum of $P < 0.05$.

2.11.2 Absolute Cell Counts and Flow Cytometry

Statistical significance of absolute cell counts and flow cytometric data was analyzed using one-way analysis of variance followed by Turkey's multiple comparison test. Error bars on bar graphs represent standard error of the mean.

2.12 REPRODUCIBILITY

All data represent a minimum of three separate experiments.

CHAPTER THREE
ESTABLISHING A MODEL FOR TRACKING THE FATE OF ANTIGEN-SPECIFIC T CELLS

3.1 INTRODUCTION

The primary goal of this thesis was to interrogate the proximal impact of transplantation tolerance-promoting antibody therapies on the fate of T cells, thus elucidating the requirements for inducing immune tolerance to a foreign antigen. As such, a specific naïve T cell population needed to be tracked through antigen-driven activation and proliferation in order to interrogate whether the presence of therapeutic antibodies changed the fate of these T cells. Therefore, a T cell adoptive transfer model was employed. This technique was initially developed as a means of accurately and precisely following the natural history of an antigen-specific T cell population through its response *in vivo* and later extended to the study of TCR-transgenic T cells (257, 258). Since the primary objective of this thesis is to compare the consequences of T cell activation under treatments with anti-LFA-1, anti-CD154, or combined anti-LFA-1/anti-CD154 therapies, the adoptive transfer of T cells enabled specific analysis of one single population of responding T cells during the course of the therapies. Importantly, detecting donor T cell proliferation during primary activation was a major outcome examined throughout this thesis. As such, adoptive transfer allowed the harvesting and processing of the T cell population to be tracked prior to transfer into the recipients to monitor subsequent *in vivo* activation. Tracking such transferred cells involved the labeling of responding T cells with various proliferation dyes and thus provided a dynamic representation of T cell reactivity during antibody treatment. To thoroughly examine the fate of T cells in the presence of therapeutic antibodies, two *in vivo* activation models were established using two types of T cell-antigen activation scenarios.

The first *in vivo* T cell activation model is monoclonal and antigen-specific. This model uses TCR transgenic T cells with congenic markers as the T cells to be tracked through activation and proliferation. As described in section

2.1.2.1, the models used were the OT-I and OT-II transgenic mice that generated CD8+ and CD4+ T cells respectively that specifically recognized the neo-antigen chicken ovalbumin (OVA) in the context of MHC H-2^b. Since the TCR transgenic T cells were monoclonal and responded solely to single OVA epitopes, this model allowed the tracking of a very specific T cell response with very low background reactivity. In other words, activating adoptively transferred OT-I or OT-II T cells *in vivo* with OVA-expressing APCs presented a very precise illustration of T cell reactivity. Therefore, the monoclonal TCR transgenic T cell model was an excellent model for interrogating general and fundamental changes in the fate of T cells.

However, while the monoclonal nature of the TCR-transgenic T cells permits clear antigen-specificity in the response, this model also presents an obstacle in accurately representing naïve T cell reactivity. That is, the study of a single antigen specificity may not accurately reflect the response of the entire polyclonal endogenous TCR repertoire. For instance, it has been shown that initial naïve T cell frequency prior to antigen exposure significantly impacted the quality of the resulting T cell response (259, 260). Since a single clone of antigen-specific T cells generally exist in exceeding low frequencies endogenously, it is very difficult to adoptively transfer a monoclonal population of T cells in comparably low cell number to recapitulate its physiological frequency and still allow robust tracking of this T cell population. Furthermore, TCR transgenic T cells were usually generated by selecting for high-affinity and high-reactivity clones amongst T cells specific for a single antigen. As such, adoptive transfer of a monoclonal TCR transgenic T cell inadvertently creates an initial naïve T cell population that is likely superior in quantity, affinity, and avidity compared to an endogenous and polyclonal antigen-specific T cell population. The representation of T cell fate using these transgenic T cells may therefore be biased. While TCR transgenic T cells allows highly specific tracking of a tracer population of T cells in a wild-type recipient, they might not be wholly representative of a typical T cell response.

To compensate for this caveat, an alternative adoptive transfer model was used in parallel with the monoclonal transgenic model to create a broader, more physiological representation of T cell response during treatments with therapeutic antibodies. Instead of using a monoclonal population of T cells as the initial tracer population, bulk polyclonal wild-type T cells were adoptively transferred into congenic recipients and challenged with allo-antigens. Adoptively transferring a wild-type, polyclonal population of T cells allowed the initial frequency of antigen-specific (alloreactive) T cells to remain physiological and unchanged, and the response to the allo-antigen would be a heterogeneous conglomerate of different allo-antigen-specific T cell clones. This prevented biasing the T cell response with artificial frequency and affinity. Furthermore, since transplantation within the same species is by nature an allogeneic response, tracking wild-type alloreactive T cells more accurately represented transplant immunity. However, due to the lack of a specific tracking marker for endogenous alloreactive T cells, one difficulty with tracking a polyclonal T cell population was the decreased precision in following "antigen-specific" T cells. Since the frequency of responding T cells was comparatively low, the analysis of T cell response was not as precise as using the monoclonal transgenic T cells. However, by using them in tandem, the two models complemented each other and created a more thorough and dynamic representation of T cell response during primary antigen activation and treatments with antibody therapies.

This chapter describes the development of the adoptive transfer model for tracking T cell primary response *in vivo*. Various perimeters of the adoptive transfer and *in vivo* antigen activation processes were examined. It also details the development of specific techniques to examine different aspects of T cell response from activation, proliferation, effector function, to apoptosis. To ensure reliable analysis of T cell response during treatments with therapeutic antibodies, every technique and model used in this thesis was thoroughly tested.

3.2 RESULTS

Monoclonal antibody therapies can prolong pancreatic islet allograft survival

Prior to examining the therapeutic mechanisms of anti-LFA-1 and anti-CD154 therapies *in vivo*, it was essential to confirm that such treatments could induce prolonged or indefinite allograft survival. This was tested using a pancreatic islet allograft model as described in section 2.9.1. Briefly, B6 recipients were rendered chemically diabetic with streptozotocin. The diabetic recipients were then transplanted with 450 BALB/c pancreatic islets under the left kidney capsule and treated with or without either anti-LFA-1 or anti-CD154 antibodies. Recipients receiving BALB/c islet allograft without any treatment consistently rejected their transplants within 21 days and reverted to hyperglycemia (Figure 3-1). When visually observed under the microscope, recipients who rejected their grafts had no observable islets remaining under the kidney capsule at the time of rejection. However, treatment with anti-LFA-1 alone allowed 40% of the recipients to achieve indefinite graft survival, and anti-CD154 prolonged graft survival in at least 70% of the recipients (Figure 3-1). These results were similar to pancreatic islet allograft prolongation observed previously in our laboratory, and anti-LFA-1 and anti-CD154 were confirmed as effective in promoting long-term allograft survival (218).

To demonstrate that the antibody-promoted allograft survival resulted in a resistance to induced allograft immunity, recipients euglycemic for a minimum of 120 days were challenged with $10x10^6$ BALB/c splenocytes intraperitoneally. All re-challenged recipients (anti-LFA-1: n=4, anti-CD154: n=6) maintained euglycemia for a minimum of two weeks post challenge with BALB/c splenocytes. Subsequently, the recipients were further challenged with intraperitoneal injections of $10x10^6$ 2C transgenic T cells, which were alloreactive CD8+ T cells with transgenic TCR specific for H-$2L^d$. Similar to the previous BALB/c splenocytes challenge, all recipients remained euglycemic for another two weeks. At the end of the two-week time period, pancreatic islet grafts were removed from all recipients by nephrectomy of the left kidney as described in section 2.9.1.5. All nephrectomized recipients reverted to hyperglycemia, thus demonstrating their previous euglycemic state as dependent of the islet allografts.

Comparison of proliferation tracking dyes

To accommodate the possibility of using various GFP reporter mice, whose GFP expression would interfere with CFSE reading using flow cytometry, two proliferation dyes on non-GFP fluorescent channels were compared against CFSE. The two proliferation dyes tested were CellTrace Violet (Invitrogen) with a peak excitation/emission spectrum of 405/450 nm, and eFluor 670 (eBioscience) with a peak excitation/emission spectrum of 647/670 nm. To test the efficacy of the proliferation dyes, polyclonal B6 lymph node and spleen lymphocytes were labeled with CFSE, CellTrace Violet, or eFluor 670 and cultured *in vitro* with the plant mitogen concanavalin A (Sigma-Aldrich) for three days. The lymphocytes were then harvested and their proliferation assessed by flow cytometry. All three dyes presented approximately 60% of the lymphocytes as having proliferated (Figure 3-2). CellTrace Violet showed the most discrete division peaks followed by CFSE and eFluor 670 (Figure 3-2). However, it also required the highest labeling concentration (10 μM) to achieve similar fluorescence intensity as CFSE (1 μM) and eFluor 670 (5 μM). Nevertheless, all three proliferation dyes were confirmed efficacious in tracking lymphocyte proliferation using flow cytometry.

Persistence of adoptively transferred TCR-transgenic T cells

The first parameter of using TCR-transgenic OT-I and OT-II T cells as an adoptive transfer model tested was dosage of T cells to mimic an appropriate initial naïve T cell frequency and the intrinsic persistence of the transgenic T cells. As described previously, initial T cell frequency has been demonstrated to change the quality of subsequent T cell responses (259, 260). It was therefore essential for an adoptive transfer model to determine a T cell tracer population dose that recreated a physiological initial T cell frequency while allowing robust tracking of the population. Furthermore, T cells generated in monoclonal transgenic mice on a RAG-knockout background may exhibit different biological properties than those produced in a polyclonal wild-type immune environment, and their ability to persist or, conversely, their intrinsic rate of attrition, after adoptive transfer is a crucial factor in designing a model to track the evolution of

their response longitudinally. Therefore, it was essential to determine the OT-I and OT-II T cell doses to achieve a reasonable initial frequency and the extent of T cell persistence in the adoptively transferred host.

To examine these two factors, I selected two different doses of OT-I or OT-II lymph node and spleen cells to adoptively transfer into B6 recipients. Subsequently, blood was collected from each recipient on days 3, 7, 14, and 21 post adoptive transfer, and frequencies of OT-I and OT-II T cells in the host circulation were assessed by flow cytometry. $5x10^6$ and $10x10^6$ adoptively transferred OT-I lymphocytes yielded similar frequencies of CD45.1+CD8+ T cells (~0.1% of total blood lymphocytes) in the recipient blood for the first 7 days post adoptive transfer (Figure 3-3). The frequencies started to taper off on day 14, which could either be due to lymphocyte attrition or homing of the T cells into the lymph nodes (Figure 3-3). Since the 0.1% of total blood lymphocyte is similar to the frequency of endogenous alloreactive T cells, $5x10^6$ OT-I lymphocytes was determined to be the optimal cell dose for adoptive transfer. In contrast, while $10x10^6$ and $20x10^6$ OT-II lymphocytes resulted in different initial CD45.1+CD4+ T cell frequencies in host circulation, neither was detectable in the circulation by 14 days post adoptive transfer (Figure 3-3). I later confirmed that the disappearance of OT-II T cells were due to intrinsic attrition rather than lymph node homing as they could not be detected in lymph nodes (Figure 3-5B). Since adoptive transfer of $10x10^6$ OT-II lymphocytes resulted in an initial T cell frequency of approximately 0.06% of total lymphocytes, and increasing the initial lymphocyte dose did not delay their attrition, this was determined to be the optimal cell dose for adoptive transfer (Figure 3-3). Taken together, both transgenic OT-I and OT-II T cells underwent attrition after adoptive transfer with OT-II T cells being particularly unstable. However, both transgenic models lasted long enough during adoptive transfer to be used as a tool to examine acute primary T cell activation and the early proximal impact of antibody therapies during this phase of antigen-specific T cell activity.

Antigen dose required for transgenic T cell activation

The second parameter of the OT-I and OT-II TCR transgenic models tested was the optimal antigen dose during primary activation. This was determined using an *in vivo* local antigen challenge model. Using adoptive transfer T cell doses as determined in the previous experiment, $5x10^6$ OT-I or $10x10^6$ OT-II lymphocytes were adoptively transferred into B6 recipients. The recipients were subsequently challenged at both hind footpads with three doses of transgenic B6-OVA splenocytes with actin-driven expression of chicken ovalbumin (OVA). The recipients' draining popliteal lymph nodes were harvested on day 3 post stimulation for OT-I and day 4 for OT-II reactivity, which was determined by the extent of T cell proliferation and up-regulation of activation marker CD44 as assessed by flow cytometry.

OT-I T cells increased in cell number to a similar magnitude when stimulated with either $0.2x10^6$ or $1x10^6$ B6-OVA splenocytes per footpad and experienced a larger expansion in cell number when challenged with $5x10^6$ B6-OVA splenocytes (Figure 3-4A). Nevertheless, all three B6-OVA splenocytes doses successfully stimulated OT-I T cell expansion. Despite the differences in OT-I T cell numbers in the draining lymph nodes, all three antigen doses stimulated equivalent rounds of T cell division, with $1x10^6$ and $5x10^6$ B6-OVA splenocytes per footpad achieving higher proportions of proliferated OT-I T cells (Figure 3-4B). In contrast, OT-II T cells did not proliferate extensively when challenged with $0.2x10^6$ B6-OVA splenocytes per footpad, and only underwent increasing T cell proliferation with $1x10^6$ and $5x10^6$ antigen dose (Figure 3-4A). Despite the differences in T cell magnitudes in the draining lymph nodes, OT-II T cells did achieve similar rounds of division when stimulated with all three antigen doses (Figure 3-4 B). However, stimulation with $1x10^6$ and $5x10^6$ B6-OVA splenocytes per footpad resulted in the highest proportions of proliferated OT-II T cells in the lymph nodes (Figure 3-4B). Both proliferated OT-I and OT-II T cells up-regulated their expression of CD44, confirming their productive interactions with cognate antigens (Figure 3-4C). Taken as a whole, since $1x10^6$ B6-OVA splenocytes per footpad was able to stimulate both OT-I and OT-II T cell

activation and proliferation, I determined this as the optimal antigen dose for my *in vivo* local antigen activation model.

Time course of transgenic T cell activation and proliferation in vivo

The third and final parameter of an *in vivo* TCR-transgenic T cell activation model established was the time frame of naïve transgenic T cell activation and proliferation. I used a T cell activation time course to determine the optimal day to harvest adoptively transferred OT-I or OT-II T cells after *in vivo* antigen challenge at the footpads. $5x10^6$ OT-I or $10x10^6$ OT-II lymphocytes were transferred into B6 recipients. The recipients were subsequently challenged with $1x10^6$ B6-OVA splenocytes per footpad, and their draining popliteal lymph nodes were harvested on days 2, 7, and 14 post antigen stimulation. Extent of OT-I and OT-II T cell proliferation was assessed by flow cytometry.

Both OT-I and OT-II T cells underwent little proliferation 2 days after initial antigen stimulation (Figure 3-5A). However, the majority of the adoptively transferred T cells had proliferated vigorously after 7 days (Figure 3-5A). Interestingly, OT-II lymphocytes could not be found in the draining popliteal lymph nodes by 14 days post antigen stimulation (Figure 3-5A). This was not a phenomenon of antigen stimulation, as OT-II lymphocytes adoptively transferred into unchallenged recipients had also disappeared from the lymph nodes (Figure 3-5B). This confirmed my previous observation that transgenic OT-II lymphocytes are intrinsically prone to attrition after adoptive transfer. OT-II T cells are therefore likely not ideal as a candidate for studying long-term T cell reactivity. However, they were still effective as a model of acute CD4+ T cell reactivity. The rationale in determining an optimal day of harvesting adoptively transferred T cells was to allow for sufficient T cell activation and proliferation at an early stage of T cell reactivity, so the initial direct impact of antibody therapies on T cell primary responses could be examined. Based upon the results of the proliferation time course, the optimal time to examine OT-I and OT-II T cell acute response appeared to be between days 2 and 7. As such, OT-I T cells were

harvested on day 3 post antigen stimulation when used in the local footpad antigen challenge model, and OT-II T cells were harvested on day 4.

Activating transgenic T cells with cognate peptides

An integral part of the analysis of T cell fate during activation in the presence of antibody therapies was the functional differentiation of activated T cells. This was determined by assessing the cytokine profiles of adoptively transferred OT-I and OT-II T cells challenged by B6-OVA splenocytes in the footpads. In this model, the T cells require a four- to five- hour *in vitro* re-stimulation with their cognate peptides to be driven into active cytokine expression for flow cytometry analysis. To find the optimal peptide concentration for this re-stimulation process, doses of OT-I and OT-II peptides were tested for their ability to induce early activation of naïve OT-I and OT-II lymphocytes. $1x10^6$ OT-I or OT-II spleen and lymph node cells were cultured *in vitro* with four different doses of their respective peptides (0.001 μM, 0.01 μM, 0.1 μM, and 1.0 μM) for six hours. The lymphocytes were subsequently assessed for the expression of early activation markers CD69 and CD25 by flow cytometry.

Expression of CD69 and CD25 by OT-I D8+ T cells were induced by all four concentrations of the OT-I peptide (Figure 3-6). The expression of activation markers increased with the peptide concentration until it plateaued at the peptide concentration of 0.1 μM (Figure 3-6). Therefore, 0.1 μM of OT-I peptide was determined to be the optimal peptide concentration for *in vitro* re-stimulation of OT-I CD8+ T cells. In contrast, OT-II CD4+ T cells did not experience up-regulation of CD69 and CD25 until incubated with 0.1 μM of OT-II peptides, and the expression of the activation markers were much stronger when OT-II lymphocytes were incubated with 1.0 μM of peptides (Figure 3-6). As such, the optimal concentration of OT-II peptide used in *in vitro* re-stimulation of OT-II CD4+ T cells was determined to be 1.0 μM. These concentrations were subsequently used in all experiments examining OT-I and OT-II T cell cytokine profiles.

Assessing apoptosis by flow cytometry

Another aspect of T cell activation examined in this thesis was T cell apoptosis both as a potentially direct result of antibody therapies or indirectly through antibodies' impact on activation-induced cell death. The two markers examined as a representation of T cell apoptosis were the apoptosis-related cysteine peptidase caspase 3 that drives apoptosis, and the anti-apoptotic B-cell lymphoma 2 protein (Bcl-2), a member of the Bcl-2 family of apoptosis regulator proteins. The expressions of these markers of apoptosis and survival were assessed by flow cytometry, and the antibodies used to label them were tested using a cultured thymocyte model.

Thymocytes naturally begin undergoing apoptosis when cultured *in vitro*. This phenomenon was used to test the efficacy of the caspase 3- and Bcl-2-specific antibodies. Thymi were harvested from wild-type B6 mice and dissociated to release thymocytes. An aliquot of the thymocytes were processed for intracellular labeling of anti-caspase 3 and anti-Bcl-2 immediately, as primary thymocyte populations generally do not contain a high number of apoptotic cells (Figure 3-7). The remaining thymocytes were cultured *in vitro* for one to three days. On each designated day after culture, thymocytes were harvested and labeled with the antibodies and assessed by flow cytometry. Thymocyte caspase 3 expression increased with amount of time in culture, while Bcl-2 first decreased then recovered in its expression by the third day of *in vitro* culture (Figure 3-7). The discrete expressions of caspase 3 and Bcl-2 as a time course of *in vitro* culturing of thymocytes demonstrated the efficacy of the caspase 3- and Bcl-2-specific antibodies as a means of interrogating T cell apoptosis.

Skin grafts as a model of transplantation

While the previous work done with the antibody therapies in this laboratory had utilized the pancreatic islet transplantation model, translating the model into the OT-OVA system encountered a major difficulty. The B6-OVA transgenic mice with actin-driven expression of ovalbumin protein had pancreata that were difficult to digest and isolate islets from. The B6-OVA pancreata were

visually different than those of the wild-type B6 mice; they were brightly white instead of slightly translucent and flesh-coloured in appearance. These pancreata could not be digested using the collagenase concentration and incubation time optimized for B6-background mice. However, even small increases in either the collagenase or the time of incubation caused extensive islet destruction. Furthermore, the islets that survived the digestion process were covered with ascinar tissues that were difficult to remove. The appearance of the pancreata, the resistance to collagenase digest, and the large amount of ascinar tissues suggested that the over-expression of ovalbumin protein on cell surfaces of B6-OVA mice was likely the cause of these anomalies. Due to these obstacles, an alternative transplantation model was necessary to accommodate the OT-OVA T cell tracking system established for tracking T cells *in vivo*.

A skin transplantation model was developed to replace pancreatic islet transplantation. Full-thickness donor trunk skin was selected as the graft rather than ear or tail skin as trunk skin was the most immunogenic of the three types of skin grafts. The finalized skin grafting protocol is detailed in section 2.9.2. As an example of the protocol, Figure 3-8A shows a combined anti-LFA-1/anti-CD154-treated B6 recipient bearing a healthy BALB/c skin graft for at least 50 days. In comparison, Figure 3-8B shows a B6-recipient treated with the same therapy starting to reject its BALB/c graft. When the skin donors had the same coat colours as the recipients, the graft was transplanted with hair growing in opposite direction from recipient natural hair growth for clear identification of donor graft. The skin transplantation model was thus successfully developed for the OT-OVA T cell tracking model in place of pancreatic islet transplantation.

Intrinsic impact of monoclonal antibody therapies on naïve immune system

Before tracking the effects of antibody therapies on the fate of antigen-specific T cells during primary activation, the intrinsic impact of these antibodies on a naïve, un-manipulated immune system was examined. Naïve wild-type B6 recipients were given transient courses of anti-LFA-1, anti-CD154 or combined anti-LFA-1/anti-CD154 therapies as outlined in section 2.3. Recipient blood,

lymph nodes (axial, brachial, inguinal, mesenteric), and spleen were harvested four days after the start of antibody treatments. This time frame was chosen to mimic the approximate time of harvest when tracking antigen-specific T cell fate in a footpad challenge model. Endogenous lymphocytes of the B6 recipients were then analyzed by flow cytometry to assess whether treatments with antibodies therapies without any other stimulation or manipulation induces phenotypic changes in the immune system.

Interestingly, while anti-CD154 treatment alone did not result in gross changes in the endogenous naïve lymphocyte phenotype, anti-LFA-1, whether alone or combined with anti-CD154, induced CD4+ and CD8+ T cell emigration from lymph nodes into the blood and spleen (Figure 3-9A). The significant decrease in T cell numbers in the lymph nodes of anti-LFA-1 or combined anti-LFA-1/anti-CD154-treated recipients corresponded with increases of T cells in both the blood and the spleen (Figure 3-9A). This was likely caused by a decrease in the level of CD62L expression on lymphocytes during anti-LFA-1 or anti-LFA-1/anti-CD154 treatments, as CD62L is a major homing receptor that directs naive T cell migration into lymph nodes (Figure 3-9B). Endogenous naïve lymphocytes lowered their CD62L expression in all three lymphoid compartments in anti-LFA-1-treated recipients, and the decrease is especially evident in the lymph nodes (Figure 3-9B). Combining anti-CD154 with anti-LFA-1 appeared to rescue CD62L expression in the recipient spleen, but the lymphocytes found in the blood of these animals still maintained distinctly reduced CD62L expression (Figure 3-9B). This change in surface marker expression was not observed with CD44 and CD25 except for a slight up-regulation of CD44 expression in lymph node cells of the anti-LFA-1-treated recipients (Figure 3-9C). It appeared that anti-CD154 treatment did not change the phenotype of naïve T cells. However, binding of anti-LFA-1 to CD11a on a naïve T cells somehow resulted in down-regulation of surface CD62L and naïve T cell emigration from lymph nodes. The intrinsic mechanism connecting antibody perturbation of CD11a and the change in CD62L expression of a naïve, un-stimulated T cell remains unclear but potentially contributes to the promotion of transplant tolerance by anti-LFA-1 therapies.

3.3 FIGURES

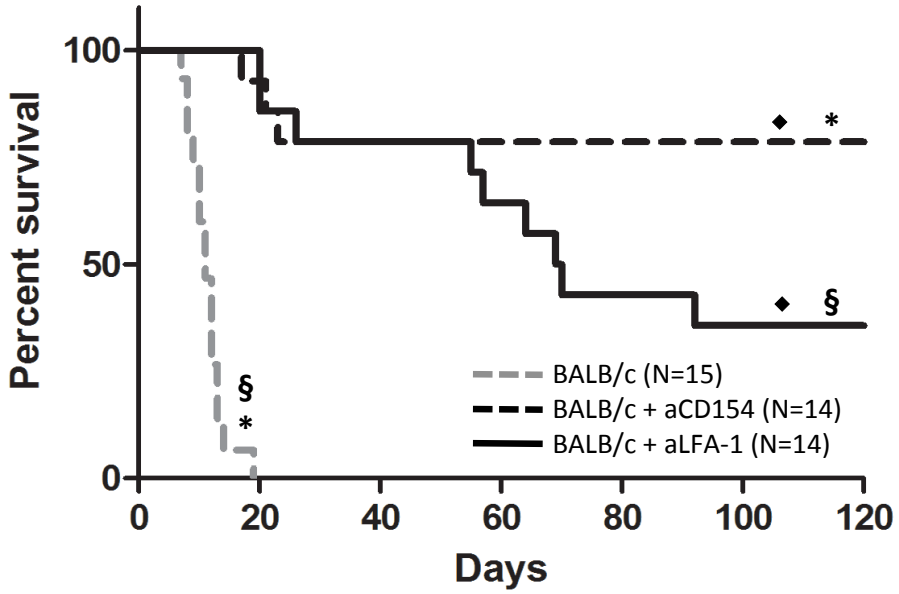


FIGURE 3-1. Impact of monoclonal antibody therapies on allograft survival. B6 recipients were transplanted with 450 BALB/c pancreatic islets as described in Materials and Methods and treated as follows: 1) no treatment, 2) anti-CD154, or 3) anti-LFA-1. Therapy-treated recipients were given antibodies based upon protocol outlined in Material and Methods. Pairs of identical symbols denote experimental groups with significant differences of at least $P < 0.05$.

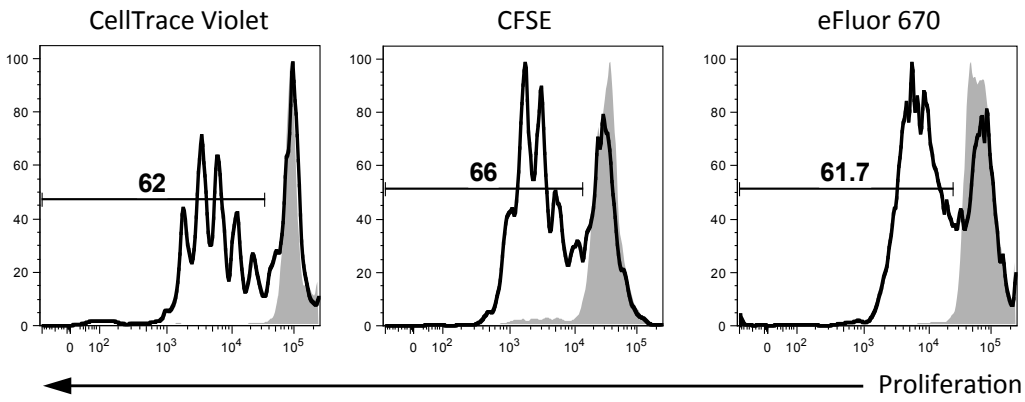


FIGURE 3-2. Efficacy of three different proliferation tracking dyes during *in vitro* T cell expansion. Polyclonal B6 lymph node and spleen cells were labeled with CellTrace Violet, CFSE, or eFluor 670 and cultured *in vitro* with concanavalin A for three days. Lymphocytes were analyzed by flow cytometry, and the efficacies of proliferation dyes in tracking lymphocyte expansion were compared. The histograms are gated on total lymphocytes.

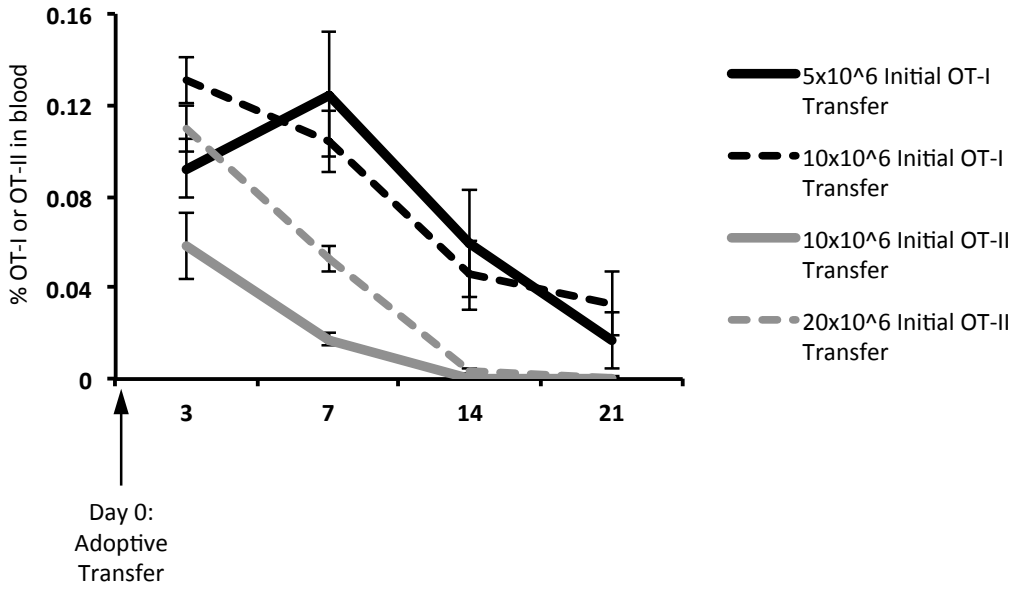


FIGURE 3-3. Persistence of adoptively transferred CD45.1 OT-I or OT-II rag-/- T cells in recipient circulation. CD45.1 OT-I or OT-II rag-/- lymphocytes were adoptively transferred into B6 recipients (n=5 receiving each cell type) at the indicated cell number on day 0. The recipients' blood was collected at days 3, 7, 14, and 21 after adoptive transfer. Lymphocytes purified from the blood were analyzed for presence of adoptively transferred cells by flow cytometry. % OT-I T cells were determined as % CD45.1+CD8+ cells in the blood, and % OT-II T cells as % CD45.1+CD4+ cells.

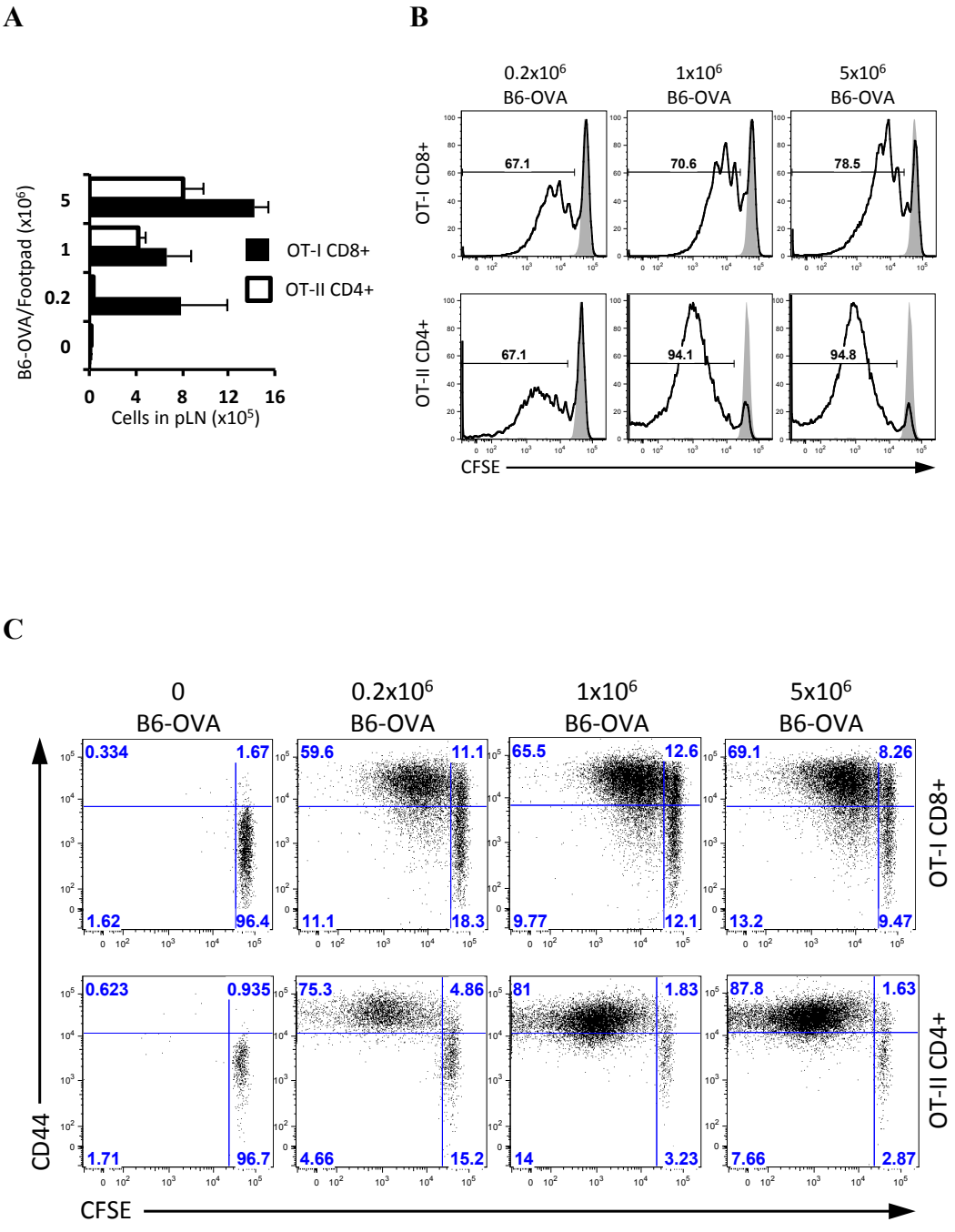


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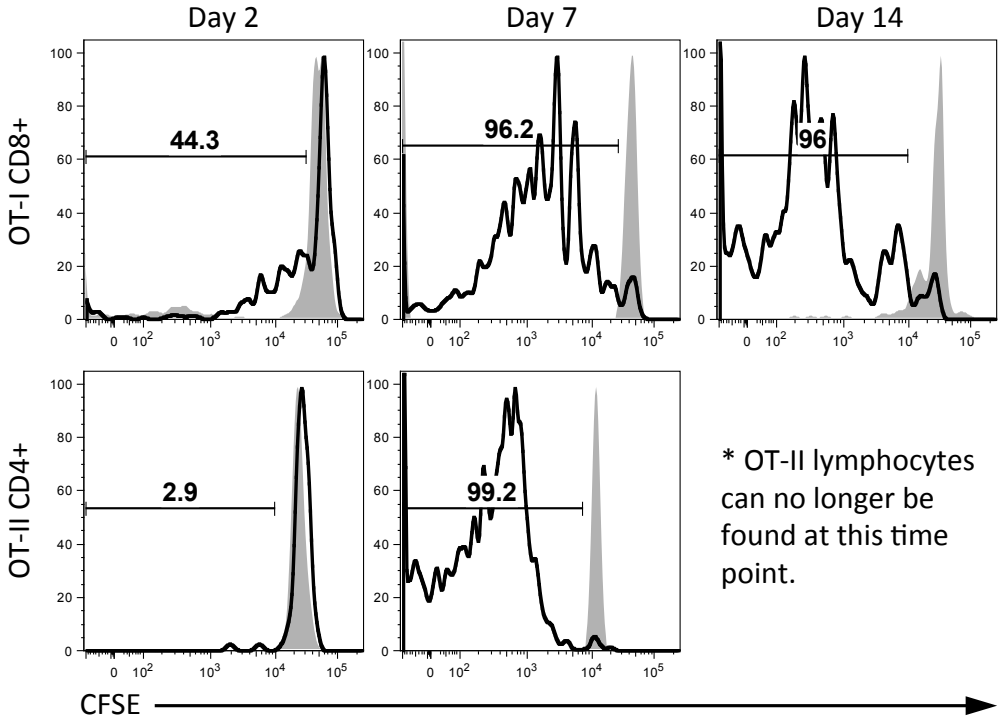
FIGURE 3-4. *in vivo* CD45.1 OT-I or OT-II rag-/- lymphocyte response to stimulation with different doses of B6-OVA splenocytes. $5x10^6$ OT-I or $10x10^6$ OT-II lymphocytes were adoptively transferred into B6 recipients. Recipients were challenged by footpad injections of three increasing doses of B6-OVA splenocytes as indicated. Recipient popliteal lymph nodes (pLN) were harvested on day 3 (OT-I) or 4 (OT-II) post stimulation and assessed by flow cytometry. *A.* Total OT-I or OT-II cells in pLN as calculated by multiplying total pLN cell count with %CD45.1+CD8+ (OT-I) or %CD45.1+CD4+ (OT-II) in pLN. *B.* Proliferation of OT-I or OT-II cells in pLN as indicated by dilution of CFSE. The histograms are gated on CD45.1+CD8+ (OT-I) or CD45.1+ CD4+ cells. *C.* Activation of OT-I or OT-II cells in pLN as indicated by up-regulation of CD44 by proliferating cells. The dot plots are gated on CD45.1+CD8+ (OT-I) or CD45.1+ CD4+ cells.

A



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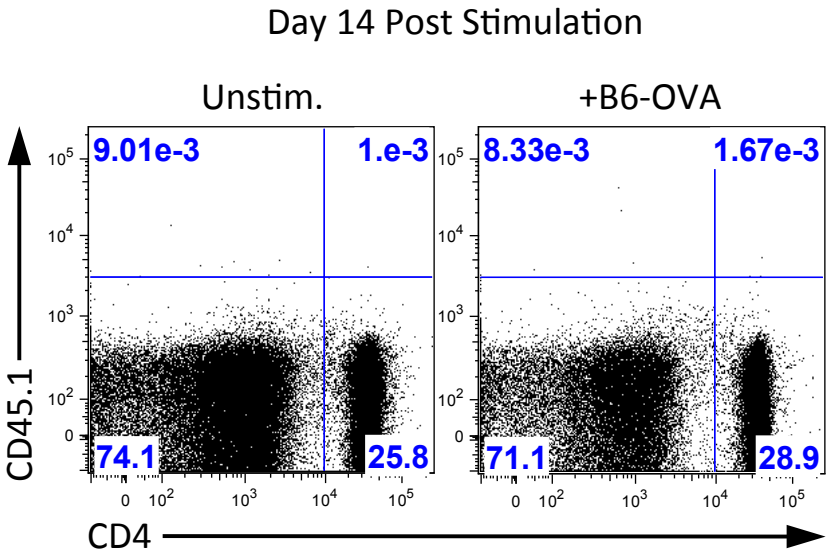

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FIGURE 3-5. Time course of OT-I or OT-II *in vivo* proliferation during footpad challenge. $5x10^6$ OT-I or $10x10^6$ OT-II lymphocytes were adoptively transferred into B6 recipients. Recipients were challenged by footpad injection of $1x10^6$ B6-OVA splenocytes per footpad at both footpads. Popliteal lymph nodes were harvested on indicated days post stimulation and analyzed by flow cytometry. *A*. Proliferation of OT-I or OT-II cells as assessed by dilution of CFSE. The histograms are gated on CD45.1+CD8+ (OT-I) or CD45.1+CD4+ (OT-II) cells. OT-II cells can no longer be found by 14 days post stimulation. *B*. Intrinsic attrition of OT-II cells by day 14 as shown by their absence in pLNs whether stimulated or not. Dot plots are gated on total lymphocytes.

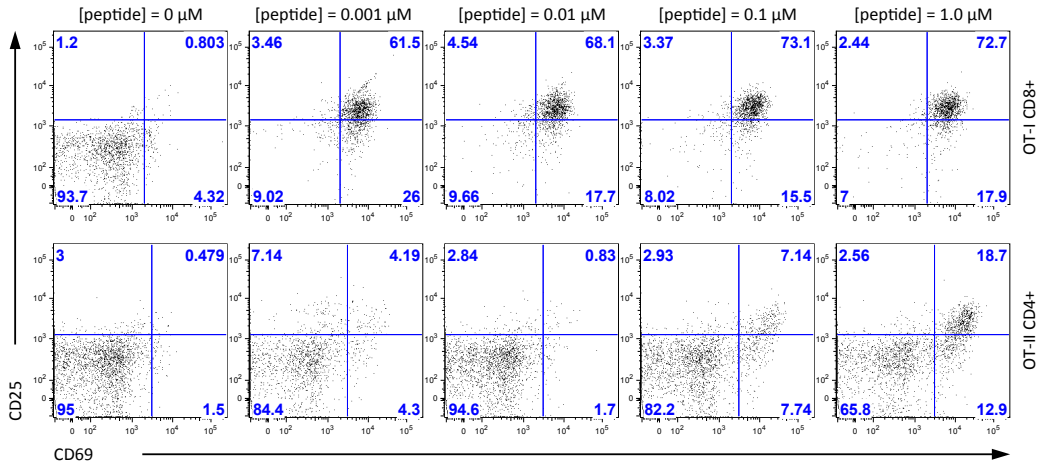


FIGURE 3-6. Stimulation of OT-I or OT-II lymphocytes by different doses of their cognate antigen peptides. OT-I lymphocytes were cultured *in vitro* with four increasing doses of $OVA_{257-264}$ peptide (SIINFEKL) and OT-II with $OVA_{329-337}$ peptide (ISQAVHAAHAEINEAGR) for six hours. OT-I and OT-II activation was determined by up-regulation of CD25 and CD69 as assessed by flow cytometry. The dot plots are gated on CD45.1+CD8+ (OT-I) or CD45.1+CD4+ (OT-II) cells.

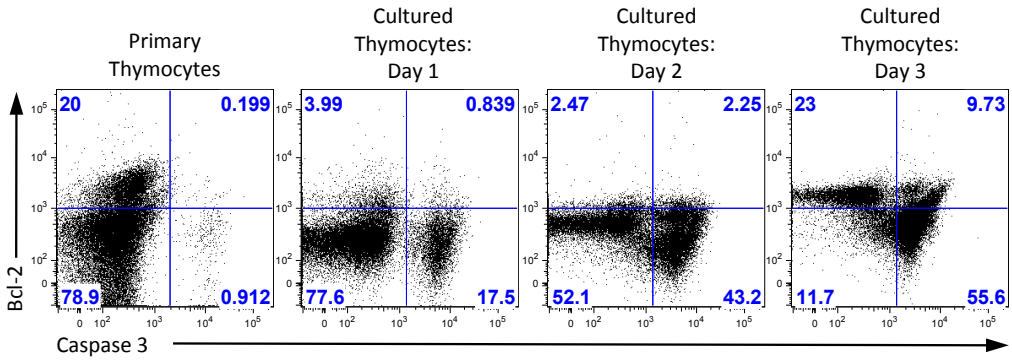


FIGURE 3-7. Efficacy of caspase 3 and Bcl-2 antibody staining for flow cytometric analysis of apoptosis. Thymocytes harvested from B6 mice were either labeled with caspase 3 and Bcl-2 antibodies and assessed by flow cytometry immediately or cultured alone *in vitro*. Cultured thymocytes were harvested on the indicated days and labeled with caspase 3 and Bcl-2 for flow cytometric analysis. The dot plots are gated on total lymphocyte.

A **B**

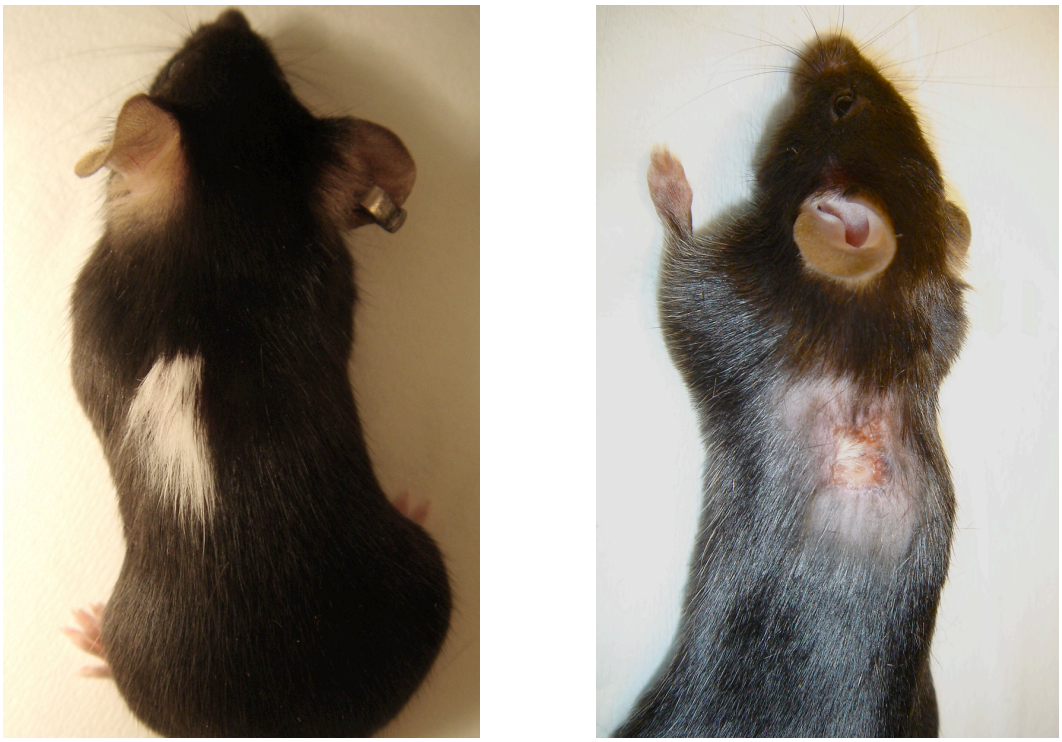
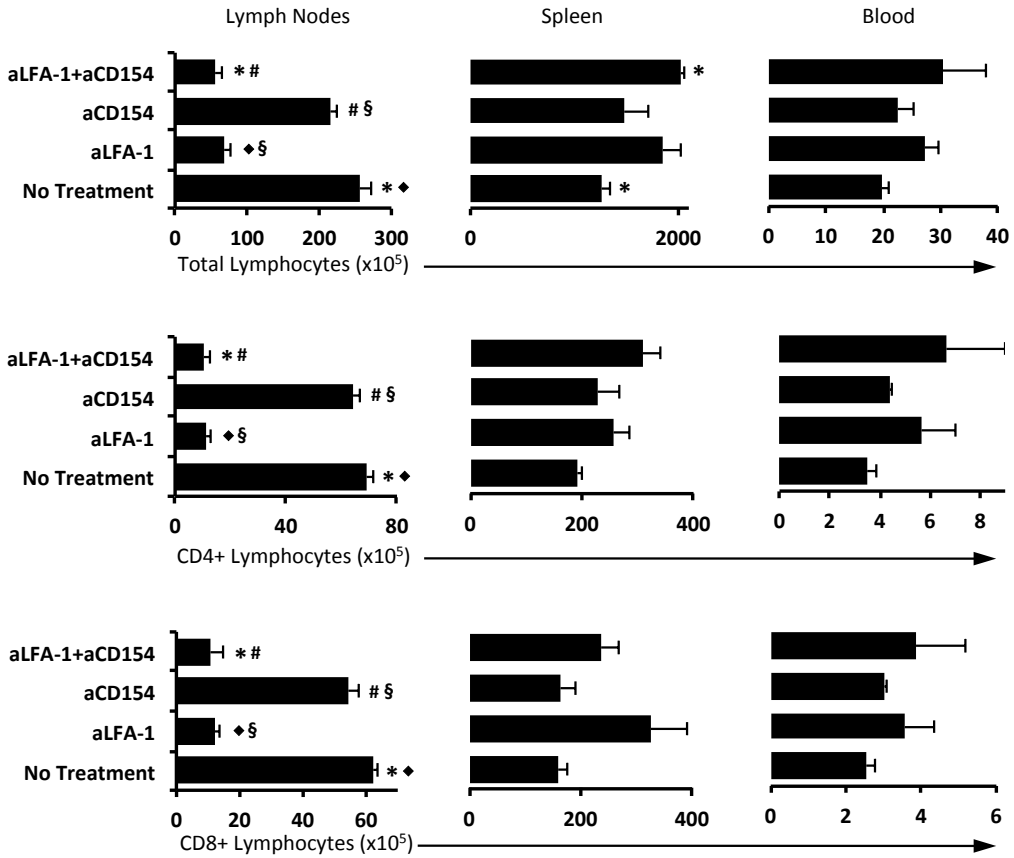


FIGURE 3-8. Skin transplantation. B6 recipients were grafted with BALB/c skin as described in Materials and Methods. *A.* Recipient treated with combined anti-LFA-1 and anti-CD154 therapy bearing healthy long-term surviving skin graft. *B.* Recipient treated with combined anti-LFA-1 and anti-CD154 therapy that started to reject skin graft.

A



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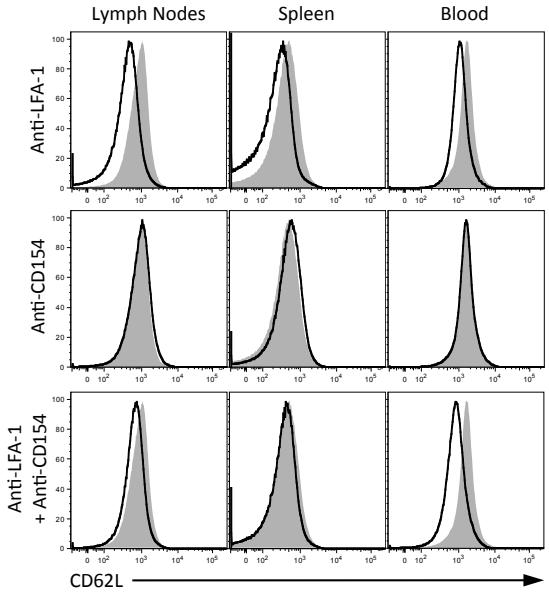

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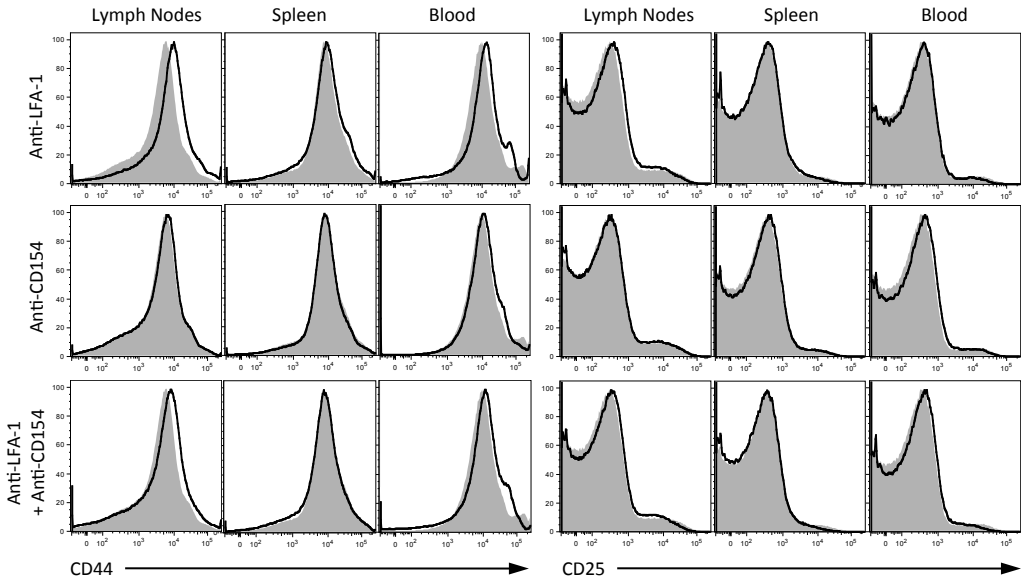


FIGURE 3-9. Proximal impact of anti-LFA-1 and/or anti-CD154 treatment on naïve lymphocytes. Naïve B6 recipients were given the following treatments: anti-LFA-1 alone, anti-CD154 alone, or anti-LFA-1 plus anti-CD154. The antibody treatments were given according to treatment protocol specified in Materials and Methods. Spleen, lymph nodes, and blood were harvested from recipients four days into the treatment protocols and analyzed by flow cytometry. *A*. Absolute lymphocyte numbers in each lymphoid compartment were counted and multiplied to % CD4+ or % CD8+ cells in the compartment to calculate total CD4+ or CD8+ cell counts. Identical pairs of symbols on the bar graphs denote experimental groups with significant differences of at least $P < 0.05$. Total lymphocyte CD62L *(B)*, CD44 *(C)*, and CD25 *(C)* expressions in each compartment was assessed by flow cytometry. The histograms are gated on total lymphocytes.

CHAPTER FOUR
PROXIMAL IMPACT OF ANTIBODY THERAPIES ON ACUTE T CELL REACTIVITY

4.1 INTRODUCTION

As described in the first chapter, transplant rejection is currently managed by chronic administration of immunosuppressive agents that non-specifically inhibit the immune system. The intrinsic toxicities of these drugs can result in organ dysfunction, and their universal inhibition of immunity leads to increased susceptibility to both infections and cancer (68–71). Thus, a fundamental goal in transplantation research is to develop a transient therapy that can result in long-term acceptance of the transplant while circumventing harmful side effects. An ideal transplant therapy induces donor-specific tolerance to the transplant while leaving the patient immune-competent against other assaults. Biologics targeting specific cell-surface immuno-receptors have garnered great interest as potential candidates for their defined rather than universal impact on the immune response (261). In particular, monoclonal antibodies disrupting the costimulatory CD40-CD154 and adhesion/costimulatory LFA-1-ICAM-1 pathways have proven successful in promoting transplant survival in both rodent and non-human primate models (210, 213, 218, 223, 239, 241, 247, 262). Using a mouse allogeneic pancreatic islet transplantation model, our laboratory has previously shown that antibodies against LFA-1 and CD154 individually induce donor-specific tolerance (218, 241). Furthermore, combining the two antibodies achieved allograft survival in all the recipients and a dominant tolerance state (218). The combination of anti-LFA-1 anti-CD154 also promoted indefinite survival and tolerance in various other transplantation models including the highly immunogenic xenogeneic islet transplants (219, 242, 246). Taken together, anti-LFA-1 and anti-CD154 treatments appear to be potent promoters of transplant survival and tolerance.

While antibody perturbation of LFA-1/ICAM-1 and CD40/40L pathways have been highly efficacious in promoting transplant survival, it has not been clear how such interventions impact the initial graft destructive T cell response. It

has been suggested that antibody perturbation of LFA-1 and CD154 pathways resulted in immediate deviation of graft-specific immune response either into deletion/anergy or induction of an alternate regulatory phenotype during T cell activation. Early studies using anti-CD154 treatments demonstrated a decrease in initial intragraft IL-2 and IFN-γ cytokine production while simultaneously increase the production of IL-4 and IL-10, suggesting T_H-1 to T_H-2 immune deviation as one possible mechanism (214, 231). Other studies indicated a rapid deletion of donor-specific CD8+ T cells or the induction of T cell apoptosis through activation-induced cell death by anti-CD154 treatments (215, 226, 263, 264). Alternatively, anti-CD154 therapies have been associated with altering naive T cell differentiation into an anergic phenotype or into CD4+Foxp3+ regulatory T cells during primary antigen challenge (227, 230). Fewer mechanistic studies have been done to elucidate the proximal impact of LFA-1-specific antibodies on naïve graft-specific T cell response. However, a recent study proposed that anti-LFA-1 induced transplant tolerance by sequestering activated, graft-specific T cells in the draining lymph nodes to prevent their infiltration into the graft site while simultaneously increasing the frequency of Foxp3+ Tregs in the draining lymph nodes (245). These studies proposed that a key mechanism of anti-LFA-1 and anti-CD154-mediated tolerance induction is an immediate deviation of graft-specific T cells toward an alternate, non-destructive phenotype during initial responses toward the transplant.

In this thesis, the impact of perturbing LFA-1 and/or CD154 during initial antigen exposure was examined using the adoptive transfer model developed and described in the previous chapter. Surprisingly, significant deviation in the fate of adoptively transferred antigen-specific CD4+ or CD8+ T cells during treatments with anti-LFA-1, anti-CD154, or combined anti-LFA-1 and anti-CD154 was not observed. Instead, rather than inducing an early dramatic alteration of T cell phenotype, anti-LFA-1 and anti-CD154 likely promoted transplant tolerance by attenuating the magnitude of graft-destructive T cell response during initial antigen encounter and therapy treatments, which then allowed early graft survival that eventually leads to the generation of long-term graft protection.

4.2 RESULTS

Antibody therapies restrained the magnitude of T cells in the draining lymph nodes.

Using the footpad challenge model as an *in vivo* representation of acute local antigen challenge, I interrogated both antigen-specific TCR transgenic and polyclonal T cell responses during anti-LFA-1 and/or anti-CD154 treatments. To examine a discrete monoclonal population of antigen-specific T cells, OT-I or OT-II lymphocytes were adoptively transferred into wild-type B6 recipients challenged at the footpads with B6-OVA splenocytes and treated with anti-LFA-1, anti-CD154, or combined anti-LFA-1/anti-CD154. When the draining popliteal lymph nodes were harvested 3 (OT-I) or 4 (OT-II) days post antigen challenge, all three therapies strikingly decreased the presence of adoptively transferred T cells in the draining lymph nodes. Importantly, while the therapies decreased total lymphocyte numbers in the nodes, they also specifically decreased the proportion of adoptively transferred antigen-specific T cells within the remaining popliteal lymph node cells (Fig. 4-1). This translated to a prominent reduction in the absolute numbers of OT-I or OT-II T cells remaining in the popliteal lymph nodes during harvest (Fig. 4-1). Anti-LFA-1 caused a significantly more pronounced reduction of antigen-specific T cells compared to anti-CD154, while combining anti-LFA-1 and anti-CD154 had the greatest impact on T cell presence in the draining lymph nodes than either antibody alone, suggesting an additive effect between the two antibodies (Fig. 4-1). The magnitude of OT-II CD4+ T cells in the draining lymph nodes seemed especially susceptible to the impact of combine anti-LFA-1/anti-CD154 therapy (Fig. 4-1B). Taken together, it appeared that the antibody therapies, especially when anti-LFA-1 was included, greatly restrained the magnitude of antigen-specific T cells in the reactive lymph node.

To compensate for any artifacts from using a monoclonal population of T cells to represent T cell reactivity, I also performed parallel experiments tracking polyclonal T cell reactivity during antibody therapies. Congenic CD45.1 B6 lymphocytes were adoptively transferred into wild-type CD45.2 B6 recipients, which allowed for the tracking of an adoptively transferred T cell population

without compromising potential variables such as naïve T cell precursor frequency and clonal variety within an antigen-specific T cell population. The recipients were challenged at the footpads with BALB/c splenocytes, and the adoptively transferred congenic T cells were assessed for their alloreactivity. All three therapies exerted similar impact on the presence of adoptively transferred polyclonal T cells in the draining popliteal lymph nodes as they did on the monoclonal transgenic T cells. In fact, polyclonal T cells experienced greater reduction in magnitude than TCR-transgenic T cells in the presence of the antibodies (Fig 4-2). In contrast to the more pronounced effect of the combined anti-LFA-1/anti-CD154 therapy in restraining the presence of antigen-specific T cells, anti-LFA-1 appeared to be as potent as the two antibodies combined in controlling both CD4+ and CD8+ T cells in the draining lymph nodes (Fig 4-2). Polyclonal CD4+ T cells also appeared to be more sensitive to the proximal effect of anti-LFA-1 therapies than CD8+ T cells, as anti-LFA-1 alone and combined with anti-CD154 decreased the number of adoptively transferred CD4+ T cells to levels below what was found in the draining lymph nodes of non-antigen-challenged recipients (Fig. 4-2). Therefore, all three therapies, but especially therapies including anti-LFA-1, significantly reduced the adoptively transferred monoclonal or polyclonal T cells from the draining lymph nodes.

Antibody therapies did not prevent T cell proliferation in the draining lymph nodes.

With such dramatic reduction in the magnitude of adoptively transferred T cells in the draining lymph node, it appeared that the antibody therapies, especially ones including anti-LFA-1, had suppressive effects on T cell reactivity. Since using a local footpad antigenic challenge model eliminated many experimental variables involved with transplantation surgeries such as tissue injury, wound healing, and graft ischemia, this observation is likely a direct consequence of proximal interaction between the therapeutic antibodies and responding T cells. If anti-LFA-1 and/or anti-CD154 directly led to the decrease in the number of responding T cells at the site of cognate antigens, this inhibitory

mechanism potentially explained therapeutic antibody-mediated transplant survival. However, when the phenotype of the T cells remaining in the draining lymph nodes was examined more closely, the impact of the therapies became less straightforward. This was also when monoclonal transgenic T cell response diverged from that of the polyclonal T cells, illustrating the complexities in delineating the fate of T cells and intrinsic variation between T cell models.

The first phenotypic parameter examined was T cell proliferative capacity in the presence of the therapeutic antibodies. Despite the prominent reduction in cell numbers, proliferation of adoptively transferred, monoclonal OT-I CD8+ T cells remaining in the draining popliteal lymph nodes was not disrupted in the presence of the antibodies (Fig. 4-3A). In fact, if not for the significant decrease in cell numbers, antibody therapies appeared to have little effect on OT-I CD8+ T cell reactivity. In contrast, the proliferative capacity of adoptively transferred OT-II CD4+ T cells appeared to be more vulnerable to the impact of therapies if both anti-LFA-1 and anti-CD154 were present (Fig. 4-3B). The proliferation of OT-II CD4+ T cells in the draining lymph nodes of the combined therapy-treated recipients appeared to lag behind untreated control by two to three divisions (Fig. 4-3B). Nevertheless, while having both antibodies present delayed the proliferation cycle of antigen-specific CD4+ T cells, the antibodies by no means grossly inhibited their antigen responsiveness. Both adoptively transferred OT-I and OT-II T cells achieved enough cycles of cell division that should have correlated to a substantial increase in T cell numbers in the draining lymph nodes. However, I have also shown the marked decrease in the number of T cells in the nodes. Therefore, the antibodies paradoxically allowed T cell proliferation but resulted in significantly diminished T cell numbers.

In contrast, the impact of antibody therapies on polyclonal T cells in the draining lymph nodes appeared less dichotomous. Adoptively transferred polyclonal T cells showed reduced proportion of proliferated cells in the presence of the therapies based on the extent of CFSE dilution in the draining lymph nodes (Fig. 4-4). Interestingly, this observation can be explained by two distinct interpretations. The first interpretation is that, unlike monoclonal T cells, antibody

therapies have higher potency in suppressing polyclonal T cell proliferation. Alternatively, another interpretation takes into account that the adoptively transferred T cell population is polyclonal with only a small proportion able to respond to an allo-antigen, and the reduction of the total number of responsive T cells were so great in this model in comparison to adoptively transferred monoclonal T cells, the additional therapy-mediated decrease in cell number within the already small proliferating population rendered the T cells seemingly non-proliferative during analysis. This interpretation is supported in the case of anti-LFA-1 single and combined treatments, where the undivided, $CFSE^{HI}$ peak were reduced alongside the proliferating, $CFSE^{LO}$ cells, suggesting a non-discriminative reduction in all T cells, whether alloreactive or not (Fig. 4-4). As there existed no markers to track polyclonal alloreactive T cells specifically, I could not distinguish whether a larger population of alloreactive T cells remained $CFSE^{HI}$ in the presence of the antibodies, which would demonstrate actual inhibition of antigen-specific T cell proliferation. While the observations may suggest that the therapies exerted a greater inhibitory effect on both polyclonal T cell proliferation and magnitude in response to an allo-antigen stimulation, taken into account the intact and vigorous proliferative capability observed in therapy-treated monoclonal T cells, I consider the second interpretation of the results more likely, and that the therapies do not completely suppress polyclonal T cell activation and proliferation.

Antibody therapies did not alter the activation phenotype of proliferated T cells in the draining lymph nodes.

I have shown the paradoxical observation that antibody therapies allowed T cell proliferation but decreased their numbers in the draining lymph nodes. The link between a seemingly productive antigen-driven proliferation and a massive decrease in cell number was puzzling. I therefore examined the activation phenotypes of the proliferated T cells for the possibility of an altered or unstable T cell activation. The primary markers used to determine the activation state of T cells were CD44 and CD62L, as up-regulation of CD44 and down-regulation of

CD62L are standard representations of an antigen-experienced T cell. OT-I and OT-II T cells proliferating in the presence of antibody therapies exhibited increasing CD44 and decreasing CD62L expression resembling T cells without therapy treatments, suggesting similarly productive engagement with cognate antigens (Fig. 4-5A and B). Regulation of activation markers on OT-II CD4+ T cells in particular did not seem to be affected by treatments with antibodies (Fig. 4-5B). Interestingly, anti-LFA-1 alone and with anti-CD154 appeared to slightly raise CD44 expression on proliferating OT-I CD8+ T cells upon antigen engagement but retained a population of CD8+ T cells that did not down-regulate their CD62L expression in the draining lymph node (Fig. 4-5A). The proliferating OT-I T cells remaining $CD62L^{HI}$ was particularly unexpected, as anti-LFA-1 has been previously shown to non-specifically down-regulate naive lymph node T cell CD62L expression in the absence of antigen stimulation (Fig. 3-8B). The overall expression of CD44 and CD62L by adoptively transferred OT-I and OT-II T cells were similar whether treated with antibody therapies or not (Fig. 4-5C and D). In fact, combined anti-LFA-1 and anti-CD154 actually increased CD44 expression on OT-I CD8+ T cells (Fig. 4-5C). Taken as a whole, antibody therapies did not prevent proliferating monoclonal T cells from acquiring a classical activation phenotype, suggesting the presence of therapeutic antibodies did not significantly interfere with antigen interaction by T cells remaining in the draining lymph nodes.

While antibody therapies had shown a greater impact on polyclonal T cell number and possibly proliferation, they did not affect activation phenotype of the responding T cells. Both proliferating polyclonal CD4+ and CD8+ T cells up-regulated CD44 and down-regulated CD62L in the presence of therapeutic antibodies (Fig. 4-6). This suggested that antibody therapies did not entirely inhibit polyclonal T cells from engaging with their antigens, and that productive antigen-driven activation and proliferation were still possible under therapy treatments despite the dramatic reduction of the T cell magnitudes in the draining lymph nodes. Combining with the observation of activation statuses in transgenic antigen-specific T cells, it seemed that all three antibody therapies allowed

productive T cell-antigen engagement and activation despite the potent reduction of T cell numbers in draining lymph nodes.

Antibody therapies differentially affected T cell effector cytokine production.

As anti-LFA-1 and anti-CD154 therapies restrained the magnitude of both monoclonal TCR-transgenic and polyclonal T cells while allowing their activation and proliferation, I next examined the antibodies' impact on T cell effector differentiation. While the reduction in draining lymph node T cell numbers could explain some of the antibodies' ability to prolong transplant survival, it is curious how the therapies control the population of T cells that still activated and proliferated. A possibility is that T cell activation in the presence of anti-LFA-1 and/or anti-CD154 alters their functional differentiation. If proliferated T cells are compromised in their ability to cause graft injury due to a deviation in effector phenotype, transplant survival will occur despite T cell activation. Therefore, using the same model of local footpad challenge, the impact of therapeutic antibodies on T cell functional differentiation, as represented by cytokine profiles, in the draining popliteal lymph nodes was investigated.

While TCR-transgenic OT-I and OT-II T cells activated and proliferated in the draining lymph nodes in the presence of anti-LFA-1 and/or anti-CD154, the antibodies appeared to selectively suppress their cytokine production. Without antibody therapies, OT-I CD8+ T cells acquired an inflammatory-type cytokine profile with high expressions of IFN-γ and granzyme B in response to B6-OVA splenocytes in the footpads (Fig. 4-7A). Anti-LFA-1 alone reduced expressions of both IFN-γ and granzyme B to approximately half of the un-modified response in the draining lymph nodes (Fig. 4-7A). In contrast, anti-CD154 did not affect the expression of IFN-γ but restrained the production of granzyme B (Fig. 4-7A). Despite the partial suppression, neither antibody alone completely inhibited the production of these inflammatory cytokines. Interestingly, when used together, anti-LFA-1 and anti-CD154 significantly decreased IFN-γ and granzyme B expressions to levels resembling un-stimulated OT-I CD8+ T cells, again showing an additive effect between the two antibodies (Fig. 4-7A). The antibodies also

impacted OT-II CD4+ T cell cytokine production. Un-modified OT-II CD4+ T cells acquired a T_H-1-like inflammatory phenotype with high expression of IFN-γ and no interleukin-4 (Fig. 4-7B, 4-7C). Anti-CD154 restrained IFN-γ expression by OT-II CD4+ T cells to a greater extent than anti-LFA-1, suggesting CD4+ T cells to be more susceptible to CD154 perturbation (Fig. 4-7B). Simultaneous treatment with both anti-LFA-1 and anti-CD154 also showed a more pronounced effect especially on reducing IFN-γ production by OT-II CD4+ T cells (Fig. 4-7B). While the antibody therapies decreased CD4+ T cell production of IFN-γ to different degrees, neither antibody alone induced interleukin-4 or a switch in T cell activation into a T_H-2-like phenotype (Fig. 4-7C). As such, the antibody therapies appear to selectively and partially suppress existing cytokine production without inducing alternative cytokine profiles in the transgenic T cells.

Similar to their impact on monoclonal transgenic T cells, the therapeutic antibodies also affected polyclonal T cells cytokine profiles. Unlike monoclonal T cells, alloreactive T cells within the polyclonal adoptively transferred population cannot be irrefutably identified by a marker. As such, the cytokine production of the polyclonal T cells were analyzed as cytokine production by T cells that had diluted CFSE fluorescence with the assumption that the majority of the $CFSE^{LO}$ population proliferated specifically in response to BALB/c splenocytes (Fig. 4-8A). Under this assumption, anti-LFA-1 appeared to suppress IFN-γ production by CD4+ T cells but not CD8+ T cells, which is contradictory to its suppression of both IFN-γ and granzyme B in monoclonal transgenic CD8+ T cells (Fig. 4-8B, Fig. 4-7A). Both polyclonal CD4+ and CD8+ T cells were susceptible to cytokine inhibition by anti-CD154 (Fig. 4-8B). Similarly to their impact on monoclonal T cells, combined anti-LFA-1 and anti-CD154 exerted greater impact on both CD4+ and CD8+ T cell IFN-γ production (Fig. 4-8B). However, the combined therapy did not suppress cytokine production by the polyclonal T cells to the same magnitude as the monoclonal transgenic T cells (Fig. 4-8B, Fig. 4-7). These observations further illustrated the variability in studying a monoclonal population of T cells versus a population including a large number of different clones. While monoclonal TCR-transgenic T cells were, by nature, expected to

respond homogenously, antigen-specific polyclonal T cells could potentially differentiate into a myriad of varied effectors (265). Therefore, the dissimilar impact of the antibodies on transgenic versus polyclonal antigen-specific T cell responses was to be expected. As a whole, the antibodies appeared to exert partial suppression to T cell effector cytokine production without inducing alternate phenotypes.

Anti-LFA-1 increased frequency but not absolute number of FoxP3-expressing CD4+ T cells in the draining lymph nodes.

While the therapeutic antibodies appeared to partially suppress draining lymph node T cell reactivity, they did not appear to alter intrinsic T cell effector phenotype. I followed up this observation by examining whether the antibodies could induce a *de novo* regulatory T cell phenotype. As described in section 1.2.1.2.3, FoxP3-expressing CD4+ T cells are a key regulatory component in self-tolerance, and have been implicated as a potential mechanism for transplant survival. A major current paradigm of how various non-depleting, therapeutic antibodies promote transplant survival and immune tolerance is through induction of FoxP3+CD4+ T regulatory cells. Therefore, I assessed the expression of FoxP3 by adoptively transferred CD4+ T cells in both monoclonal and polyclonal footpad stimulation models, the presence of which would indicate the antibodies' ability to drive naïve T cells into acquiring regulatory phenotype.

As transgenic OT-II mice were also on a rag gene-knockout background, naïve OT-II CD4+ T cells did not express FoxP3. This was shown by the low background levels of FoxP3 expression among un-stimulated OT-II CD4+ T cells (Fig. 4-9). While anti-CD154 alone did not change the proportion of FoxP3+ OT-II T cells in the draining lymph nodes, treatments with anti-LFA-1 alone or combined with anti-CD154 resulted in large increases in the percentages of Foxp3+ OT-II CD4+ T cells (Fig. 4-9). This appeared to support the paradigm that therapeutic antibodies preferentially skew naïve T cell phenotype into a tolerogenic lineage. However, when the absolute numbers of FoxP3+ OT-II CD4+ T cells were calculated, this seeming increase in the magnitude of Tregs

disappeared (Fig. 4-9). In fact, anti-LFA-1 and combined therapy prominently reduced the number of Foxp3+ OT-II T cells in draining lymph nodes (Fig. 4-9). This was likely a result of the overall reduction in the total number of OT-II T cells as a result of these two therapies (Fig. 4-1B). Therefore, while anti-LFA-1 and combined anti-LFA-1/anti-CD154 resulted in a higher frequency of FoxP3+ OT-II T cells in the draining lymph nodes, the absolute number of FoxP3+ OT-II T cells revealed that the antibodies did not cause a preferential *de novo* generation of regulatory T cells.

Similar to the trend observed in monoclonal TCR-transgenic CD4+ T cells, the presence of anti-LFA-1 and combined anti-LFA-1/anti-CD154 during T cell activation increased the percentage of FoxP3+ CD4+ T cells in the draining lymph nodes (Fig. 4-10). The increase in percentage with the combined antibody therapy was significantly higher compared to FoxP3 expression in non-therapy-treated CD4+ T cells and anti-CD154-treated T cells (Fig. 4-10). Nevertheless, neither therapy caused significant increase in the absolute number of FoxP3+ CD4+ T cells despite the increases in frequencies (Fig. 4-10). As the polyclonal T cells originated from wild-type, rag gene-sufficient donors, the FoxP3-expressing CD4+ T cells observed could include both natural regulatory T cells already expressing FoxP3 and induced regulatory T cells activated into a FoxP3-expressing lineage upon antigen stimulation. Therefore, in the polyclonal model of T cell activation, anti-LFA-1 and combined anti-LFA-1/anti-CD154 did not appear to induce generation of new FoxP3+ CD4+ T cells or preferential recruitment of existing FoxP3+ CD4+ T cells based on the lack of increase in T cell numbers (Fig. 4-10). Taken together, both the monoclonal transgenic T cell and polyclonal T cell data indicate that the presence of therapeutic antibodies during T cell activation did not preferentially generate new regulatory T cells in the responding CD4+ T cell population.

Antibody therapies did not increase T cell apoptosis in the draining lymph nodes.

I have so far established that anti-LFA-1 and/or anti-CD154 significantly restrained the number of reactive T cells in the draining lymph nodes while still allowing their activation, proliferation, and differentiation. Surprisingly, the antibodies, while partially suppressing effector cytokine production, did not induce alternate cytokine profiles or trigger *de novo* generation of FoxP3+ regulatory T cells. I have thus returned to the original question I set out to answer: what is the proximal impact of the therapeutic antibodies on T cells that allows transplantation tolerance? As of now, the most striking effect of the antibody therapies was the decrease in the number of T cells in the draining lymph nodes. This raised the possibility that the antibodies facilitated activation-induced cell death and led to the deletion of responding T cells. To explore this possibility, I assessed the expressions of the pro-apoptotic marker caspase 3 and the anti-apoptotic marker Bcl-2 by adoptively transferred, footpad-challenged TCR-transgenic and polyclonal T cells directly *ex vivo*. If anti-LFA-1 and/or anti-CD154 significantly increased T cell expression of the pro-apoptotic caspase 3 or decreased the pro-survival Bcl-2 expressions, it could potentially explain the paradox of T cell activation and proliferation leading to decreased T cell number in the draining lymph nodes, and lend support to a deletional mechanism for generating transplantation tolerance.

The results were, unfortunately, anti-climatic. The therapeutic antibodies did not significantly increase the expression of caspase 3 by proliferating monoclonal OT-I and OT-II T cells in the draining lymph nodes (Fig. 4-11A). Both OT-I CD8+ and OT-II CD4+ T cells activated and proliferated without antibody treatment started showing presence of apoptotic, caspase 3-expressing cells at approximately three to four rounds of cell division, suggesting a baseline activation-induced cell death mechanism in this model (Fig. 4-11A). Anti-LFA-1 treatment alone appeared to accelerate the appearance of caspase 3-expressing OT-I and OT-II T cells earlier, and might suggest some effect on accelerated T cell death following activation (Fig. 4-11A). However, the percentages of total caspase-3 expression by proliferating transgenic T cells in the presence of anti-LFA-1 were not strikingly different from the non-therapy-treated T cells (Fig. 4-

11A). As for Bcl-2, which promotes T cell survival when expressed, activated T cells automatically down-regulate Bcl-2 during the acute expansion phase, and the trend of Bcl-2 down-regulation did not appear to vary in the presence or absence of the antibodies (Fig. 4-11B). The same observations were seen in proliferating polyclonal alloreactive T cells (Fig. 4-12). Therefore, while antibody-driven apoptosis and deletion would explain the rapid disappearance of responding T cells from the draining lymph nodes, *ex vivo* assessment of caspase 3 and Bcl-2 did not indicate this as a direct impact of anti-LFA-1 and/or anti-CD154 on antigen-specific T cells.

Antibody therapies allowed T cell persistence and reactivity during secondary antigen exposure.

However, while expression of apoptotic proteins is a solidly reliable indicator of T cell death *in vitro*, it is notoriously difficult to examine apoptosis *in vivo*, since apoptotic cells are rapidly cleared from the immune microenvironment. Furthermore, studies have shown that expression of apoptotic markers *ex vivo* may not be restricted to apoptotic cells (266–270). As such, I used an alternative method to indirectly investigate T cell deletion as a mechanism of anti-LFA-1 and/or anti-CD154. Instead of looking for immediate up-regulation of apoptosis, I tested the persistence of adoptively transferred, antigen-activated, and therapy-treated T cells. This was done by systemically challenging recipients adoptively transferred with OT-I, OT-II, or polyclonal B6 with B6-OVA or BALB/c splenocytes with or without therapy regimens. Recipients' spleens were harvested minimum three weeks after initial antigen challenge, and the splenocytes were given secondary antigen challenge *in vitro*. The results from this approach answered two questions. 1) If the adoptively transferred T cells could be recovered, then deletion was unlikely a mechanism of anti-LFA-1 and/or anti-CD154. 2) If T cells were recovered and could respond to the secondary challenge, then clonal anergy was further demonstrated to not be a mechanism of the therapies.

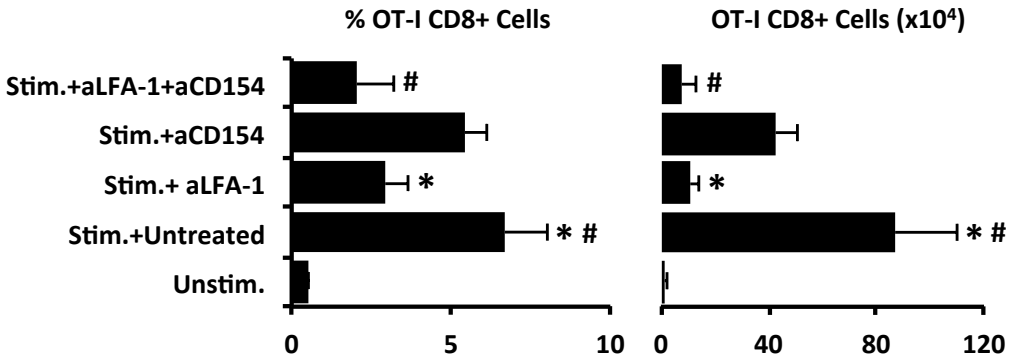
Unfortunately, this experiment was difficult to carry out with the monoclonal TCR-transgenic T cells. As shown in chapter three, adoptively transferred OT-II CD4+ T cells underwent complete attrition within two weeks after transfer (Fig. 3-4). As a confirmation of that observation, they could not be found at three weeks post primary antigen challenge in this experiment. OT-I CD8+ T cells fared slightly better, and a small population could still be recovered from recipients' spleens, and these persistent transgenic CD8+ T cells proliferated vigorously during *in vitro* secondary challenge whether the recipients were given antibody therapies or not (Fig. 4-13A). This showed that antibody therapies induced neither clonal deletion nor anergy of antigen-specific CD8+ T cells during initial antigen encounter and therapy treatments (Fig. 4-13A). Interestingly, while none of the therapeutic antibodies eliminated the presence of persistent, antigen-experienced, $CD44^{HI}$ OT-I T cells in the spleens, OT-I T cells activated initially in the presence of anti-LFA-1 showed reduced expression of IFN-γ during secondary antigen challenge (Fig. 4-13B). However, if the T cells were activated in the presence of both anti-LFA-1 and anti-CD154 initially, the persistent T cells expressed IFN-γ normally upon secondary challenge (Fig. 4-13B). Taken as a whole, antibody therapies did not cause deletion or anergy of monoclonal antigen-specific CD8+ T cells during initial antigen encounter, and the persistent T cells were able to proliferate and acquire effector phenotype in response to a secondary antigen challenge.

Similar results were observed with polyclonal T cells, in which adoptively transferred B6 T cells could still be detected three weeks after primary challenge with BALB/c splenocytes. The persistent T cells underwent proliferation upon secondary BALB/c challenge whether therapeutic antibodies were present during primary antigen encounter or not (Fig. 4-14A). Furthermore, both persistent CD4+ and CD8+ T cells retained their abilities to produce IFN-γ and granzyme B (Fig. 4-14B). Polyclonal CD4+ T cells initially activated in the presence of anti-CD154 appeared to have a reduced but not inhibited capacity to express IFN-γ (Fig. 4-14B). Interestingly, CD8+ T cells activated in the presence of anti-LFA-1 appeared to actually have enhanced production of granzyme B upon secondary

challenge (Fig. 4-14B). Despite the modest fluctuations in effector cytokine production, antibody therapies did not prevent adoptively transferred polyclonal T cells to differentiate into effector phenotypes during secondary antigen challenge (Fig. 4-14B). Furthermore, the therapies did not result in significant up-regulation of FoxP3 or expression of the anti-inflammatory cytokine interleukin-10 in CD4+ T cells during secondary challenge (Fig. 4-14C). In fact, adoptively transferred CD4+ T cells remaining after initial treatment with anti-CD154 and combined anti-LFA-1/antiCD154 therapies showed reduced expression of these regulatory markers during secondary challenge (Fig. 4-14C). Similar to observations made with monoclonal OT-I CD8+ T cells, treatment with antibody therapies did not appear to cause universal deletion and inactivation of reactive T cells, and the recipients retained T cells that could be activated into functional, non-regulatory effectors during subsequent exposures to their antigens.

4.3 FIGURES

A



B

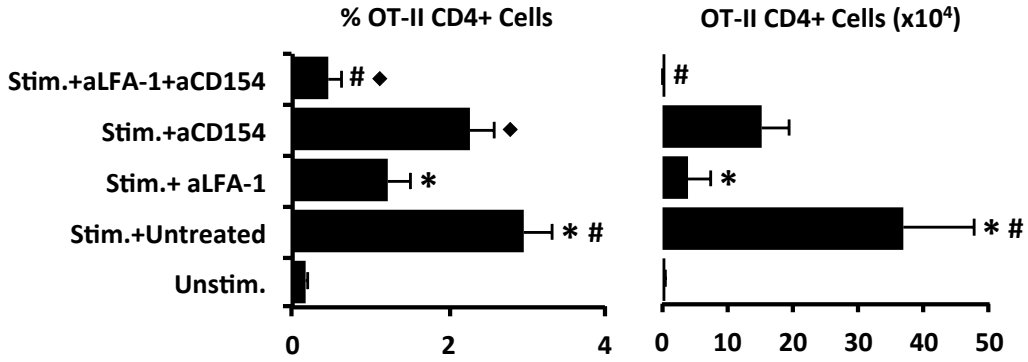


FIGURE 4-1. Antibody therapies restrained the number of antigen-specific T cells in draining lymph nodes. $5x10^6$ CD45.1 OT-I or $10x10^6$ CD45.1 OT-II lymphocytes were adoptively transferred into CD45.2 B6 recipients. Recipients were challenged at the footpads with $1x10^6$ B6-OVA splenocytes with or without anti-LFA-1, anti-CD154, or combined anti-LFA-1/anti-CD154 treatments. Draining popliteal LNs were harvested 3 (OT-I) or 4 (OT-II) days post footpad challenge and analyzed by flow cytometry. Percentages of CD45.1+CD8+ (OT-I) or CD45.1+CD4+ (OT-II) T cells were their proportion among total popliteal LN cells. Absolute cell numbers were calculated by multiplying the percentages to total number of popliteal LN cell. Pairs of identical symbols on bar graphs denote data groups with significant differences of at least $P < 0.05$. *A.* Percentage and absolute number of OT-I CD8+ T cells in popliteal LNs. *B.* Percentage and absolute number of OT-II CD4+ T cells in popliteal LNs.

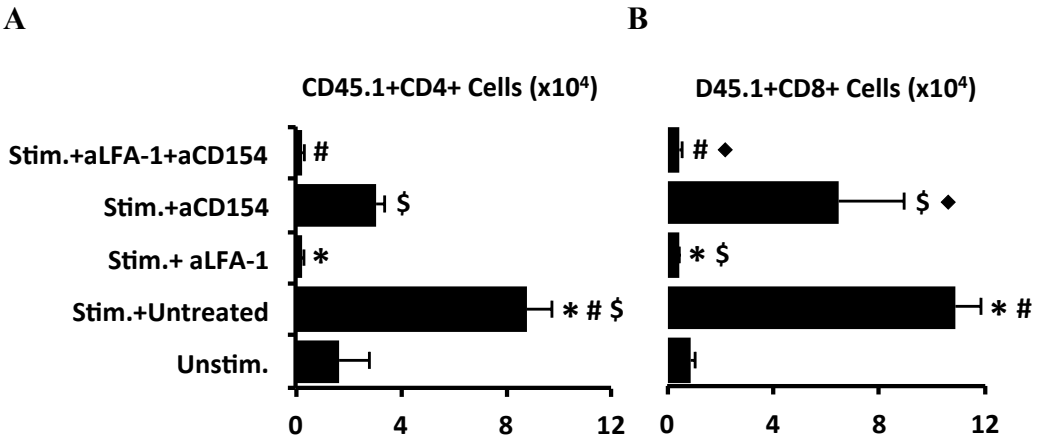
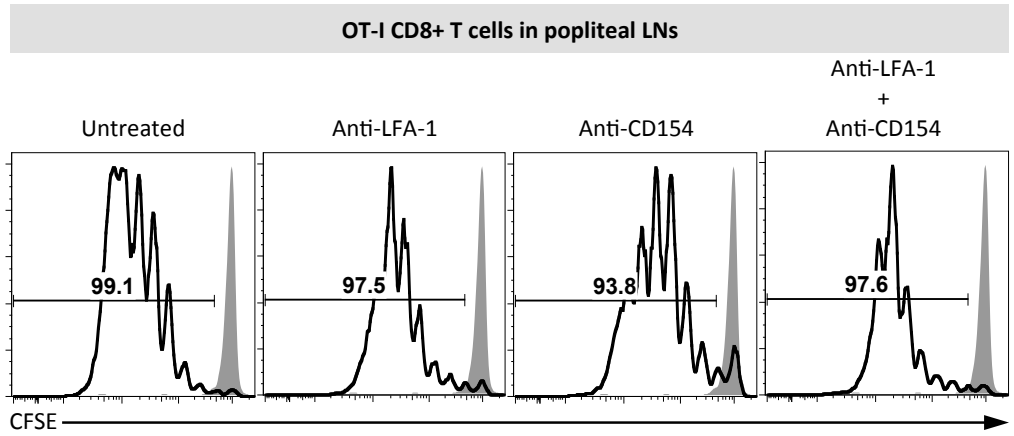


FIGURE 4-2. Antibody therapies restrained the number of polyclonal T cells in draining lymph nodes. $30x10^6$ CD45.1 B6 lymphocytes were adoptively transferred into CD45.2 B6 recipients. Recipients were challenged at the footpads with $1x10^6$ BALB/c splenocytes with or without anti-LFA-1, anti-CD154, or combined anti-LFA-1/anti-CD154 treatments. Draining popliteal LNs were harvested 5 days post footpad challenge and analyzed by flow cytometry. Absolute cell numbers were calculated by multiplying the percentages of CD45.1+CD4+ or CD45.1+CD8+ T cells to the total number of popliteal LN cell. Pairs of identical symbols on bar graphs denote data groups with significant differences of at least $P < 0.05$. *A.* Absolute number of adoptively transferred CD4+ T cells in popliteal LNs. *B.* Absolute number of adoptively transferred CD4+ T cells in popliteal LNs.

A



B

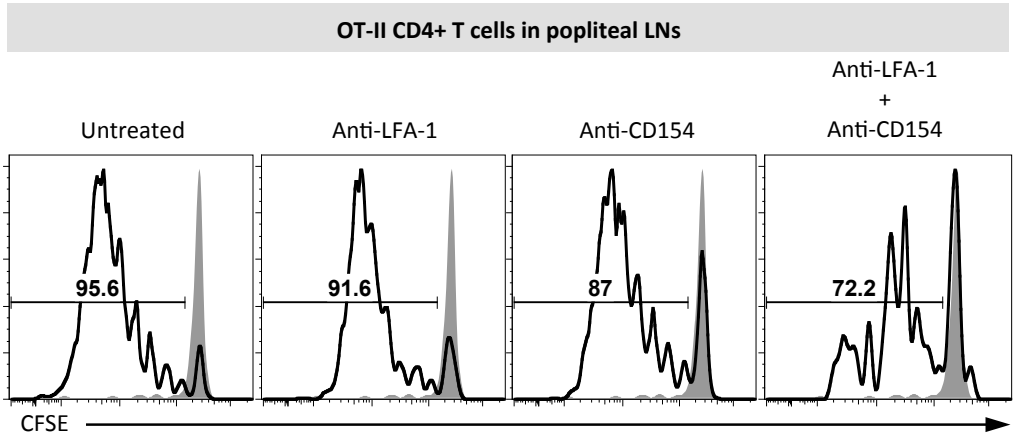


FIGURE 4-3. Antibody therapies did not inhibit antigen-specific T cell proliferation in draining lymph nodes. Mice were treated as described in Figure 4-1. Popliteal LN cells were analyzed for activation proliferation by CFSE dilution using flow cytometry. *A.* OT-I T cell proliferation in popliteal LNs. Histograms represent CD45.1+CD8+ -gated lymphocytes. *F.* OT-II T cell proliferation in popliteal LNs. Histograms represent CD45.1+CD4+ -gated lymphocytes.

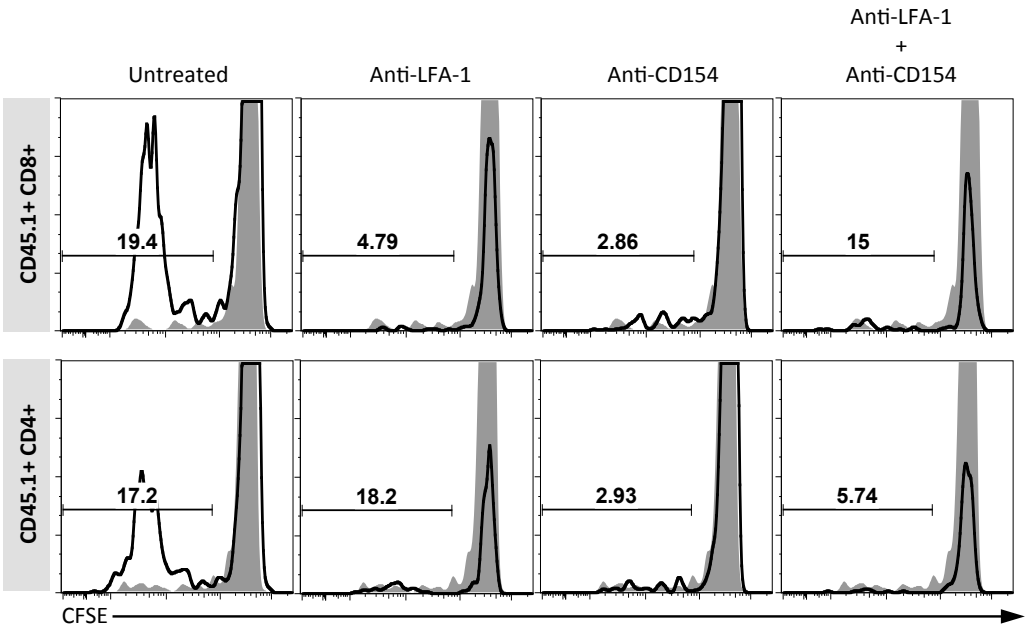


FIGURE 4-4. Antibody therapies differentially restrained the presence of proliferated polyclonal T cells in draining lymph nodes. Mice were treated as described in Figure 4-2. Popliteal LN cells were analyzed for proliferation by CFSE dilution using flow cytometry. Histograms are gated on CD45.1+CD8+ or CD45.1+CD4+ T cells in popliteal LNs.

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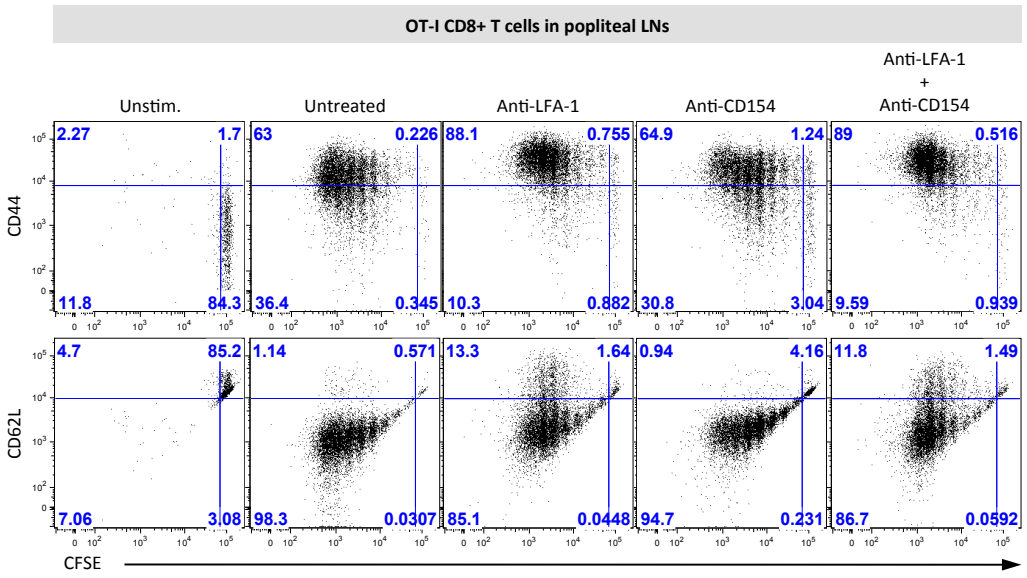

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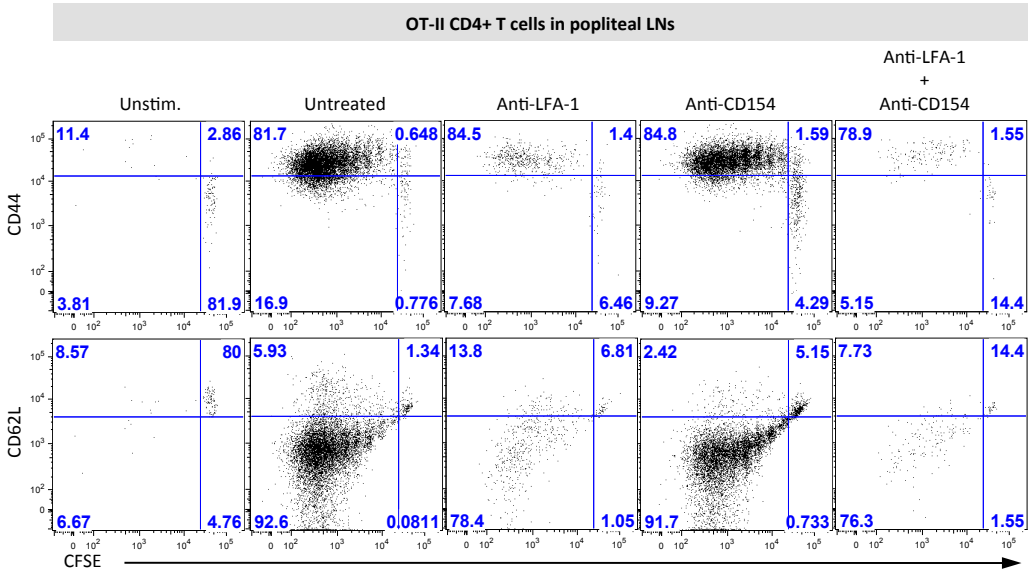

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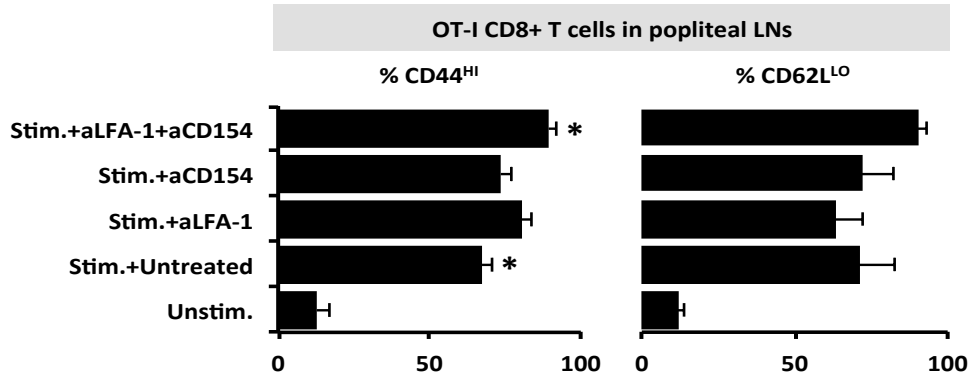

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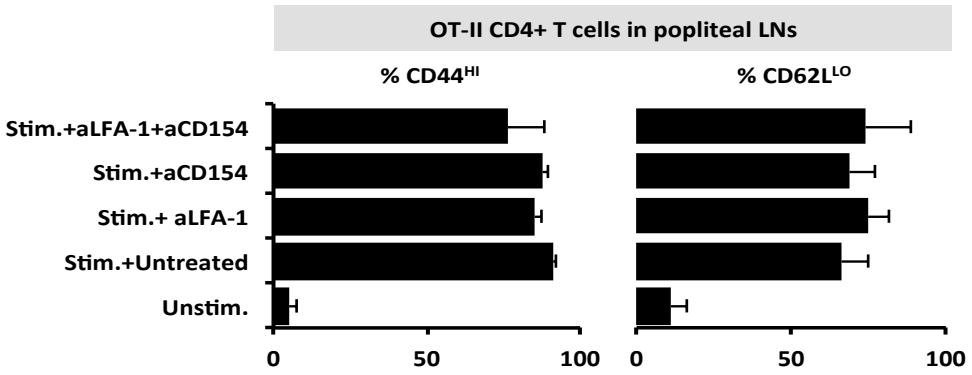

FIGURE 4-5. Antibody therapies did not inhibit antigen-specific T cell activation in the draining lymph nodes. Mice were treated as described in Figure 4-1. Popliteal LN cells were analyzed for activation ($CD44^{HI}$, $CD62L^{LO}$) by flow cytometry. *A.* Up-regulation of CD44 and down-regulation of CD62L by proliferating OT-I T cells. Dot plots were gated on CD45.1+CD8+ cells in popliteal LNs. *B.* Up-regulation of CD44 and down-regulation of CD62L by proliferating OT-II T cells. Dot plots were gated on CD45.1+CD4+ cells in popliteal LNs. *C.* Overall expression of activation markers by OT-I (CD45.1+CD8+) T cell in popliteal LNs. *D.* Overall expression of activation markers by OT-II (CD45.1+CD4+) T cells in popliteal LNs. Pairs of identical symbols on bar graphs denote data groups with significant differences of at least $P < 0.05$.

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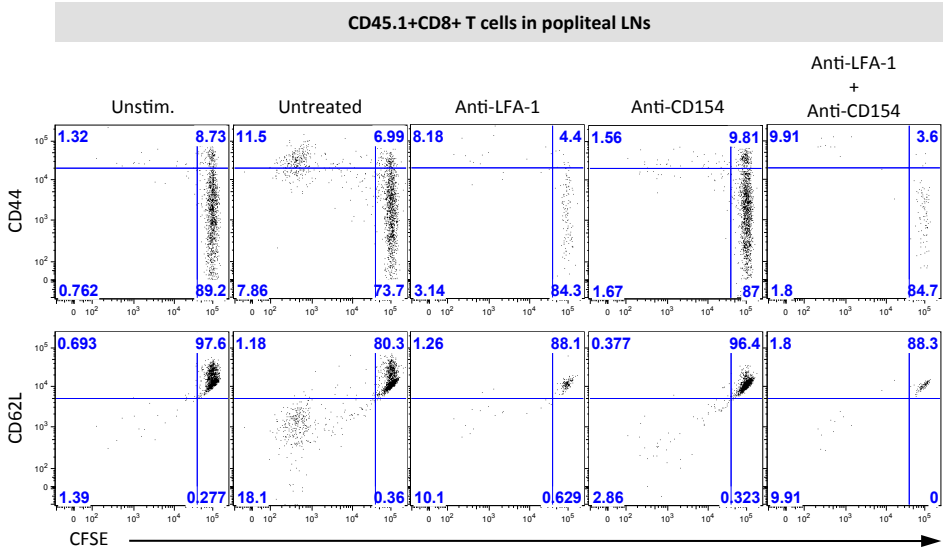

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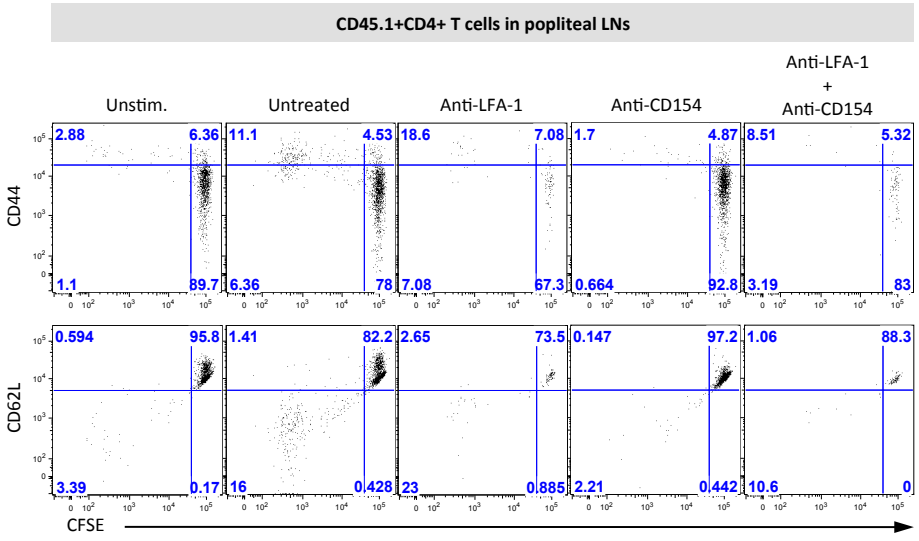

FIGURE 4-6. Antibody therapies differentially allowed activation of polyclonal T cells in the draining lymph nodes. Mice were treated as described in Figure 4-2. Popliteal LN cells were analyzed for activation ($CD44^{HI}$, $CD62L^{LO}$) by flow cytometry. *A.* Up-regulation of CD44 and down-regulation of CD62L by adoptively transferred and proliferating CD8+ T cells. Dot plots were gated on CD45.1+CD8+ cells in popliteal LNs. *B.* Up-regulation of CD44 and down-regulation of CD62L by adoptively transferred and proliferating CD4+ T cells. Dot plots were gated on CD45.1+CD4+ cells in popliteal LNs.

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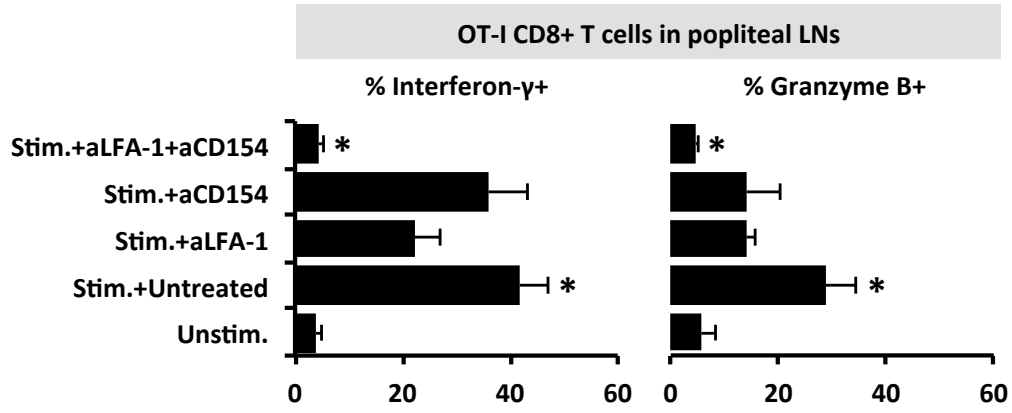

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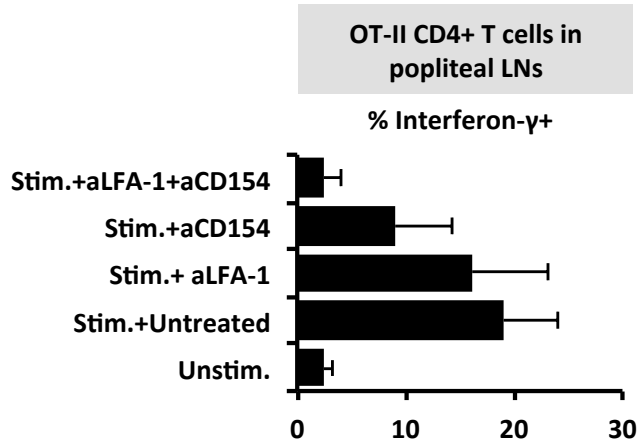

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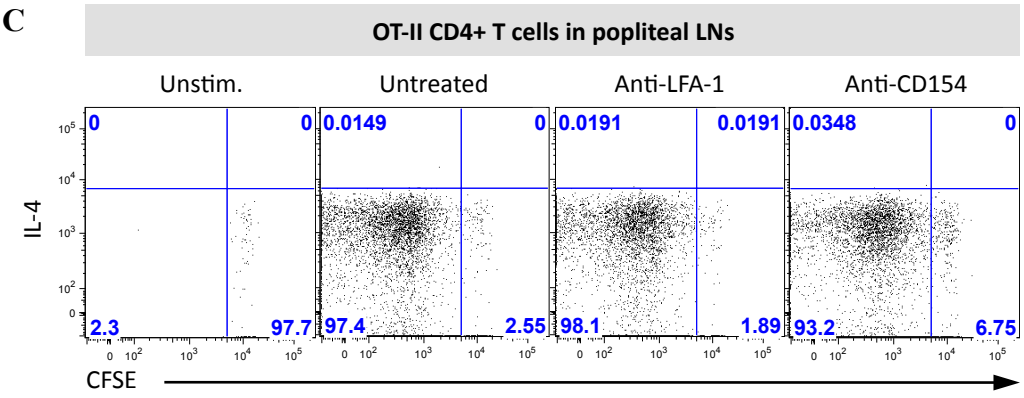

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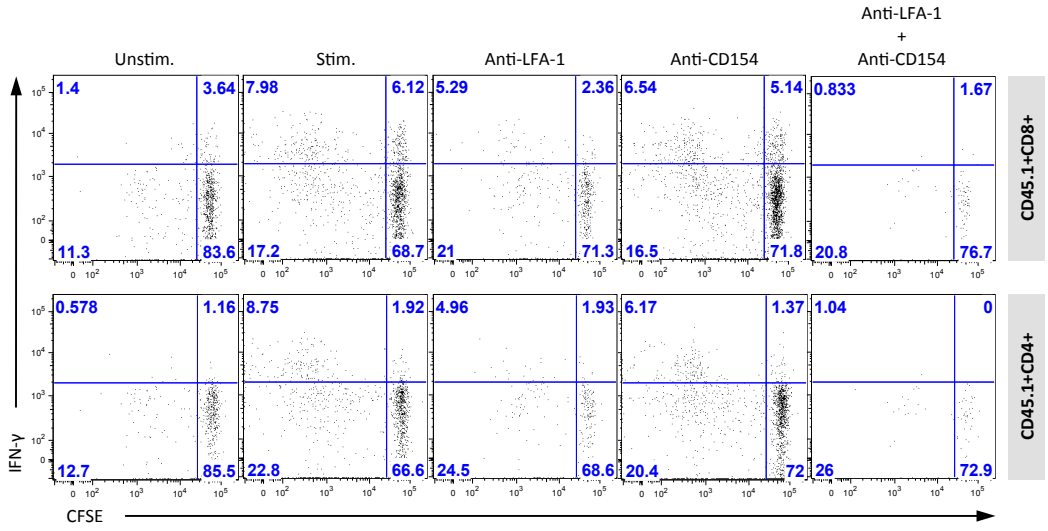
FIGURE 4-7. Combined therapy with anti-LFA-1 and anti-CD154 suppressed effector cytokine production by antigen-specific CD4+ and CD8+ T cells in draining lymph nodes. Mice were treated as described in Figure 4-1. T cell cytokine profile was determined by intracellular antibody labeling and flow cytometry after 4 hours of *in vitro* re-stimulation with OT-I or OT-II peptides and brefeldin A. Data shown are cytokine production by OT-I (gated on CD45.1+CD8+) and OT-II (gated on CD45.1+CD4+) T cells in popliteal LNs. Pairs of identical symbols on bar graphs denote data groups with significant differences of at least $P < 0.05$. *A.* IFN-γ and granzyme B production by OT-I T cells in popliteal LNs. *B.* IFN-γ production by OT-II T cells.

A



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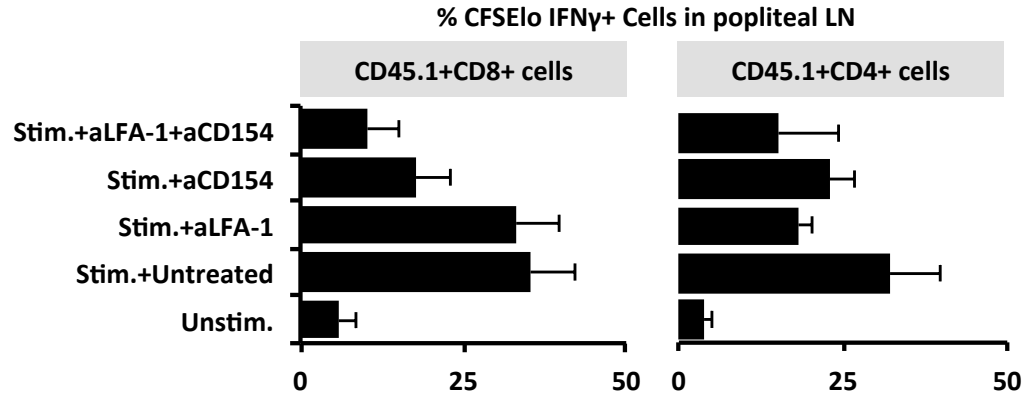


FIGURE 4-8. Combined therapy with anti-LFA-1 and anti-CD154 suppressed effector cytokine production by polyclonal CD8+ T cells in the draining lymph nodes. Mice were treated as described in Figure 4-2. T cell cytokine profile was determined by intracellular antibody labeling and flow cytometry after 4 hours of *in vitro* re-stimulation with PMA/ionomycin and brefeldin A. *A*. IFN-γ production by adoptively transferred and proliferating CD8+ (gated on CD45.1+CD8+) and CD4+ (gated on CD45.1+CD4+) T cells in popliteal LNs. *B*. Overall IFN-γ production by adoptively transferred and proliferated CD8+ (gated on CD45.1+CD8+CFSE^{LO}) and CD4+ (gated on CD45.1+CD4+CFSE^{LO}) T cells in popliteal LNs.

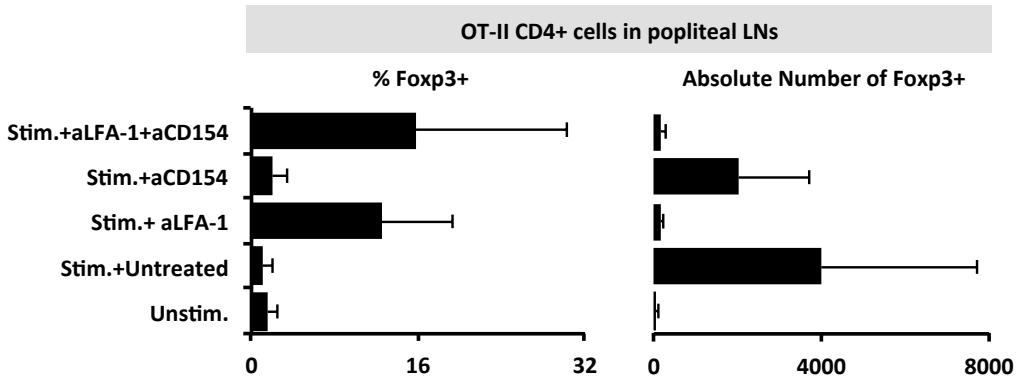


FIGURE 4-9. Treatments with anti-LFA-1 alone or anti-LFA-1 combined with anti-CD154 increased proportion but not absolute number of Foxp3+ antigen-specific CD4+ T cells in draining lymph nodes. Mice were treated as described in Figure 4-1. Foxp3 expression by adoptively transferred OT-II T cells in popliteal LNs was determined by intracellular antibody labeling and flow cytometry. Percentages of Foxp3+ OT-II T cells were Foxp3-expressing cells within the CD45.1+CD4+ gate in the popliteal LNs. Absolute numbers of Foxp3+ OT-II T cells were determined by multiplying the percentage of Foxp3-expressing cells by total number of CD45.1+CD4+ T cells (as described in Figure 4-1) in popliteal LNs.

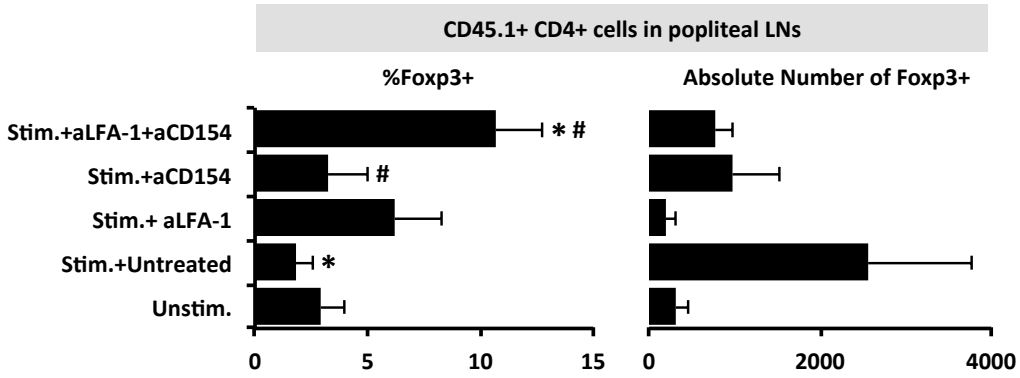
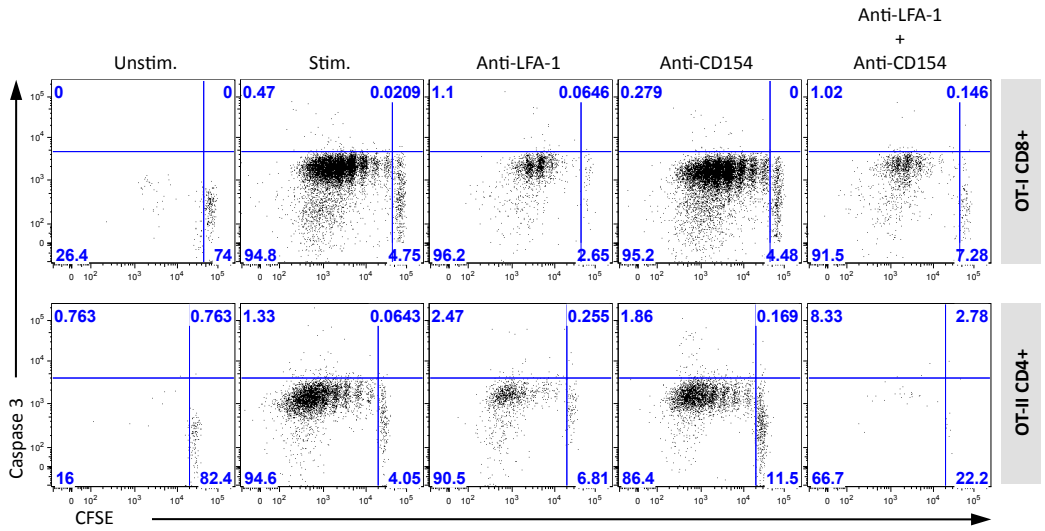


FIGURE 4-10. Treatments with anti-LFA-1 alone or anti-LFA-1 combined with anti-CD154 increased proportion but not absolute number of Foxp3+ polyclonal CD4+ T cells in draining lymph nodes. Mice were treated as described in Figure 4-2. Foxp3 expression by adoptively transferred CD4+ T cells in popliteal LNs was determined by intracellular antibody labeling and flow cytometry. Percentages of Foxp3+ T cells from the adoptively transferred population were Foxp3-expressing cells within the CD45.1+CD4+ gate in the popliteal LNs. Absolute numbers of Foxp3+ T cells were determined by multiplying the percentage of Foxp3-expressing cells by total number of CD45.1+CD4+ T cells (as described in Figure 4-2) in popliteal LNs.

A



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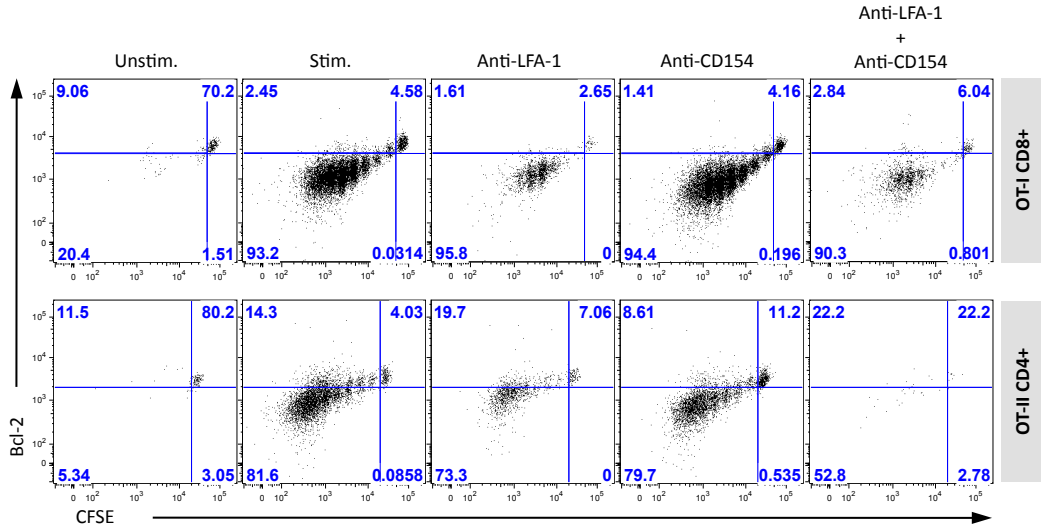

FIGURE 4-11. Antibody therapies did not significantly induce antigen-specific T cell apoptosis in the draining lymph nodes. Mice were treated as described Figure 4-1. Caspase 3 and Bcl-2 expression by OT-I (gated on CD45.1+CD8+) and OT-II (gated on CD45.1+CD4+) T cells in draining popliteal LNs were determined by *ex vivo* intracellular antibody labeling and flow cytometry. *A.* Caspase 3 expression in popliteal LNs. *B.* Bcl-2 expression in popliteal LNs.

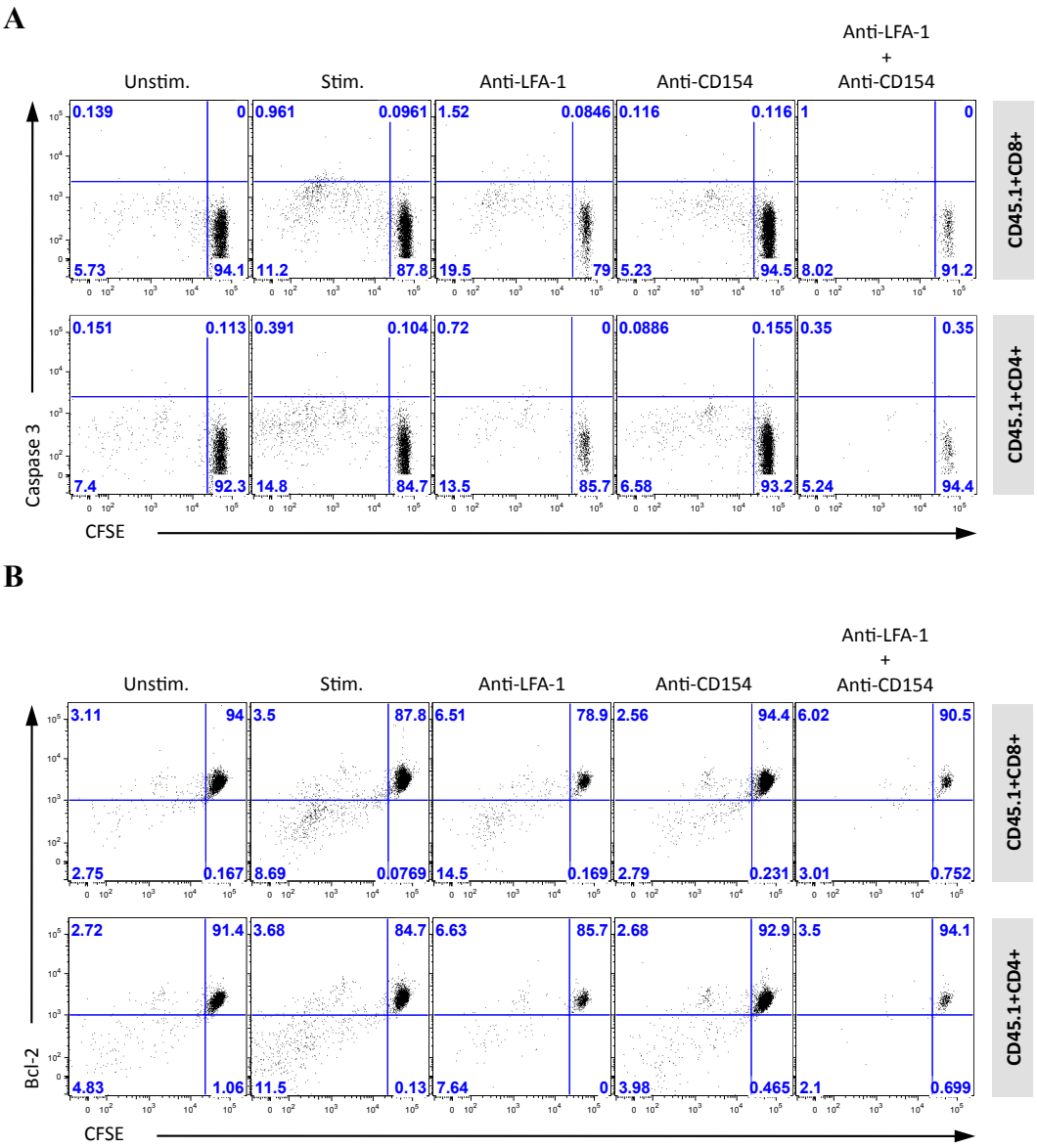

FIGURE 4-12. Antibody therapies did not significantly induce polyclonal T cell apoptosis in the draining lymph nodes. Mice were treated as described Figure 4-1. Caspase 3 and Bcl-2 expression by CD45.1 B6 CD8+ (gated on CD45.1+CD8+) and CD4+ (gated on CD45.1+CD4+) T cells in draining popliteal LNs were determined by *ex vivo* intracellular antibody labeling and flow cytometry. *A.* Caspase 3 expression in popliteal LNs. *B.* Bcl-2 expression in popliteal LNs.

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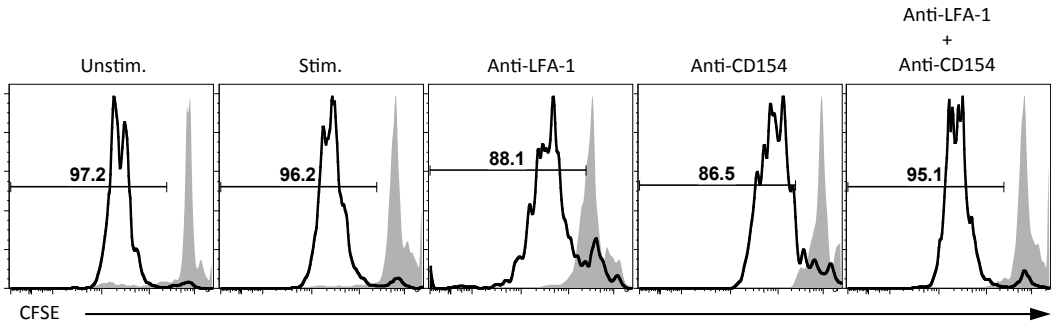

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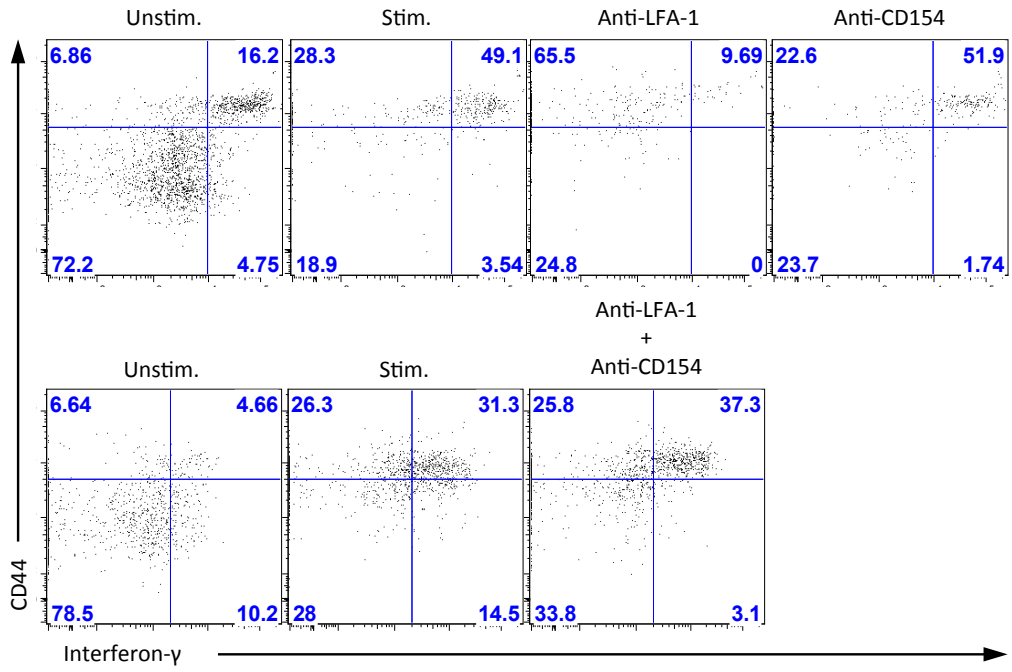

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FIGURE 4-13. Anti-LFA-1 and anti-CD154 therapies allowed antigen-specific CD8+ T cell persistence and maintenance of reactivity. $5x10^6$ OT-I lymphocytes were adoptively transferred into B6 recipients. Recipients were challenged with $5x10^6$ B6-OVA splenocytes intraperitoneally with or without antibody treatments. Recipient spleens were harvested after a minimum of 21 days post primary challenge. *A*. Proliferation of persisting adoptively transferred OT-I T cells upon secondary B6-OVA challenge. Recipient splenocytes were cultured *in vitro* with B6-OVA splenocytes for three days and proliferation measured by CFSE dilution. Histograms shown are gated on CD45.1+CD8+ T cells. *B*. CD44 expression and IFN-γ production by persisting adoptively transferred OT-I (gated on CD45.1+CD8+) T cells. Recipient splenocytes were cultured *in vitro* with OT-I peptide and brefeldin A for 5 hours prior to intracellular antibody labeling.

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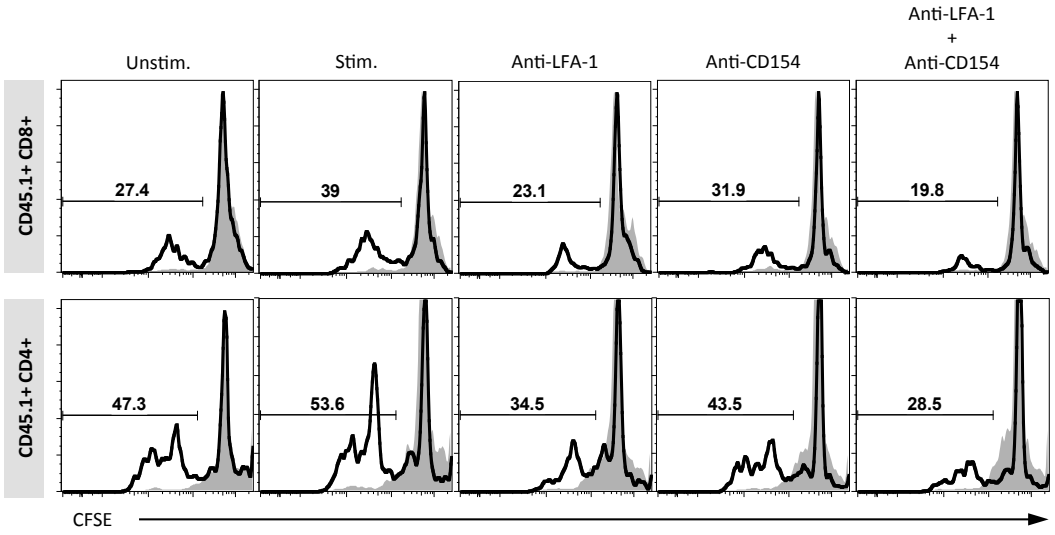

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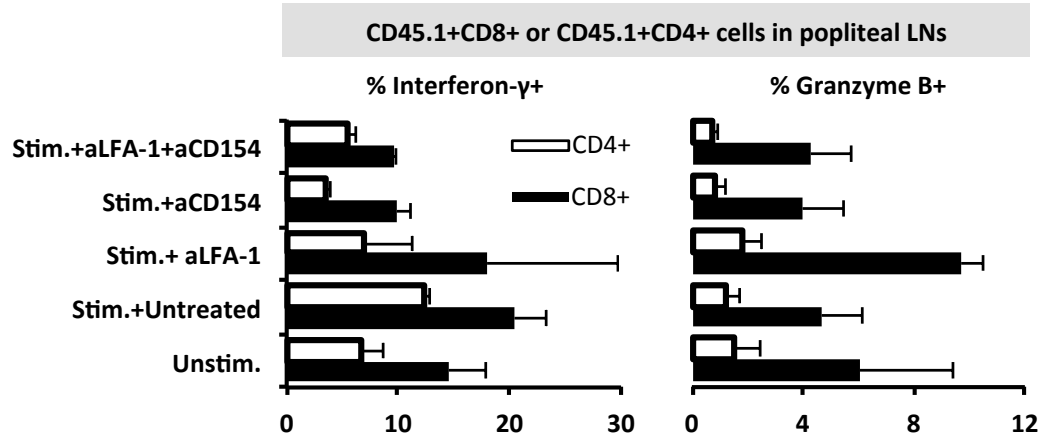

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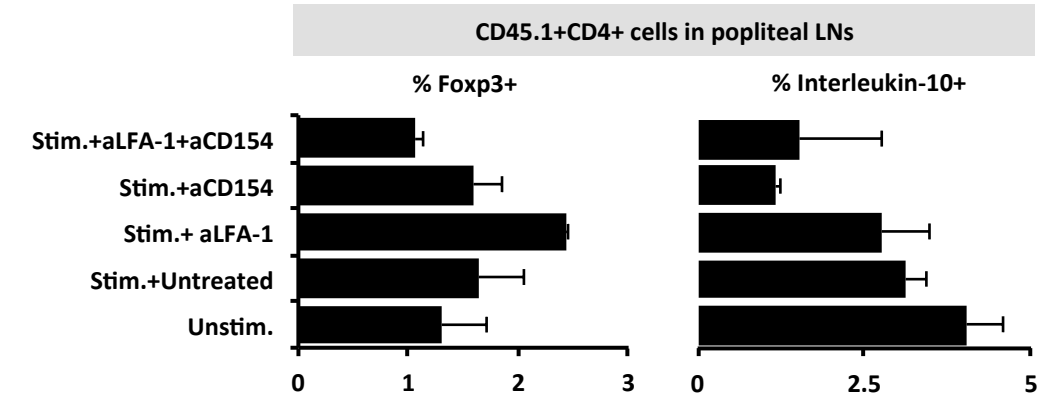

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FIGURE 4-14. Anti-LFA-1 and anti-CD154 therapies allowed polyclonal T cell persistence and maintenance of reactivity. $30x10^6$ CD45.1 B6 lymphocytes were adoptively transferred into CD45.2 B6 recipients. Recipients were challenged with $5x10^6$ BALB/c splenocytes intraperitoneally with or without antibody treatments. Recipient spleens were harvested after a minimum of 21 days post primary challenge. *A*. Proliferation of persisting adoptively transferred T cells upon secondary BALB/c challenge. Recipient splenocytes were cultured *in vitro* with irradiated BALB/c splenocytes for three days and proliferation measured by CFSE dilution. Histograms shown are gated on either CD45.1+CD4+ or CD45.1+CD8+ T cells. *B.* Effector cytokine production by persisting adoptively transferred (gated on CD45.1+CD8+ or CD45.1+CD4+) T cells. Recipient splenocytes were cultured *in vitro* with PMA/ionomycin and brefeldin A for 5 hours prior to intracellular antibody labeling. *C.* Foxp3 expression and interleukin-10 production by persisting adoptively transferred CD4+ (gated on CD45.1+CD4+) T cells after *in vitro* re-stimulation as described in *B*.

CHAPTER FIVE
REGULATION OF NAÏVE GRAFT-SPECIFIC T CELLS DURING MAINTENANCE OF TRANSPLANT SURVIVAL

5.1 INTRODUCTION

To my immense surprise, while many previous studies suggested direct alteration of early T cell reactivity by anti-LFA-1 and anti-CD154 therapies as their mechanism of transplantation tolerance, I had not observed a significant induction of this phenomenon. In my models, anti-LFA-1 and/or anti-CD154 did not induce clonal anergy, clonal deletion, or alternate effector and/or regulatory phenotypes in the responding T cell populations. Unlike the studies that proposed immediate induction of tolerogenic T cell phenotypes, I did not detect any overt change in T cell activation and differentiation in the presence of anti-LFA-1 and/or anti-CD154 that could suggest a dominant and self-perpetuating T cell tolerance being generated. While the therapeutic antibodies did significantly decrease the number of T cells in the draining lymph nodes and partially suppressed their cytokine production, these T cells did not demonstrate any major phenotype alterations that suggested the generation of a tolerance mechanism that could mediate long-term transplant survival. In other words, in contrast to the current paradigms hypothesizing the tolerizing antibodies to actively change graft-destructive T cells into tolerance-prone phenotypes, my results so far showed a distinct lack of such early induction of alternate, non-destructive T cell responses. The proximal impact of these tolerance-promoting antibody therapies on responding T cells appeared unexpectedly modest.

However, a large amount of data from the past twenty years has showed irrefutable evidence indicating the efficacy of transient anti-LFA-1 and anti-CD154 therapies in mediating transplant survival and donor-specific tolerance. For instance, our laboratory had tested the antibodies alone and in combination in a variety of murine transplant models including pancreatic islets and hearts in both allogeneic and xenogeneic donor-recipient combinations (218, 219, 241, 248). Clearly, treatments with the two antibodies undeniably lead to

transplantation tolerance. Furthermore, many studies have demonstrated that donor-specific tolerance generated by anti-LFA-1 and anti-CD154 therapies was in fact maintained by a dominant, T cell-dependent regulatory mechanism that was generated at some point during therapy treatments (212, 216, 218, 225, 263). This was puzzling, since I had so far not observed any such generation of *de novo* T cell tolerance during initial T cell activation in the presence of the antibodies. Therefore, I decided to investigate whether such regulatory/tolerogenic mechanism was reliably generated in time.

I used a neo-antigen skin transplant model with combined anti-LFA-1 and anti-CD154 therapy as detailed in section 2.9.2.2. I generated B6 recipients bearing long-term surviving B6-OVA skin grafts, and subsequently challenged the grafts by adoptively transferring naïve OT-I or OT-II T cells into the recipients. The transferred T cells were carefully tracked and interrogated for their ability to respond to the transplant antigen. Interestingly, while T cell reactivity did not appear to undergo significant changes during initial treatment period with antibody therapies, recipients bearing long-term surviving B6-OVA grafts actively altered the reactivity of naïve graft-specific T cells. Therefore, a dominant regulatory mechanism had been established during the maintenance phase of transplant survival and tolerance in recipients that had been given transient anti-LFA-1/anti-CD154 treatments

5.2 RESULTS

Combined anti-LFA-1 and anti-CD154 therapy prolonged skin graft survival.

As the skin transplant model is recognized for its stringent nature due to the high immunogenicity of skin grafts and the difficulty in achieving prolonged skin transplant survival, it was used to test the efficacy of the antibody therapies. The fully allogeneic model in which BALB/c skin grafts were transplanted onto B6 recipients could not achieve indefinite survival with any of the three antibody therapies (Fig. 5-1A). However, combined anti-LFA-1 and anti-CD154 therapy (median day to rejection: 57) did significantly prolong the survival of full MHC-mismatched skin graft compared to non-treated (median day to rejection: 10),

anti-LFA-1- (median day to rejection: 20), or anti-CD154-treated grafts (median day to rejection: 17) (Fig. 5-1A). This suggests a synergistic effect of combining the two antibodies in preventing transplant rejection. Although the therapies did not achieve indefinite survival of fully MHC-mismatched skin grafts, combined anti-LFA-1 and anti-CD154 treatment did significantly delay transplant rejection in this stringent model.

The antibody therapies were also tested on a neo-antigen-mismatch skin transplant model in which B6 recipients were given B6-OVA skin. Interestingly, while anti-CD154 (median day to rejection: 24) failed to prevent graft rejection in this model, anti-LFA-1 alone (median day to rejection: 40) significantly delayed B6-OVA skin rejection (Fig. 5-1B). This is especially interesting considering that anti-CD154 is a more superior single therapy compared to anti-LFA-1 in the pancreatic islet transplant model, suggesting the intrinsic nature of the transplant as a potentially significant variable in the study of transplantation tolerance (Fig. 3-1). Similar to the fully allogeneic model, combining anti-LFA-1 and anti-CD154 excelled in prolonging B6-OVA skin graft survival comparing to either therapy alone. Furthermore, in contrast to the MHC-mismatch skin grafts, the combined therapy reliably achieved indefinite survival (>60 days post transplantation) of the B6-OVA grafts in approximately 70% of the recipients (Fig. 5-1B). Therefore, the neo-antigen-mismatch model of B6-OVA skin transplantation was used in the following experiments to test naïve graft-specific T cell response in a recipient bearing a long-term surviving transplant.

Naïve graft-specific T cells responded to long-term surviving graft with altered activation and effector phenotype.

Since the antibody therapies did not appear to significantly alter antigen-specific T cell activation or induce alternate T cell phenotypes during the treatment period, it was curious whether the proximal impact of the antibodies were exerted on the transplant instead. A potential explanation of prolonged graft survival when no significant tolerogenic phenomenon was observed in the responding T cells was that the graft might have decreased immunogenicity.

Alternatively, since the most prominent effect of the antibody therapies observed was the decreased magnitude of T cells in the draining lymph nodes, it was also possible that the therapeutic antibodies prevented T cells from directly responding with the transplants, thus preventing their rejection via T cell ignorance. To test these two possibilities, naïve graft-specific OT-I or OT-II T cells were adoptively transferred into combined anti-LFA-1/anti-CD154-treated recipients bearing healthy B6-OVA grafts for a minimum of sixty days (Fig. 5-2A, Fig. 5-3A). Skin draining lymph nodes were harvested and analyzed for OT-I or OT-II T cell response to the surviving graft as compared to their response to a newly transplanted B6-OVA graft in a naïve recipient without therapy (Fig. 5-2A, Fig. 5-3A). The objective was to test whether naïve T cells responded to antibody therapy-treated, long-term surviving transplant differently than to an untreated transplant bearing their cognate antigens.

The long-term surviving B6-OVA skin grafts were neither non-immunogenic nor ignored by graft-specific T cells. Both naïve, adoptively transferred OT-I CD8+ and OT-II CD4+ T cells accumulated in skin draining lymph nodes to similar extents as the accumulation of graft-specific T cells in the draining lymph nodes of newly transplanted, non-therapy-treated B6-OVA skins (Fig. 5-2B, Fig. 5-3B). Furthermore, both CD8+ and CD4+ T cells have proliferated vigorously in the skin draining lymph nodes of the therapy-treated recipients, suggesting productive T cell-antigen engagements with the B6-OVA grafts (Fig. 5-2B, Fig. 5-3B). The adoptively transferred OT-I and OT-II T cells did not induce acute graft rejection in the therapy-treated recipients. This observation, combined with the intact ability of OT-I and OT-II T cells to proliferate in response to the graft, suggests that combined anti-LFA-1 and anti-CD154 therapy prolonged graft survival without diminishing its immunogenicity.

Interestingly, while recipients bearing long-term surviving skin grafts allowed productive activation and proliferation of naïve graft-specific T cells, the T cells exhibited altered activation and effector phenotype. Although naïve OT-I CD8+ T cells underwent similar rounds of division in the two recipients, CD44 expression by proliferating OT-I T cells showed a two-fold decrease in response

to therapy-treated recipients bearing "protected" skin grafts comparing to non-therapy-treated recipients with fresh B6-OVA skins (Fig. 5-2C). Strikingly, interferon-γ production by proliferating OT-I T cells showed a ten-fold decrease when responding to long-term surviving B6-OVA grafts (Fig. 5-2C). The two-fold decrease in CD44 expression was also observed in proliferating OT-II CD4+ T cells responding to long-term skin grafts. Interestingly, while combined anti-LFA-1/anti-CD154 did not appear to induce alternate generation of FoxP3+ OT-II T cells during initial antigen exposure, long-term B6-OVA skin grafts in recipients previously treated with the combined therapy drove a population of naïve OT-II CD4+ T cells to up-regulate FoxP3 expression early in their proliferation (Fig. 5-3C). Taken as a whole, it appeared that while anti-LFA-1 and anti-CD154 did not induce alternative T cell phenotype during initial antigen exposure, skin transplants with prolonged survival after treatment with antibodies remained fully immunogenic but let to an altered graft-specific T cell effector phenotype.

Naïve graft-specific T cells responded to peripheral challenge in recipients bearing long-term surviving graft with altered effector phenotype.

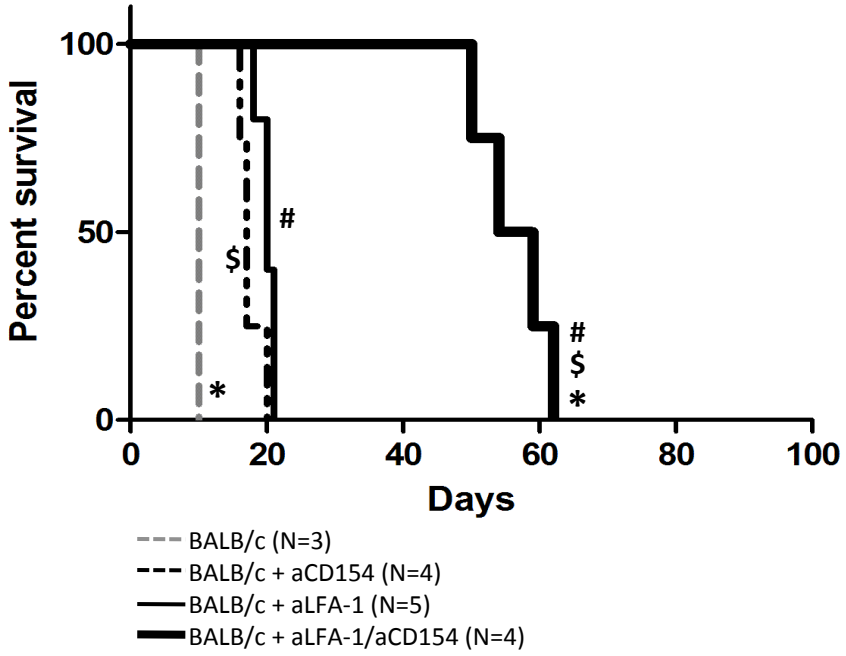
Although the antibody therapies demonstrated a lack of immediate generation of T cell tolerance, recipients with long-term surviving skin grafts after anti-LFA-1 and anti-CD154 treatment actively altered naïve graft-specific T cell activation and differentiation phenotypes. This suggested that the therapy did indeed lead to active T cell regulation later in time despite the lack of such phenomenon during initial antigen exposure. If an active regulatory mechanism was thus developed, it would suggest a systemic regulation of T cell reactivity towards donor antigens that was not limited to the graft site. To explore this concept, naïve OT-I or OT-II T cells were adoptively transferred into combined anti-LFA-1/anti-CD154-treated recipients bearing healthy B6-OVA skin for minimum of sixty days. Naïve graft-specific T cells were also concurrently transferred into untreated recipients who had rejected B6-OVA grafts minimum sixty days prior to transfer and recipients bearing syngeneic B6 skin for the same

amount of time (Fig. 5-4A, Fig. 5-5A). The recipients were then given a peripheral graft antigen challenge in the form of footpad injection with B6-OVA splenocytes (Fig. 5-4A, Fig.5-5A). T cell reactivity in both the popliteal lymph nodes and the skin draining lymph nodes were analyzed to assess whether the altered phenotype observed in naïve T cells activated by long-term skin grafts could be seen when the T cells were responding to graft antigens in general (Fig. 5-4A, Fig. 5-5A). In other words, I wanted to investigate whether the antibody therapy mediated the generation of a systemic, donor-antigen-specific change in T cell reactivity.

After the peripheral graft antigen challenge, both OT-I CD8+ and OT-II CD4+ T cells trafficked into and proliferated vigorously in both the popliteal and the skin draining lymph nodes in recipients bearing long-term surviving B6-OVA grafts (Fig. 5-4B, Fig. 5-5B). This again demonstrated that while the therapy had prevented rejection of the B6-OVA skin grafts, naïve graft-specific T cells could still engage productively both directly with the graft or with graft antigen given peripherally. Significantly, OT-I CD8+ T cells showed the same decrease in interferon-γ as previously observed both in the skin draining lymph nodes and the popliteal lymph nodes, suggesting this altered effector function as a fundamental and systemic change in the recipients' entire anti-OVA T cell response rather than a phenomenon restricted to T cells activated directly by the transplant (Fig. 5-4C). The down-regulation of interferon-γ was also present in proliferating OT-II CD4+ T cells in both the skin and the popliteal lymph nodes, albeit to a lesser extent (Fig. 5-5C). Interestingly, the induction of FoxP3+ expression exclusively in naïve OT-II CD4+ T cells responding to long-term B6-OVA skin observed earlier was not evident when peripheral graft antigen was given. In this case, FoxP3 expression was ubiquitously induced in OT-II T cells early in their proliferation phase in recipients bearing long-term skin, rejected skin, and syngeneic skin (Fig. 5-5C). Taken together, combined anti-LFA-1/anti-CD154 therapy appeared to eventually generate a fundamental change in graft-specific T cell reactivity if indefinite graft survival was achieved despite the lack of immediate immune deviation during initial antibody treatments.

5.3 FIGURES

A



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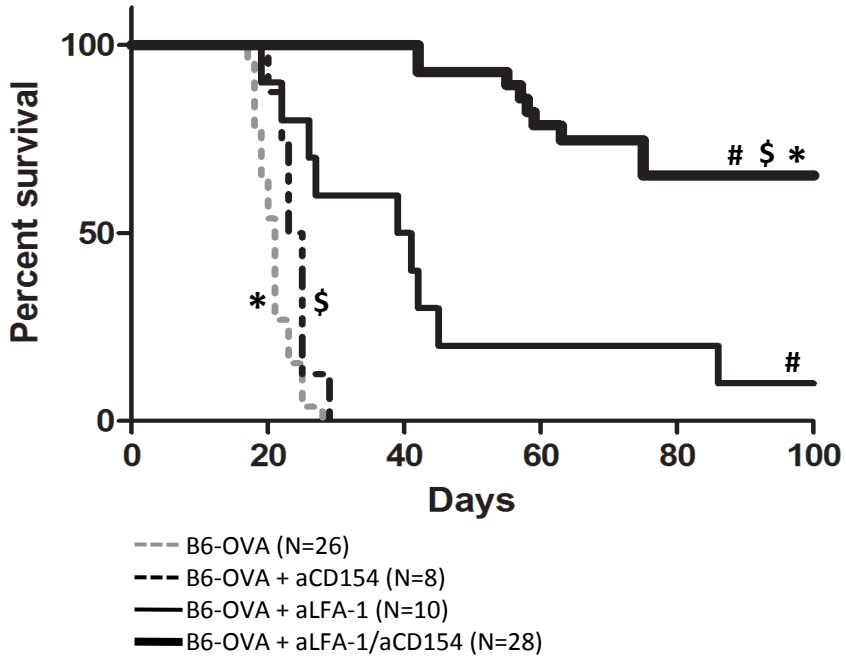

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FIGURE 5-1. Combined anti-LFA-1 and anti-CD154 therapy prolonged skin graft survival. Skin grafts were performed as described in chapter two. Pairs of identical symbols denote statistical significance of minimum P <0.05. *A*. B6 recipients were transplanted with full-thickness skin as follows: BALB/c alone (n=3), BALB/c with anti-CD154 (n=4), BALB/c with anti-LFA-1 (n=5), BALB/c with combined anti-LFA-1/anti-CD154 therapy (n=4). *B*. B6 recipients were transplanted with full-thickness skin as follows: B6-OVA alone (n=26), B6-OVA with anti-CD154 (n=8), B6-OVA with anti-LFA-1 (n=10), B6-OVA with combined anti-LFA-1/anti-CD154 therapy (n=28).

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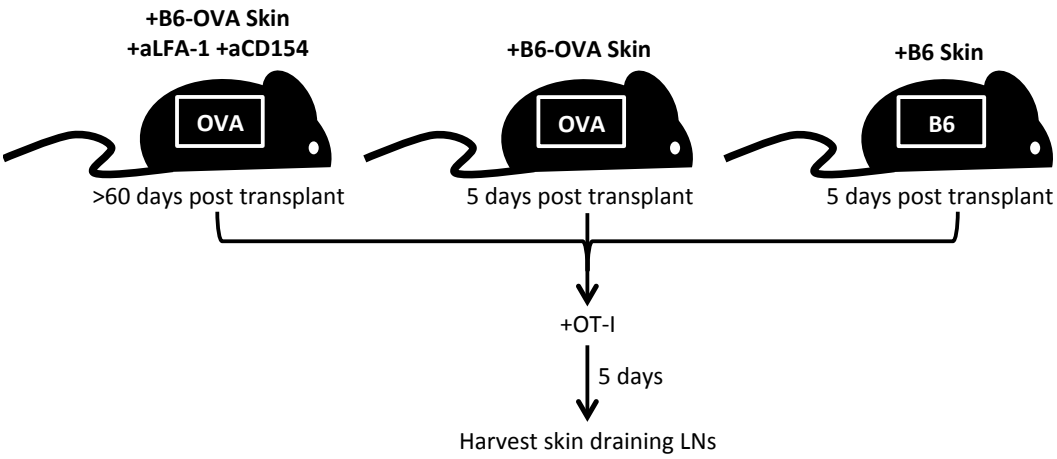

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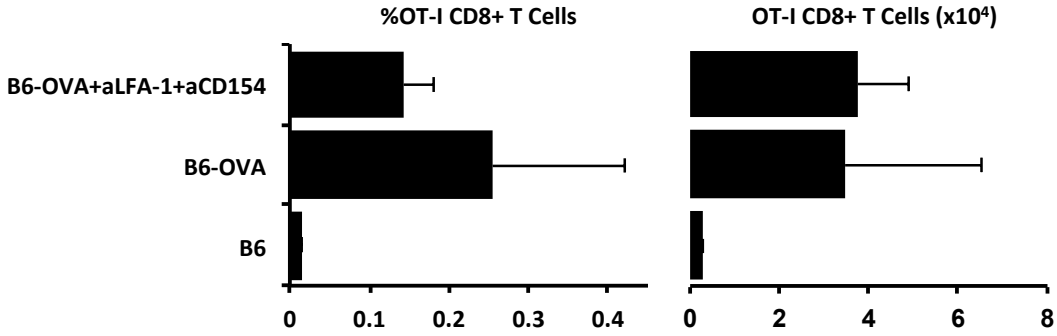

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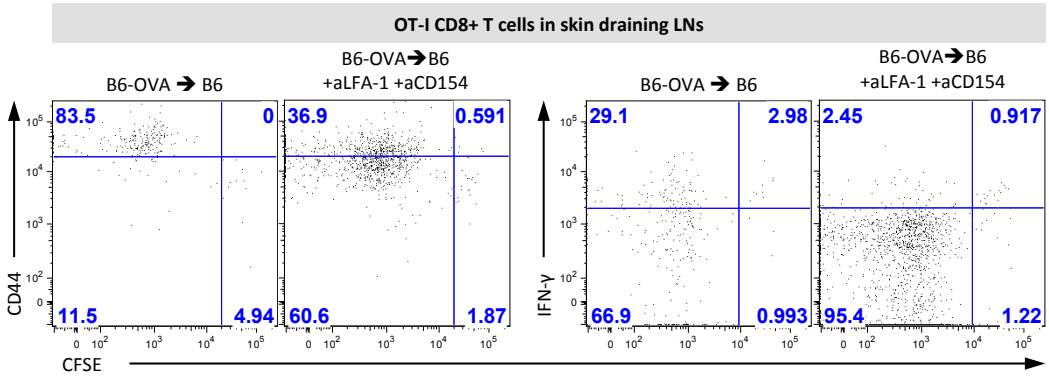

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FIGURE 5-2. Long-term surviving skin graft allowed naïve graft-specific CD8+ T cell proliferation but altered activation phenotype and effector function. *A.* Naïve CD45.1 OT-I ($1x10^4$) lymphocytes were adoptively transferred into CD45.2 B6 recipients bearing the following: 1) B6-OVA skin surviving minimum of 60 days after combined anti-LFA-1/anti-CD154 treatment, 2) newly transplanted B6-OVA skin, and 3) newly transplanted B6 skin. Skin draining LNs were harvested 5 days post transfer and analyzed by flow cytometry. *B.* Proportion (%CD45.1+CD8+ live lymphocytes) and absolute numbers (%CD45.1+CD8+ x total skin LN cell count) of OT-I CD8+ T cells in skin draining LNs. *C.* CD44 and interferon-γ expression by proliferating OT-I CD8+ T cells in skin draining LNs. Plots are gated on CD45.1+CD8+ live lymphocyte population.

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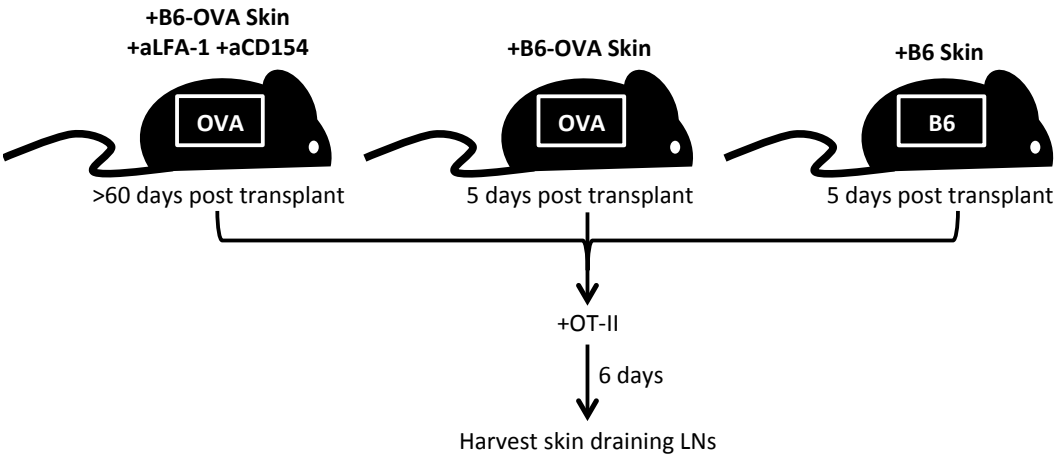

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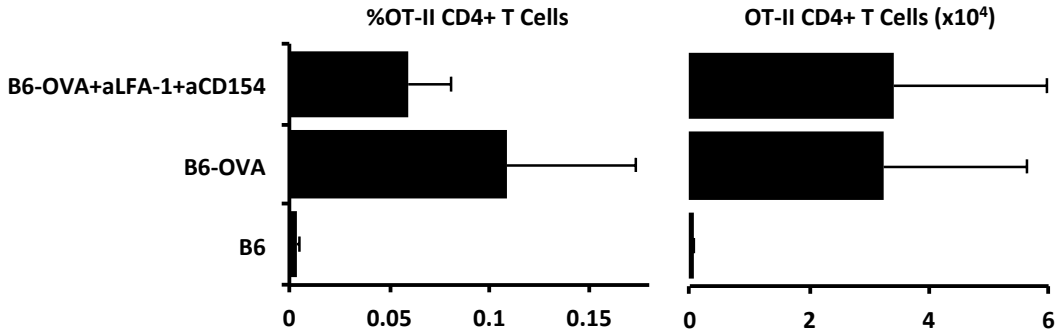

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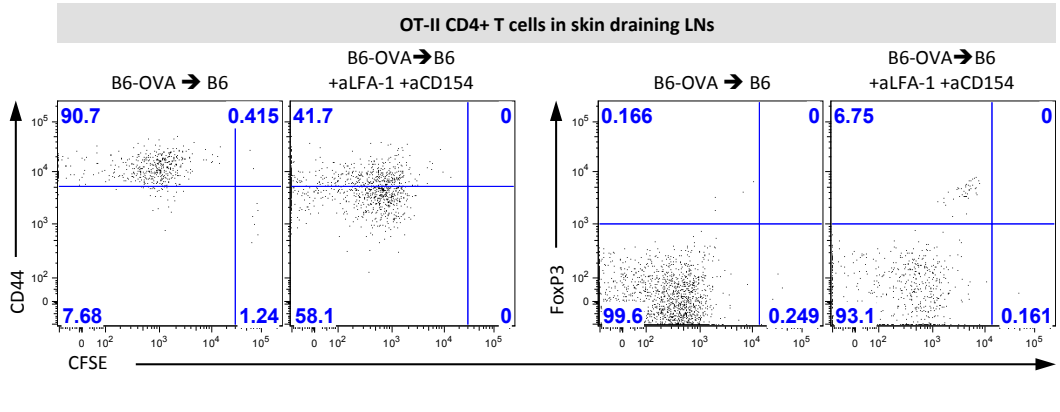

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FIGURE 5-3. Long-term surviving skin graft allowed naïve graft-specific CD4+ T cell proliferation and acquisition of regulatory phenotype. *A*. Naïve CD45.1 OT-II ($2x10^4$) lymphocytes were adoptively transferred into CD45.2 B6 recipients bearing the following: 1) B6-OVA skin surviving minimum of 60 days after combined anti-LFA-1/anti-CD154 treatment, 2) newly transplanted B6-OVA skin, and 3) newly transplanted B6 skin. Skin draining LNs were harvested 6 days post transfer and analyzed by flow cytometry. *B*. Proportion (%CD45.1+CD4+ live lymphocytes) and absolute numbers (%CD45.1+CD4+ x total skin LN cell count) of OT-II CD4+ T cells in skin draining LNs. *C*. CD44 and FoxP3 expression by proliferating OT-II CD4+ T cells in skin draining LNs. Plots are gated on CD45.1+CD4+ live lymphocyte population.

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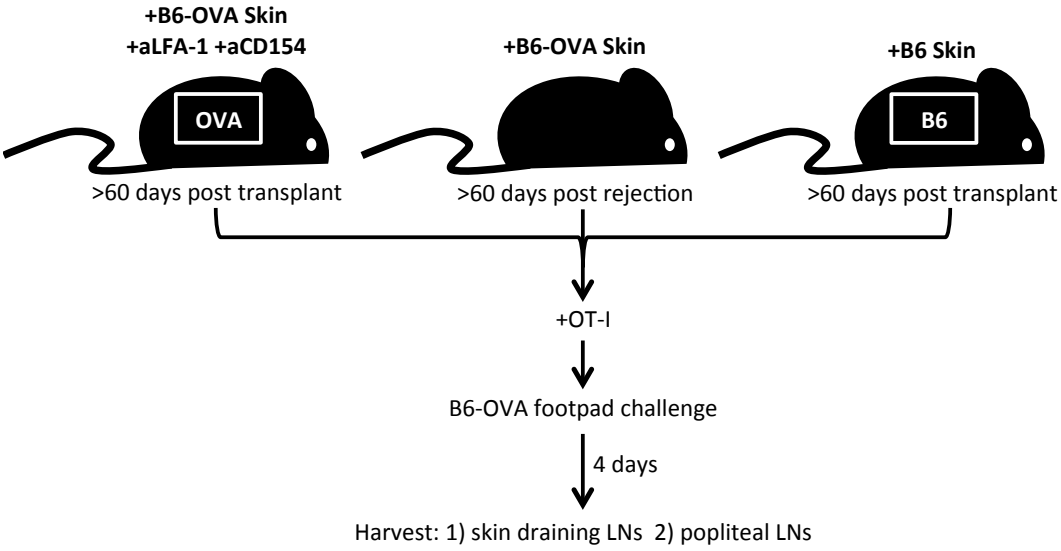

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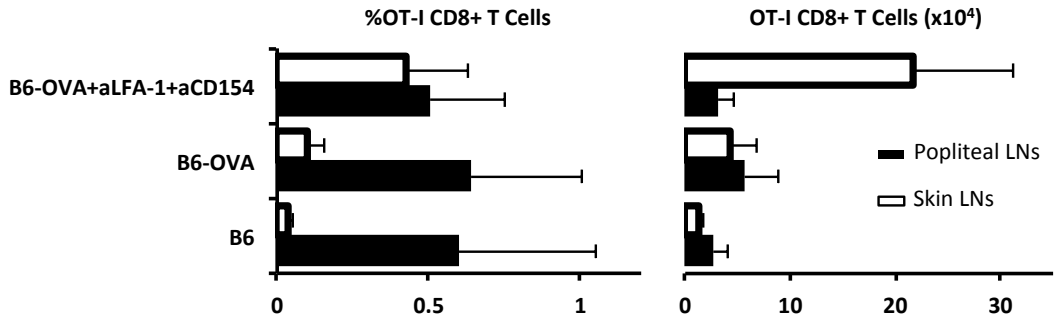

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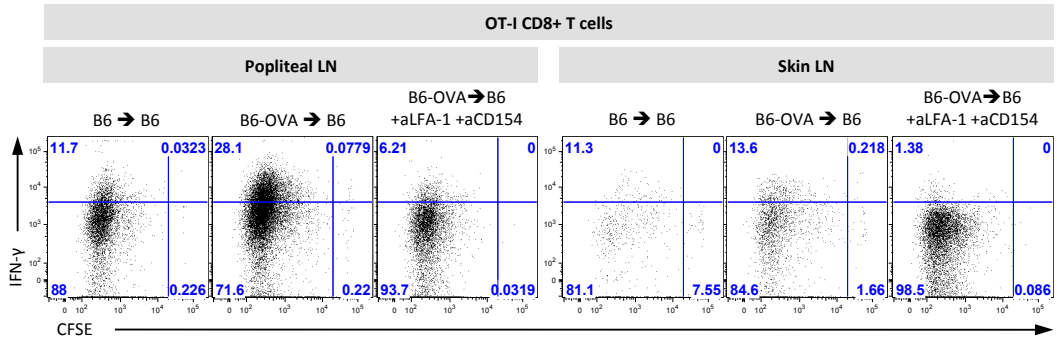

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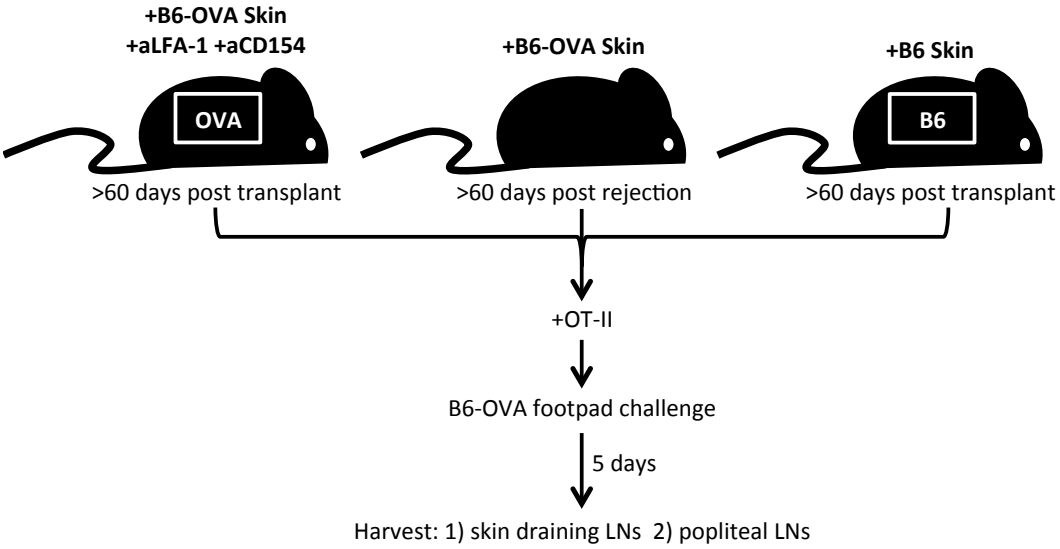
FIGURE 5-4. Recipients bearing long-term surviving skin graft allowed naïve graft-specific CD8+ T cell proliferation but altered effector function in both the skin and popliteal lymph nodes upon peripheral graft-antigen footpad challenge. *A.* Naïve CD45.1 OT-I ($1x10^4$) lymphocytes were adoptively transferred into CD45.2 B6 recipients: 1) B6-OVA skin surviving minimum of 60 days after combined anti-LFA-1/anti-CD154 treatment, 2) B6-OVA skin rejected minimum 60 days prior to challenge , and 3) B6 skin for minimum 60 days. Recipients were challenged at the footpads with $1x10^6$ B6-OVA splenocytes post adoptive transfer. Skin draining LNs and popliteal LNs were harvested 4 days post footpad challenge and analyzed by flow cytometry. *B.* Proportion (%CD45.1+CD8+ live lymphocytes) and absolute numbers (%CD45.1+CD8+ x total skin LN cell count) of OT-I CD8+ T cells in popliteal and skin draining LNs. *C.* Interferon-gamma production by proliferating OT-I CD8+ T cells in popliteal and skin draining LNs. Plots are gated on CD45.1+CD8+ live lymphnocytes.

A



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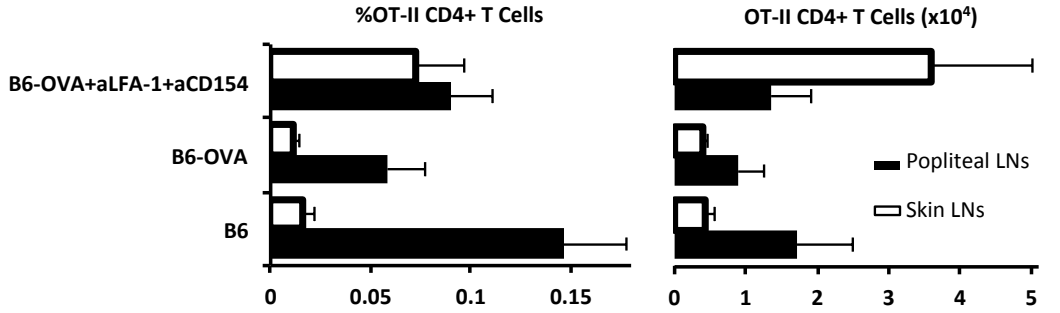

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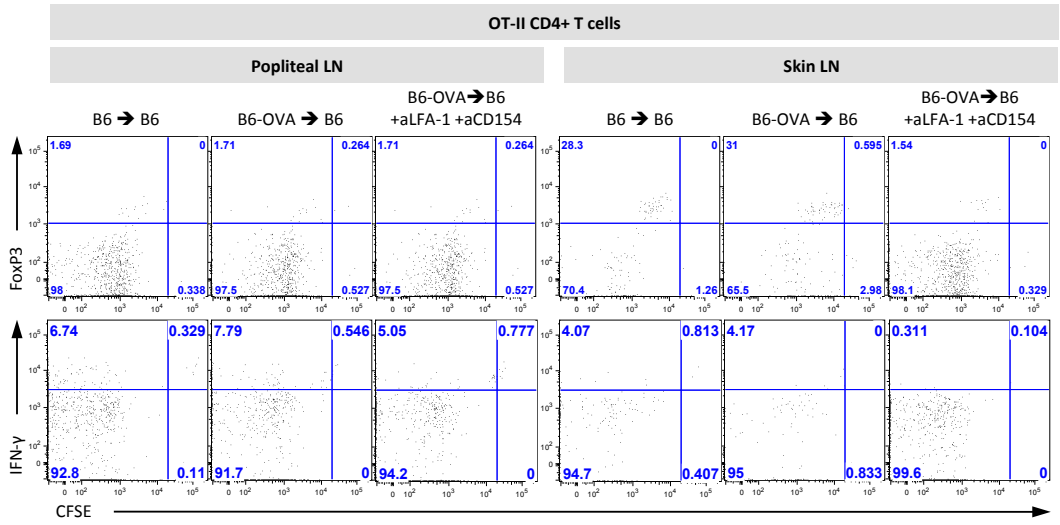

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FIGURE 5-5. Recipients bearing long-term surviving skin graft allowed naïve graft-specific CD4+ T cell proliferation but altered effector function in the skin draining lymph nodes upon peripheral graft-antigen footpad challenge. *A*. Naïve CD45.1 OT-II ($2x10^4$) lymphocytes were adoptively transferred into CD45.2 B6 recipients with the following: 1) B6-OVA skin surviving minimum of 60 days due to combined anti-LFA-1/anti-CD154 treatment, 2) B6-OVA skin rejected minimum 60 days earlier, and 3) B6 skin for minimum 60 days prior to adoptive transfer. Recipients were challenged at the footpads with $1x10^6$ B6-OVA splenocytes post adoptive transfer. Skin draining LNs and popliteal LNs were harvest 5 days post footpad challenge and analyzed by flow cytometry. *B.* Proportion (%CD45.1+CD4+ live lymphocytes) and absolute numbers (%CD45.1+CD4+ x total skin LN cell count) of OT-II CD4+ T cells in popliteal and skin draining LNs. *C.* Interferon-gamma and FoxP3 expression by proliferating OT-II CD4+ T cells in popliteal and skin draining LNs. Plots are gated on CD45.1+CD4+ live lymphocytes.

CHAPTER SIX
DISCUSSION

6.1 INTRODUCTION

6.1.1 T Cells and Transplant Rejection

While transplantation is still the most effective treatment for end-stage organ failure, immune-mediated transplant rejection remains the primary barrier to favourable long-term patient outcomes. Amongst the immune cells participating in transplant rejection, T cells are a critical mediator in inducing graft injury and facilitating destructive responses from other cell types. T cells can recognize donor antigens when presented both in the context of donor MHC and self MHC. As such, T cells possess great flexibility in detecting and responding to transplant antigens, thus rendering protection of transplant from T cell surveillance a difficult and complex task. CD8+ and CD4+ T cells can both independently and synergistically instigate transplant destruction. Activated CD8+ CTLs are adept at causing direct graft damage through diverse mechanisms including apoptosis-promoting granules and pro-inflammatory cytokines (20–27). CD4+ T cells, on the other hand, can simultaneously facilitate direct graft injury and enhance anti-transplant activities of other immune mediators such as B cells and CD8+ T cells (33, 34). Therefore, effective control of multi-faceted T cell reactivity is a critical hurdle in advancing transplantation as a medical treatment.

6.1.2 Suppressing Immunity versus Inducing Tolerance

Currently, the only clinically available option for controlling transplant rejection is immunosuppressants. These agents completely and non-discriminately inhibit immune activities, including T cell reactivity. The two most prevalent types of clinically available immunosuppressants are small-molecule pharmaceutical agents derived from microbial products that interfere with the mechanics of cell cycle and T cell-specific depleting antibodies that bind to and lyse T cells. While immunosuppressants are effective in globally inhibiting T cell activity and thus preventing acute cell-mediated transplant injury, they carry

severe adverse side effects. The immunodeficiency induced by immunosuppressants, while allowing for transplant survival, leaves patients highly susceptible to pathogenic infection and malignancies (68–71). Furthermore, immunosuppressive agents are intrinsically toxic, and life-long dependence on them critically compromises patient health and, ironically, the viability of the transplant itself. For instance, the small-molecule pharmaceutical agents cause multiple organ injuries and complications including nephrotoxicity, neurotoxicity, and post-transplantation diabetes mellitus, while depleting antibodies generate an acute cytokine storm as part of their mechanism of action and result in skewed immune composition (47, 62). Moreover, since immunosuppressive agents are routinely used as a combination therapy, the patients suffer from the cumulative toxicities of these agents but are dependent on them to prevent transplant rejection.

Given the damage caused by chronic usage of immunosuppressive agents, prevention of transplant rejection becomes paradoxically a limiting factor for long-term graft and patient survival. Therefore, a critical goal in improving patient and transplant outcome is to focus the development of transplant therapies on generating donor-specific immune tolerance rather than inhibiting global immune reactivity. The key to balancing the restraint of graft-destructive immune responses and the prevention of therapy-intrinsic adverse affects is developing a therapy that is capable of inducing transplant tolerance with a transient course of treatment. This induced immune tolerance, unlike immunodeficiency mediated by immunosuppression, should be a donor antigen-specific response and thus permits the patient to retain a competent immune system. Therefore, the ideal transplant therapy will allow the patient to indefinitely accept the transplant while still preserve protective immunity against pathogenic and mutagenic assaults.

6.1.3 Non-Depleting Antibodies as Tolerance-Inducing Agents

As studies into peripheral T cell tolerance suggested that TCR signal strength during antigen recognition determines the consequence of naïve T cell activation, and this signal is a combination of costimulation and adhesion along

with the specific TCR-antigen-MHC interaction, manipulating T cell activation signals became a viable possibility for inducing a tolerogenic rather than pathogenic T cell response. Monoclonal, non-depleting antibodies specific for T cell surface adhesion and costimulation molecules were therefore developed to execute precise interference with these pathways. The specific nature of monoclonal antibody therapies allows them to target selective immune surface molecules, thus reducing the dangers associated with comprehensive immune paralysis generated by immunosuppressants. Furthermore, many of these monoclonal antibodies have already shown great efficacy in prolonging allograft survival and generating donor-specific tolerance as transient therapies in animal models. As such, monoclonal non-depleting antibodies targeting T cell surface molecules involved in adhesion and costimulation appear to be promising candidates for transplant tolerance-promoting therapies.

6.2 INTERPRETATION OF THESIS RESULTS RELATIVE TO CURRENT PARADIGMS

Amongst the plethora of therapeutic monoclonal antibodies that have been tested as transplant therapies, antibodies targeting the adhesion and costimulation molecule, LFA-1 (CD11a), and the costimulation molecule, CD154 (CD40L), have been particularly efficacious in generating donor-specific transplant tolerance in mouse and non-human primate models (223, 239, 241, 247, 262). Significantly, these two antibodies can mediate indefinite graft survival as transient therapies. This leads to the major question as to how a short-term administration of antibodies targeting a costimulatory molecule or an adhesion molecule can result in long-term maintenance of transplant survival and tolerance. Although the two antibodies, particularly anti-CD154, have since been widely studied in multiple animal transplant models for their ability to promote tolerance, their specific mechanisms of restraining graft-specific T cell from mediating injuries remain vague. Despite the ambiguity concerning the proximal impact of the antibodies on graft-specific T cells, in the case of anti-CD154, various models have been hypothesized through the years to explain its mechanism of tolerance

induction. However, the large variety of hypothesized paradigms plus little overlap in mechanisms proposed by different research groups indicate that there lacks a cohesive and systematic examination into the link between an antibody binding to a T cell costimulatory molecule and long-term graft survival and donor-specific tolerance. Similarly, anti-LFA-1, while widely and repeatedly tested for its ability to induce transplant tolerance and excellent compatibility in synergizing with different therapeutics, has not been studied as closely with regard to its early impact on T cells. As such, the mechanism of tolerance induction by anti-LFA-1 is even less defined than that of anti-CD154. Importantly, improved understanding of the processes involved in restraining graft-specific T cell immunity and generating donor-specific tolerance by therapeutic antibodies will allow the rational design of a safe and effective transplant therapy. To address this gap in knowledge and determine the impact of anti-CD154 and/or anti-LFA-1 on antigen-specific T cells, models of tracking the fate of T cells were established in this thesis to methodically examine the proximal impact of anti-LFA-1 and/or anti-CD154 on T cell reactivity.

Since the key question is the connection between a transient therapy with antibodies targeting T cell surface molecules and long-term transplant tolerance, it is perhaps unsurprising that the current hypothesized paradigms of anti-LFA-1- and anti-CD154-mediated tolerance focus on an early and drastic change in T cell fate that would generate immediate graft tolerance. As detailed in chapter one, the exploration of induced transplantation tolerance progressed at a parallel pace with the discovery of self-tolerance. In fact, most identified mechanisms of central and peripheral self-tolerance have been applied to the generation of transplantation tolerance. Therefore, deviations from expected immune responses during naïve T cell activation that result in peripheral self-tolerance have been the models in explaining transplantation tolerance generated by the short-term use of biologics and therapeutic antibodies. In chapter one, three principal mechanisms of altered T cell activation fate in peripheral self-tolerance were described: induction of clonal anergy, induction of clonal deletion and death, and generation of altered, regulatory or non-destructive T cell phenotypes. These mechanisms form the

primary paradigms currently proposed as the proximal impact of anti-CD154 and anti-LFA-1 on graft-specific T cells (Fig. 6-1). Therefore, my thesis results will be discussed relative to these paradigms in the following sections.

6.2.1 Induction of Clonal Anergy as a Route of Transplantation Tolerance

Clonal anergy is one of the earliest identified mechanisms of peripheral immune tolerance to self-antigens. Anergy is defined as a state of hyporesponsiveness in which T cells are alive and not deleted, but are functionally inactive (112). Generally, an anergic T cell is recognized as one that is refractory to proliferation and production of IL-2 upon encountering its cognate antigen, and this is likely due to T cell activation with insufficient signal strength (114). In other words, T cell clonal anergy is considered a consequence of failed T cell activation. In the context of peripheral self-tolerance, clonal anergy provided a means for controlling autoreactive T cell reactivity. If autoreactive T cells are interacting with self-antigens either presented on tissue parenchymal cells lacking costimulatory ligands or immature APCs that have yet to up-regulate their costimulatory signals, the induction of anergy would prevent these T cells from being activated against self. In the context of tolerance induction to a transplant then, it seems logical that a possible mechanism of generating transplant tolerance is driving graft-destructive T cells into clonal anergy. If a transplant therapy facilitates donor-specific T cells to interact with graft antigens with subpar activation signals, these T cells should become anergic and lose their ability to proliferate and differentiate in response to the donor graft. More specifically, if a therapeutic antibody targeting T cell costimulatory or adhesion molecules can modulate activation strength, it may be capable of utilizing clonal anergy as a mechanism of generating donor-specific transplantation tolerance.

Indeed, clonal anergy or T cell hyporesponsiveness has been observed in multiple studies using anti-CD154 or anti-LFA-1 as transplant tolerance-inducing therapies. These studies demonstrated loss of host T cell activation, clonal expansion, and/or effector functional differentiation in response to donor antigens when treated with the therapeutic antibodies. For instance, by using an adoptive

transfer model, Quezada *et al.* showed anti-CD154 rendering antigen-specific T cells refractory to activation and proliferation by tracking the fate of CD4+ TCR transgenic TEa-Tg T cells recognizing allo-antigen Eα in the context of class II MHC I-A^b in response to stimulation with donor-specific transfusion (DST) (227). While DST predictably induced proliferation and expansion of TEa-Tg T cells in the lymph nodes, the presence of anti-CD154 abrogated TEa-Tg T cell expansion (227). Furthermore, TEa-Tg T cells harvested from anti-CD154-treated recipients lacked an activated phenotype ($CD44^{HI}CD62L^{LO}CD69^{HI}$) and exhibited reduced production of IL-2, a hallmark of T cell anergy (227). Anti-CD154 also abrogated adoptively transferred TCR transgenic AND CD4+ T cell clonal expansion in an antigen-specific bone-marrow chimerism model (271). Similarly, anti-LFA-1 inhibited T cell clonal expansion *in vitro* in an allogeneic mixed lymphocyte reaction (248). All told, induction of T cell clonal anergy is one possible mechanism allowing anti-CD154 and anti-LFA-1 to generate long-term transplant survival and donor-specific tolerance.

The theory of clonal anergy induced by T cell interaction with anti-LFA-1 or anti-CD154 as a mechanism of generating transplant tolerance is supported by the studies showing the loss of T cell activation of proliferation in the presence of these antibodies. I first investigated this phenomenon using a monoclonal antigen-specific model of adoptively transferred, CFSE-labeled, OT-I CD8+ or OT-II CD4+ T cells into wild-type B6 recipients challenged locally at the footpads with B6-OVA splenocytes. Surprisingly, despite the decrease in the number of T cells in the draining lymph nodes, the presence of the antibodies did not impede OT-I or OT-II T cell proliferation (Fig. 4-2). Furthermore, proliferated OT-I or OT-II T cells in the presence of all three therapies also showed activation phenotypes similarly to T cells proliferated in the absence of antibodies (Fig. 4-5). In the case of monoclonal antigen-specific T cells, my data did not indicate inhibition of activation and proliferation as a consequence of anti-CD154 and/or anti-LFA-1 treatments and did not support clonal anergy as a mechanism of tolerance induction by these antibodies.

Interestingly, the proximal impact of anti-CD154 and/or anti-LFA-1 on early T cell activation and proliferation in a polyclonal B6-anti-BALB/c footpad challenge model proved to be less straightforward. Frequency of proliferated T cells distinctly decreased in the polyclonal model depending on the therapy used (Fig. 4-4). Anti-LFA-1 and anti-CD154 down-regulated the presence of proliferated CD8+ T cells in the draining popliteal lymph nodes, while anti-CD154 and combined anti-CD154/anti-LFA-1 affected CD4+ T cells similarly (Fig. 4-4). Nevertheless, the proliferated cells showed "normal" expression of activation phenotype (Fig. 4-6). At a glance, this did resemble a partial inhibition of T cell proliferation, and thus partly supported clonal anergy as a mechanism of anti-CD154 or anti-LFA-1-induced tolerance. However, without being able to track antigen-specific polyclonal alloreactive T cells, I could not determine whether the non-proliferating pool of T cells contained a higher number of alloreactive T cells in the presence of antibodies, which would correlate to a suppressed ability to proliferate in response to antibody treatments. Also, considering the significant loss of both proliferating and non-proliferating T cells from the draining lymph nodes and the ability of the adoptively transferred T cells to proliferate upon secondary activation, rather than the induction of anergy, I hypothesize instead that the decrease in the frequency of proliferated polyclonal T cells was due to a global decrease of T cell number in the lymph nodes rather than a specific inhibition of T cell proliferation (Fig. 4-2, 4-14). This observation and hypothesis will be discussed in further detail later in this discussion.

Several factors could explain the contradiction between my data and the previous studies showing inhibition of T cell activation and proliferation in the presence of anti-CD154 or anti-LFA-1. While Quezada *et al.* and Kurtz *et al.* observed T cell anergy with DST/anti-CD154 treatment using the TEa-Tg and AND TCR transgenic models respectively, I showed intact T cell proliferative ability with the OT-I/OT-II model (227, 271) (Fig. 4-3). However, the nature of the TCR transgenic model may be a key reason for the difference observed. TCR transgenic T cells represent one single clone within a population of antigen-specific T cells. However, an endogenous naïve antigen-specific T cell population

consists of upwards to a hundred different T cell clones, and each clones responds uniquely to the antigen (265). Therefore, a wild-type, unmodified "antigen-specific" response is in reality a cumulative representation of many distinct T cell responses (265). In contrast, monoclonal TCR transgenic T cells only portray one single discrete response towards an antigen. In other words, the therapeutic antibodies could potentially induce anergy in some endogenous T cell clones but not others. Therefore, it is possible that the TEa-Tg and AND T cells are clones prone to therapy-induced anergy while OT-I and OT-II T cells are not, thus demonstrating contradictory reaction to anti-CD154 treatment. Having considered this limitation of the transgenic model, I have tracked the fate of wild-type polyclonal T cell response in parallel to the transgenic T cells. Perhaps unsurprisingly, polyclonal T cell activation and proliferation appeared to be more sensitive to the presence of the antibodies and might have been rendered partially anergic. As such, it is possible that anti-CD154 and anti-LFA-1 are both capable of inducing clonal anergy in some T cells but not others. Nevertheless, considering the drastic decrease in both proliferated and un-proliferated T cell number in the lymph node in the polyclonal model and the intact ability of the T cells to proliferate during secondary antigen exposure, I would postulate that anergy, if indeed one of the many consequences of anti-CD154 and anti-LFA-1 treatments, is not a primary mechanism of tolerance-induction compared to the disappearance of T cells from the draining lymph nodes.

6.2.2 Induction of T Cell Death or Deletion as a Route of Transplantation Tolerance

Another mechanism involved in peripheral self-tolerance is the deletion of autoreactive T cells. Unlike clonal anergy, which inactivates a T cell upon antigen stimulation but leaves them alive and potentially capable of regaining their responsiveness, certain T cell activation events lead to outright death and elimination of the cell. For instance, activation-induced cell death is well established as a part of the standard consequence of T cell activation as a means of controlling the sudden increase in antigen-specific T cells during clonal

expansion. In the context of peripheral self-tolerance, repeated and chronic T cell exposure to its cognate antigen could potentially initiate death pathways (106). Deletion or death of an autoreactive T cell in the periphery occurs through apoptosis not unlike thymocyte death during negative selection (106). Essentially, as a mechanism of self-tolerance, the induction of apoptosis removes autoreactive T cells from the periphery and thus prevents injury to self. Translating this concept into transplantation tolerance suggests that eliminating anti-donor T cells by death and deletion would be an effective and beneficial mechanism of protecting the transplant from destructive T cell responses. While anti-LFA-1 has not been examined as closely in this respect, anti-CD154 has been observed to mediate early deletion of donor-specific T cells in many studies. In this regard, death and deletion of T cells appear to be a potential mechanism of antibody-mediated transplant tolerance.

Deletion of graft-specific T cells has been reported by multiple studies as a major early mechanism of therapies with anti-CD154. T cell apoptosis was first demonstrated as a necessary mechanism for DST/anti-CD154-mediated transplant tolerance in two distinct models. The first model utilized a transgenic mouse strain with constitutive expression of Bcl-xL in T cells (264). Bcl-xL is a pro-survival protein of Bcl-2 family (272). It blocks apoptosis by binding to and interfering with the function of pro-apoptotic executioner proteins, BAX and BAK, and inducer BH3-only proteins (272). T cells in the transgenic Bcl-xL mice therefore are protected from apoptosis. While DST and anti-CD154 achieved long-term allogeneic cardiac transplant survival in 100% of the wild-type recipients, survival dwindled to approximately 20% in transgenic Bcl-xL recipients, suggesting the loss of T cell apoptosis negatively impacted transplant tolerance induction (264). The second model tracked proliferation and apoptosis of naïve C3H/He (H-2^k) lymphocytes adoptively transferring into irradiated BALB/c (H-2^d) recipients and treated with a tolerance-inducing regimen of combined anti-CD154 and CTLA-4-Ig (215). During primary activation, anti-CD154 and CTLA-4-Ig up-regulated Annexin V staining of proliferating C3H/He CD4+ T cells, suggesting activation-induced cell death as an intrinsic mechanism

of the therapy (215). The role of T cell death in anti-CD154-induced tolerance was further investigated by Iwakoshi *et al.* using an adoptive transfer model in which H-2K^b-specific KB5 DES+ CD8+ transgenic T cells were tracked in recipients given H-2^b skin grafts (226). It was shown that combined DST and anti-CD154 significantly decreased the number of transferred KB5 DES+ CD8+ T cells recovered from recipient lymph nodes seven days after skin transplantation (226). T cell deletion by apoptosis during anti-CD154 therapies has also been demonstrated in murine bone-marrow transplantation (209, 271). Proximal impact of anti-LFA-1 therapies on T cell death and deletion has not been examined as extensively as anti-CD154. However, OT-I CD8+ T cells adoptively transferred into anti-LFA-1-treated recipients given B6-OVA skin grafts showed increased staining of Annexin V during initial T cell activation, implying an increase in apoptotic cells in the presence of anti-LFA-1 (245). Taken as a whole, these observations suggest that anti-CD154 and anti-LFA-1 facilitate early T cell death and deletion as a means of generating transplantation tolerance.

My data initially appeared to support T cell deletion as a mechanism of anti-CD154 and/or anti-LFA-1 therapies. Both the monoclonal TCR transgenic model and the polyclonal allogeneic model showed an approximately two-fold decrease in adoptively transferred T cells in the draining popliteal lymph nodes when challenged with their cognate antigen in the footpads in the presence of anti-CD154 (Fig. 4-1, 4-2). Even more dramatically, anti-LFA-1 and combined anti-CD154/anti-LFA-1 therapies led to more than a ten-fold decrease in the number of draining lymph node T cells (Fig. 4-1, 4-2). As the disappearance of T cells from the draining lymph nodes appeared to recapitulate previous studies supporting T cell death and deletion as a mechanism of tolerance, I looked for T cell apoptotic markers in the draining lymph nodes as a confirmation of the mechanism. However, when examined directly *ex vivo*, none of the therapies showed particularly increased presence of apoptotic T cells as determined by the expression of pro-apoptotic caspase 3 protein (Fig. 4-11, 4-12). As the rapid clearance of apoptotic cells renders assessing cell death difficult *ex vivo*, I used a secondary method to confirm whether T cell deletion occurred. I allowed

adoptively transferred T cells to be systemically activated then assessed the presence and activity of transferred T cells over three weeks later, the rationale being that, if T cell deletion is a necessary mechanism of tolerance, I should not be able to recover the adoptively transferred T cells, especially the monoclonal TCR transgenic T cells by this time point. Surprisingly, both adoptively transferred OT-I CD8+ T cells and polyclonal T cells were present three weeks after initial antigen exposure and remained responsive and proliferative to secondary antigen challenge (Fig. 4-13, 4-14). Therefore, while anti-LFA-1 and/or anti-CD154 therapies resulted in striking decrease in the magnitude of T cells in the draining lymph nodes during initial T cell activation, this does not appeared to be caused by increased T cell deletion and death driven by the therapeutic antibodies.

The lack of increased early anti-LFA-1- and/or anti-CD154-driven T cell deletion and death indicated by my data is directly contradictory to the previous studies described above. Two factors may explain the discrepancy between the results. The first one is the method used in determining T cell apoptosis. In the previous studies mentioned, apoptotic cells were assessed by staining draining lymph node T cells with Annexin V. Annexin V binds to phosphatidylserine on the cell surface, and extracellular exposure of phosphatidylserine is recognized as a hallmark of cells undergoing apoptosis and a signal for macrophage clearance of the apoptotic cells (266, 270). However, phosphatidylserine exposure is not exclusive to apoptotic cells, and can be observed on a range of viable, apoptotic, and necrotic cells *ex vivo* (266). In fact, *ex vivo* labeling of viable cells with Annexin V has been repeatedly observed (267, 268, 270). As such, Annexin V-positive cells may not actually proceed to apoptosis. Furthermore, CD8+ T cells have been shown to transiently expose phosphatidylserine during rapid antigen-driven clonal expansion without entry into death, so an *ex vivo* snapshot in time showing increased Annexin V-positive T cells in the draining lymph nodes during acute primary T cell activation may simply be an indicator of vigorous T cell proliferation (269). Granted, my use of caspase 3 as an alternative indicator of apoptosis may not have been entirely representative, either, as caspase 3 was also

shown to be transiently expressed by non-apoptotic T cells during acute proliferation (269). Regardless, as I did not observe significant increase in caspase 3-expressing T cells in the draining lymph nodes during primary activation and antibody treatments, my data did not support T cell apoptosis as a significant mechanism of transplant tolerance induction by anti-CD154 and/or anti-LFA-1.

Another factor contributing to the difference observed in T cell deletion in the presence of therapeutic antibodies between my data and previous studies is the potential role of trafficking as a mechanism of antibody therapies. The previous studies searched for T cells in the draining lymph nodes only, and concluded a decrease in the magnitude of T cells in the nodes as a result of deletion. My data also showed dramatic decrease in the number of adoptively transferred T cells in the draining lymph nodes during primary activation. However, these T cells could be recovered from the recipients' spleens three weeks after primary activation and respond productively to secondary antigen challenge. This paradoxical observation suggests that rather than deletion, the T cells might have simply been absent in the draining lymph nodes during primary activation. If anti-CD154 and/or anti-LFA-1 can dictate T cell migration, they can potentially diminish the presence of T cells in the draining lymph nodes without deleting them. In other words, the therapeutic antibodies could temporarily "hide" the T cells from their antigens and prevent full-scale immune response, thus mimicking a deletional phenotype in the lymph nodes. This hypothesis is supported by my earlier observation that administering anti-LFA-1 alone or with anti-CD154 to naïve wild-type recipients without immune stimulation was sufficient to alter CD62L expression on endogenous T cells and relocate them from the lymph nodes into the circulation and the spleen (Fig. 3-9). Therefore, while Annexin V staining may not represent T cell death, decrease in the number of T cells in the draining lymph nodes may not represent deletion. As such, my data do not support death and deletion as a proximal impact of anti-CD154 and anti-LFA-1 on T cell activation.

6.2.3 Alteration of T Cell Phenotypes as a Route of Transplantation Tolerance

Aside from T cell anergy and deletion, a third mechanism of controlling destructive T cell reactivity is to allow T cell activation but alter their differentiation into a non-destructive phenotype. In the context of peripheral self-tolerance, an example of this would be the induction of regulatory T cells, especially FoxP3-expressing CD4+ regulatory T cells. As detailed in section 1.2.1.2.3, T cells of the regulatory lineage presumably recognize self-antigens, especially the natural T regulatory cells postulated to develop from thymic negative selection and enter the periphery already committed to the FoxP3 lineage (130, 131). However, unlike destructive autoreactive T cells, regulatory T cells do not recognize self-antigens to cause injury. On the contrary, regulatory T cells suppress other effector T cells through multiple direct and indirect mechanisms, such as by secreting their hallmark cytokines, IL-10 and TGF-β, which down-regulate immune responses. In the case of transplantation tolerance, a second type of FoxP3-expressing regulatory T cells, induced T regulatory cells, is of particular interest. Induced Tregs phenotypically resemble a naïve effector CD4+ T cell until antigen encounter and, at which point, possess the plasticity to differentiate into either effector T cells or a regulatory T cells depending on the immune environment during its activation (130, 131). Since these CD4+ T cells presumably emigrate from the thymus as naïve T cells that have survived negative selection, they potentially recognize a wide range of non-self antigens (131). These naive peripheral CD4+ T cells could potentially be activated to become protective of rather than destructive toward their cognate antigen. As such, induction of induced, graft-specific regulatory T cells could hypothetically be a mechanism of anti-CD154 and anti-LFA-1-mediated transplantation tolerance.

Aside from generating FoxP3-expressing regulatory T cells, another example of controlling destructive immunity through altered T cell differentiation is by inducing a non-inflammatory effector response instead of a pro-inflammatory one. This was primarily observed in naïve CD4+ T cell effector differentiation. The classic paradigm of T_H cell differentiation postulates that a

naïve CD4+ T cell could be activated to become one of two primary types of effector T cells. When encountering a bacterial, viral, or other intracellular pathogen, T_H-1 effectors with signature cytokine profile of IL-12 and IFN-γ are generated (273, 274). T_H-1 effectors then proceed to initiate an inflammatory response and amplify the activation of other pro-inflammatory immune cells such as CD8+ CTLs and macrophages (273, 274). On the other hand, if the pathogen is extracellular, such as intestinal parasites, T_H-2 effectors with cytokines IL-4, IL-5, and IL-13 are induced to mediate a non-inflammatory humoural response by augmenting B cell reactivity (273, 274). Importantly, T_H-2 cytokines suppress T_H-1 differentiation and vice versa, thus ensuring the presence of one predominant effector CD4+ T cell response in the immune microenvironment. The significance of this phenomenon in regulating undesirable T cell immunity was observed in models of chronic inflammatory diseases. Parasitic helminths potently induce a T_H-2 response, and this strong T_H-2 phenotype has been shown to ameliorate existing T_H-1-mediated inflammation in the cases of inflammatory bowel diseases, chronic mycobacterium infection, and rheumatoid arthritis (275–278). A deliberate induction of T_H-2 immunity has also been implicated as potential treatments for inflammatory autoimmune diseases such as Type-1 diabetes and multiple sclerosis (279). Considering CD4+ T cell-mediated inflammatory response is one of the major mechanisms of transplant failure as discussed in section 1.1.1.2, deviation from a pro-inflammatory T_H-1 response to a non-inflammatory T_H-2 one by anti-CD154 or anti-LFA-1 therapies could potentially be a mechanism to protect transplant from injuries mediated by inflammation.

Acute induction of alternate T cell phenotypes by anti-CD154 has been reported in contradictory results. Van Maurik *et al.* showed that transfer of splenocytes from mice treated with DST and anti-CD154 four weeks prior into a secondary recipient could not delay graft rejection, while splenocytes from similarly treated recipients bearing >100-day surviving transplant could (280). This suggested that direct interaction of anti-CD154 with donor-specific T cells did not induce protective regulatory T cells. However, using an antigen-specific adoptive transfer model, Ferrer *et al*. observed increased percentage of FoxP3+

population in transferred OT-II CD4+ T cells in B6-OVA skin grafted recipients treated with either anti-CD154 alone or anti-CD154 plus DST (230). As this FoxP3 expression was seen within fourteen days of transplantation and therapy treatments, Ferrer *et al.* concluded that anti-CD154, whether alone or in combination therapy, directly altered naïve CD4+ T cell differentiation toward a regulatory lineage (230). Nevertheless, induction of regulatory T cells during initial antigen exposure in the presence of anti-CD154 appears to be controversial.

Deviation of immune response between pro-inflammatory T_H-1 and non-inflammatory T_H-2 response has also been attributed to anti-CD154 therapies. Hancock *et al*. demonstrated that anti-CD154 and DST therapy resulted in early mononuclear infiltrates within cardiac allografts expressing IL-4 and IL-10 instead of the IL-2 and IFN-γ found in control rejecting grafts (231). Using a F1 islet transplant model in which H-2^d grafts were transplanted into H-$2^{b/k,d}$ recipients, Zheng *et al.* also found significantly increased intragraft IL-4 transcripts in recipients treated with DST and anti-CD154 (214). Both studies suggested the skewing toward a T_H-2 effector function to be a potential mechanism of anti-CD154 that favoured transplant survival and tolerance.

In comparison to anti-CD154, therapies with anti-LFA-1 have not been extensively interrogated for their ability to directly generate alternate T cell phenotypes. Reisman *et al.* showed in the draining lymph nodes of a BALB/c to B6 allogeneic skin graft model that anti-LFA-1 decreased the frequency of inflammatory-type CD8+ T cells expressing both IFN-γ and TNF-α while increasing the frequency of FoxP3-expressing CD4+ T cells (245). However, whether this was due to anti-LFA-1 inducing an alternate phenotype or facilitating trafficking and differential accumulation of T cells in the lymph nodes was unclear. Despite the contradictory and sometimes ambiguous results, inducing non-graft-destructive T cell phenotypes is still considered a major paradigm explaining the efficacy of therapeutic antibodies in facilitating donor-specific transplantation tolerance.

Interestingly, my data both supported and refuted induction of alternate T cell phenotypes as a mechanism of anti-LFA-1- or anti-CD154-mediated

transplantation tolerance. When the frequency of FoxP3-expressing CD4+ T cells was assessed, both the TCR transgenic model and the polyclonal alloreactive model showed significantly increased percentages of FoxP3+ CD4+ T cells in the draining lymph nodes when the recipients were treated with anti-LFA-1 or combined anti-LFA-1/antiCD154 (Fig. 4-9, 4-10). Unlike the observation reported by Ferrer *et al.*, however, anti-CD154 did not significantly raise the frequency of Tregs in the draining lymph nodes (230) (Fig. 4-9, 4-10). However, since an "induction" of regulatory T cells implies *de novo* generation of FoxP3+ Tregs in the presence of therapeutic antibodies, and thus should correlate to an increase in FoxP3+ T cell number on top of frequency, I considered the better indicator of Treg induction to be a change in the absolute number of Tregs in the lymph nodes. When the absolute numbers of FoxP3-expressing CD4+ T cells were calculated, the dramatic increase in Tregs during anti-LFA-1 or anti-LFA-1/anti-CD154 therapies became insignificant (Fig. 4-9, 4-10). In fact, anti-LFA-1- or combined anti-LFA-1/anti-CD154- treated recipients had fewer FoxP3+ T cells than non-therapy-treated, control activated recipients in both the TCR transgenic and the polyclonal alloreactive models (Fig. 4-9, 4-10). In other words, while anti-LFA-1 and anti-LFA-1/anti-CD154 therapies certainly changed Treg-to-T-effector ratio as indicated by the striking increase in the frequency of FoxP3+ T cells in the draining lymph nodes, they did not appear to have induced significant generation of new Tregs. Considering that I have observed anti-LFA-1 and combined anti-LFA-1/anti-CD154 diverting naïve T cells out of lymph nodes into the circulation and spleen by changing their CD62L expression, an observation supported by Reisman *et al.*, it is possible that effector T cells are more susceptible than FoxP3+ regulatory T cells to anti-LFA-1-driven trafficking changes, thus the frequency but not number of Tregs increased due to reduced presence of effector T cells (245) (Fig. 3-8, 4-9, 4-10). Regardless of the mechanism, my data do not support *de novo* induction of regulatory T cells as a mechanism of anti-LFA-1 and/or anti-CD154.

As I did not observe induction of regulatory T cells during initial antigen exposure and therapy treatments, I assessed the cytokine profiles of the adoptively

transferred T cells recovered from the draining lymph nodes to determine whether a switch between effector T cell phenotypes could be a mechanism of antibody-mediated transplantation tolerance. Contrary to the studies reporting an increased presence of IL-4 in the graft sites, I did not find any indication of an alternate cytokine profile generated in the presence of the therapies. All three therapies decreased CD4+ and CD8+ T cell cytokine (IFN-γ, granzyme B) expression to different extents in both the TCR transgenic and polyclonal models, but none induced generation of alternate cytokines (Fig. 4-7, 4-8). As such, the therapies, especially combined anti-LFA-1/anti-CD154, appeared to down-regulate expression of T cell effector cytokine release without changing their intrinsic cytokine profiles. Taken together, anti-LFA-1 and/or anti-CD154 seemed to exert partial suppressive effect on T cell effector function rather than inducing an alternate effector phenotype.

My data contradicted previous studies proposing induction of regulatory T cells as a mechanism of anti-CD154. A major factor explaining the difference is the method of data analysis. In the study by Ferrer *et al.*, which was one of the first reports that concretely hypothesized Treg induction as an early direct consequence of anti-CD154 treatments by showing increased presence of FoxP3+ CD4+ T cells in the draining lymph nodes (230). However, this increase in Tregs was reported as an increase in FoxP3+ T cell frequency and not absolute number. When looking strictly at T cell frequencies, my data also appeared to show a dramatic increase in the regulatory T cells after treatments with anti-LFA-1 or combined anti-CD154/anti-LFA-1. However, by calculating the absolute number of Tregs, I then determined that this increase in percentage of FoxP3+ T cells did not translate to an increase in absolute number. In fact, the absolute cell number data would suggest that the antibodies had no effect on the generation of FoxP3+ Tregs. Therefore, as Ferrer's study showed only increase in the percentage of Tregs, it is difficult to determine whether induction of Tregs was really observed as a mechanism of anti-CD154. Whether a skewing of the frequency of Tregs versus that of T effectors is enough to impart transplant tolerance without active generation of Tregs is unknown. It is possible that the increase in percentage of

Tregs likely caused by a decrease in effector T cells in the presence of anti-LFA-1 or combine anti-LFA-1/anti-CD154 could be sufficient in driving a tolerogenic rather than destructive response. In this case, the antibodies could be thought of as having "induced" an alternate T cell phenotype or response. However, since the two therapies also largely diminished the presence of T cells in the draining lymph nodes, there were likely too few Tregs to be able to single-handedly execute transplantation tolerance despite their higher percentage. In fact, my data supported the observation of van Maurik *et al.*, which saw no evidence of acute induction of regulatory lymphocytes during the first month of DST/anti-CD154 (280). My data so far have not shown an immediate change in T cell differentiation into either a regulatory or an alternative effector phenotype as a consequence of anti-CD154 and/or anti-LFA-1 treatment. On the contrary, the therapeutic antibodies appeared to partially inhibit existing T cell effector function rather than generating novel T cell phenotype and function.

6.2.4 Maintenance of Tolerance

To my surprise, my interrogation into the proximal impact of anti-LFA-1 and/or anti-CD154 on T cell activation unexpectedly showed no clonal anergy, clonal deletion, or induction of alternate phenotype during T cell differentiation. My observations largely contradicted previous studies proposing direct and immediate changes in T cell fate as mechanisms of transplantation tolerance mediated by anti-LFA-1 and anti-CD154. In contrast, while my data showed a change in T cell trafficking associated with anti-LFA-1, T cells remaining in the draining lymph nodes engaged their cognate antigen and proliferated. Besides from the reduced magnitude of T cells in the draining lymph nodes, the other major effect of the antibodies seemed to be a partial inhibition of T cell effector cytokine production. However, major alterations during T cell activation that could indicate the beginnings of a tolerogenic response were not observed. Unexpectedly, the proximal impact of two such potent tolerance-inducing therapeutic antibodies on T cells appeared to be startlingly unremarkable.

Nevertheless, a large body of data from the last twenty years has repeatedly demonstrated that transient anti-LFA-1 and anti-CD154 treatments during the early peri-transplantation period promoted powerful long-term graft survival and donor-specific tolerance. Previous data from our laboratory alone have shown the two antibodies to both independently and synergistically promote transplantation tolerance to allogeneic cardiac and allogeneic and xenogeneic pancreatic islet grafts in the murine model (218, 219, 241, 248). Furthermore, many studies emphatically demonstrated that the maintenance of this transplant tolerance is contingent on the presence of a dominant, T cell-dependent regulatory response (212, 216, 218, 225, 263). In other words, regardless of whether anti-LFA-1 and anti-CD154 therapies induced early tolerogenic T cell responses immediately upon treatments, T cell-dependent mechanisms are responsible for the maintenance of transplant tolerance once long-term graft survival has been achieved. Since, contrary to my predicted results, I did not observe immediate induction of tolerogenic T cell phenotypes in the presence of anti-LFA-1 and/or anti-CD154, I investigated the fate of naïve graft-specific T cells during the maintenance phase of transplant survival to see whether a regulatory mechanism resulted from the seeming lack of one during the early phase of transplantation.

Interestingly, while my previous data did not indicate early immune deviation towards a tolerogenic response in the presence of anti-LFA-1 and anti-CD154, recipients bearing long-term surviving B6-OVA skin grafts changed functional phenotypes of naïve, adoptively transferred OT-I or OT-II T cells. At sixty days post transplantation, combined anti-LFA-1/anti-CD154-treated recipients maintained their B6-OVA skin grafts while the antibodies had presumably left their immune system. Interestingly, adoptively transferred OT-I or OT-II T cells accumulated in the skin draining lymph nodes and proliferated vigorously, suggesting that the graft remained fully immunogenic and antigenic (Fig. 5-2, 5-3). However, IFN-γ production by OT-I CD8+ T cells was suppressed despite the vigorous proliferation (Fig. 5-2). Significantly, a population of FoxP3+ CD4+ T cells was generated in the pool of proliferating OT-II T cells in the tolerant recipients (Fig. 5-3). It appeared that, at the maintenance phase of

anti-LFA-1/anti-CD154-generated graft survival and tolerance, a regulatory mechanism had taken form and was capable of controlling naïve graft-specific T cells. Furthermore, this apparent regulatory mechanism was not limited to T cells reacting to the transplant itself. When donor antigens were given as a peripheral challenge at the footpads of recipients bearing long-term B6-OVA skin grafts, IFN-γ production by OT-I CD8+ T cells was similarly suppressed in spite of the intact proliferative response (Fig. 5-4). Therefore, while I did not observe immediate induction of tolerogenic T cell responses during early treatment period when anti-LFA-1 and anti-CD154 were present, an active regulatory mechanism capable of altering naïve graft-specific T cell reactivity was eventually established during the maintenance of transplant survival and tolerance. It could thus be inferred from my observations that it was not the initial interaction of the antibodies with the T cells that "induced" transplantation tolerance, but rather a consequence of the immune activities that occurred after the early period of therapy treatments.

6.3 A PROPOSED MODEL OF TRANSPLANTATION TOLERANCE

In this thesis, I have shown that treatments with anti-LFA-1 and/or anti-CD154 surprisingly resulted in no immediate induction of overt T cell tolerance. I did not observe clonal deletion or anergy of activated T cells in the presence of the therapeutic antibodies, nor did I detect preferential generation of alternate non-destructive T cell phenotypes. In the presence of the antibodies, T cells productively engaged with their antigens by acquiring an activation phenotype and proliferative capability. The most significant impact of the antibody therapies appeared to the striking decrease in the number of T cells in the draining lymph nodes and partial inhibition in their effector cytokine production. Nevertheless, when prolongation of transplant survival was achieved, a regulatory mechanism was present to dominantly control naïve T cell activation and differentiation. Therefore, while transient antibody therapies promoted long-term transplant survival, they did not induce early generation of tolerogenic T cell responses when they were present in the recipient immune system. In other words, my

results demonstrate an actively maintained transplant survival as a consequence of a lack of early immune deviation during anti-LFA-1 and/or anti-CD154 treatments. Based upon these observations, I hypothesize that, rather than altering the initial graft-specific T cell response, anti-LFA-1 and anti-CD154 act to partially inhibit the magnitude of T cell reactivity to prevent immediate graft destruction. This in turn allows the transplant and its relatively quiescent immune microenvironment to gradually generate a tolerant T cell response that then continues to maintain transplant survival.

6.3.1 Therapeutic Antibodies versus Transplant: Who is the Tolerogen?

The concept of a temporarily suppressed initial immune response that allows for gradual subsequent generation of transplantation tolerance is similar to the concept of APC-depleted, "cultured", grafts. Studies have shown that transplants depleted of APCs create an environment of "indifference" that allowed initial graft survival in adult immune-competent recipients by neither activating nor tolerizing graft-specific T cells (281). However, over time, T cell-dependent and donor-specific transplant tolerance ultimately developed from this early state of T cell non-reactivity (282–285). In other words, while the APC-depleted transplants did not immediately facilitate either destructive or tolerogenic T cell responses, the T cells were not entirely ignorant of the transplant. In fact, a certain degree of T cell interaction with the transplant led to the generation of T cell-dependent donor-specific tolerance (283). This implies that tolerance, not immunity, is the dominant response of long-term T cell interaction with an APC-depleted graft when given enough time. This concept can be translated to anti-LFA-1 and/or anti-CD154-generated transplant tolerance based upon my observations of their mechanism of action. The consequence of transient anti-LFA-1 and/or anti-CD154 has been demonstrated in multiple animal and transplant models to facilitate long-term graft survival and donor-specific tolerance maintained by a dominant T cell regulatory mechanism. On the other hand, I have shown that the early impact of the antibodies on activated T cells is partial inhibition of reactivity without the induction of clonal anergy, clonal

deletion, or generation of regulatory phenotypes. In other words, although anti-LFA-1 and/or anti-CD154 reliably generate transplant tolerance, their presence at the initial stage of transplantation induced neither overt destructive responses nor tolerogenic regulation. Therefore, correlating my observations to previous studies on the phenomenon of APC-depleted allograft tolerance, I propose that tolerance induction by anti-LFA-1 and/or anti-CD154 occurs in **three stages**: 1) **immediate partial suppression** of T cell reactivity to dampen early T cell-mediated injury and allow initial transplant survival, 2) as the presence of the antibodies wanes, **recovery of T cell reactivity** in the presence of a transplant lacking inflammation or other extrinsic stress and stimulatory signals due to dampened early T cell responses, and 3) **gradual and time-dependent generation of T cell tolerance** by allowing T cells to respond to the transplant in a quiescent immune environment.

It is important to note that the initial state of attenuated immune responses caused by the presence of anti-LFA-1 and/or anti-CD154 is not universal inhibition of T cell reactivity. Anti-LFA-1 and anti-CD154 restrained but did not block productive T cell interaction with their cognate antigens. Moreover, the long-term skin graft survival generated by the antibodies was not a state of clonal ignorance, and the graft retained its immunogenicity and ability to interact with but not rejected by graft-specific T cells. This is demonstrated by previous studies showing that while APC-depleted islet allografts transplanted in immune-competent recipients gradually generated dominant, transferable, and CD4+ T cell-dependent tolerance, the same grafts given to immune-deficient recipients without adaptive immunity were unable to generate tolerance when the recipients were reconstituted with T cells (281–283). Other studies have also shown that allografts given to immune-deficient recipients, even when allowed time to heal and reach a quiescent state prior to reconstituting the recipient immune systems, could not induce tolerance and, in some cases, were promptly rejected after immune reconstitution (286, 287). This suggests that the lack of a host immune response prevented the time-dependent generation of tolerance toward the transplant, even when the graft itself has reached an immune quiescent state.

Another illustration of the importance of an intact immune system in the generation of tolerance is the body of evidence showing that immunosuppressants globally paralyzing the immune system, such as calcineurin-inhibitors and immune cell depletion therapies, block tolerance induction experimentally and inhibit regulatory T cell activity clinically (288–291). Furthermore, if tolerance-promoting antibody therapies are given in a manner that excessively restrains T cell response, their efficacy actually markedly decreases. This was illustrated by a study demonstrating that combined anti-LFA-1 and anti-ICAM-1 treatment resulted in greater reduction of T cell proliferation than either therapy alone, but was paradoxically the weakest therapy of the three in inducing long-term pancreatic islet allograft survival (248). While relative down-regulation of T cell immunity appeared to be crucial for anti-LFA-1- and/or anti-CD154-mediated transplantation tolerance, the antibodies do not act as immunosuppressants. Therefore, aside from the immune microenvironment surrounding a transplant, continuous host immune recognition of the transplant is equally essential as time in the evolution of a successful donor-specific tolerogenic response.

To summarize, I hypothesize that anti-LFA-1 and anti-CD154 therapies promote long-term transplant survival by a mechanism similar to that of APC-depleted grafts. The antibodies diminish graft-specific T cell responses enough to prolong initial graft survival while still permitting T cell-antigen interaction; the transplant then gradually induces tolerance to itself, which then actively maintains long-term transplant acceptance. To reiterate my proposed model in the context of peripheral self-tolerance, previously proposed paradigms of anti-LFA-1- or anti-CD154-generated tolerance assign mechanisms of self-tolerance, such as clonal anergy, deletion, or induction of regulation, as intrinsic consequences of the interaction between the therapeutic antibodies and the graft-specific T cells. **In contrast, my model hypothesizes that the ability to generate tolerance lies not with the therapeutic antibodies but with the transplant itself**. The antibodies facilitate early survival of the transplant in a relatively immunologically quiescent state, which subsequently allows the transplant to activate T cells towards a non-destructive response. The transplant therefore mimics a self-antigen encountered

by an autoreactive T cell in the periphery, and mechanisms of peripheral self-tolerance likely occur at this stage to generate T cell tolerance to the transplant. Thus, the transplant itself, rather than the therapeutic antibodies, is the tolerogen and promoter of T cell tolerance.

6.3.2 Implications of Time-Dependent Evolution Transplantation Tolerance

Instead of a therapy-induced, immediate skewing of the immune responses towards tolerance, I hypothesized a model of transplantation tolerance that instead requires a gradual and time-dependent evolution into donor-specific tolerance by virtue of maintaining a relatively immunologically quiescent environment surrounding the transplant (Fig. 6-2). This model requires time to achieve a stable mechanism that can eventually maintain transplant survival, and it suggests that the transplant itself acts as a peripheral self-antigen promoting the generation of such tolerogenic response. Based upon these criteria, this model carries several significant implications that modify our understanding of transplantation tolerance and immune tolerance in general.

6.3.2.1 Designing a Transplantation Tolerance Therapy

Since my proposed model of transplantation tolerance assigns the transplant as the tolerogen rather than the therapeutic antibodies, this implies that the exact nature of the antibodies is not key to generating tolerance. Instead, this model suggests that there is a universal sequence of events in the induction of transplantation tolerance that, as long as they occur, tolerance can be generated regardless of the exact therapeutic agents used. To facilitate transplantation tolerance, I hypothesized that the initial inflammation and destructive response towards the graft needs to be dampened to ensure graft survival but a threshold level of T cell activity needs to remain and interact with the transplant. This is then followed by T cells activated by the transplant in a quiescent environment similar to an autoreactive T cell engaging a self-antigen in a non-autoimmune individual. This interaction then gradually establishes a donor-specific tolerance response towards the transplant itself. Therefore, according to this model, the

basis to finding tolerance-permissive therapy is developing therapeutic agents that suppress initial innate and adaptive immune response enough to prevent acute transplant rejection and allow the presentation of donor transplant antigens in a quiescent manner, but still permit a baseline level of T cell reactivity to actively generate donor-specific tolerance. Since a number of different biologic agents currently qualify this description, this perhaps explains why many agents, especially antibodies targeting a myriad of distinct T cell surface molecules, all result in transplantation tolerance. After all, if the therapeutic agents are not required to themselves be able to intrinsically induce T cell tolerogenic responses, the exact nature and identity of the therapy are not as crucial as their ability to achieve early graft survival without gross suppression of all T cell reactivity.

If the solution to designing a tolerance-generating therapy is finding a therapeutic agent that can dampen the initial immune response surrounding the transplant to both ensure immediate graft survival and allow donor antigens to be presented in a tolerance-promoting context, what are the key criteria this agent should have? From the large body of data acquired in the studies of peripheral self-tolerance, it is generally accepted that if an antigen is presented 1) without costimulatory signals, 2) without sufficient affinity or avidity, 3) at too high or too low a density, or 4) without environmental factors such as inflammation, pathogen infection, and tissue distress, then the presented antigen is likely to promote a tolerogenic rather than a immunogenic response. Of these criteria, it appears that inflammation in particular plays an essential role in accelerating transplant rejection and preventing the induction of transplant tolerance. While pathogen infection has been accepted as a potent barrier to tolerance induction, the exact factors interfering with tolerance brought about by an infection remained unclear until recently. The Chong group showed that *Listeria monocytogenes* infection prevented skin and cardiac allograft acceptance by anti-CD154 and DST in mice (248). Interestingly, they also discovered that this disruption of tolerance was not universal to all pathogen infections. *Pseudomonas aeruginosa*, another common opportunistic pathogen in the clinics, did not affect anti-CD154/DST-mediated graft prolongation and tolerance (292). This difference

was attributed to the specific immune responses elicited by these two pathogens. *P. aeruginosa* typically triggers a strong humoural response with a dominant T_H-2 phenotype (293, 294). On the other hand, *L. monocytogenes* infection elicits a powerful inflammatory response from both the innate immune cells and CD8+ CTLs (295). Unsurprisingly, *L. monocytogenes*-mediated disruption of tolerance induction turned out to be dependent on pro-inflammatory cytokines IL-6 and type-I interferons (292, 296). One potential explanation of the potent inhibitory effect of inflammation on tolerance induction is that inflammation acts similarly to pathogen-associated molecular patterns from microbial byproducts and damage-associated molecular patterns released during tissue distress to stimulate dendritic cell maturation through toll-like receptors, thus allowing their increased effectiveness in activating T cells (297). Based on these observations, it appears that inflammation is a key environmental determinant of the success in generating transplantation tolerance. This is especially relevant in the clinical setting, as human patients, unlike mice kept in specific pathogen-free facilities, are constantly exposed to a large variety of pathogens, and thus will require careful monitoring during the peri-transplant period. Therefore, to permit the transplant to promote a tolerogenic response, it is necessary for the initial therapy treatment to especially suppress inflammation around the graft tissue and allows presentation of the donor antigens in the absence of additional immune activation due to inflammation and tissue distress.

6.3.2.2 What Is the Role of Regulatory T Cells?

Since my hypothesized model of time-dependent transplant tolerance generation calls for an initial down-regulation of anti-graft immune response followed by gradual generation of transplant-specific tolerance, and my data had not shown specifically increased generation of *de novo* FoxP3+CD4+ Tregs, one might infer from this discussion that regulatory T cells play a limited role in this model of transplant tolerance. However, this is clearly not the case. As stated in section 6.2.4, many studies have shown that antibody therapy-induced transplantation tolerance is reliant on the presence of T cell-mediated regulation.

For instance, treatment with anti-CD4 in thymectomized mice bearing long-term surviving allogeneic skin promptly induced transplant rejection, and depletion of CD4+ T cells prior to transplantation in this model also abrogated graft survival (212). Furthermore, depletion of CD4+ T cells during the peri-transplantation period reduced the effectiveness of combined anti-CD154 and anti-LFA-1 therapy in a pancreatic islet allograft model (218). Specific depletion of CD4+FoxP3+ Tregs also demonstrated that Tregs were needed constantly during transplant tolerance induction and maintenance (298). All these evidence highlight the importance of T cell-mediated regulation in transplantation tolerance. The question is therefore when and where this regulatory activity develops during the generation of tolerance to a transplant.

According to my data, I hypothesize that while antibody therapies may not up-regulate the generation of regulatory T cells, they facilitate their eventual development. The early increase in Treg-to-T-effector ratio observed in this thesis suggested that anti-LFA-1 therapies preferentially maintained regulatory T cells rather than effector T cells at the site of antigen challenge. This initial sparing of existing regulatory T cells may allow early transplant survival, which will then facilitate active expansion and generation of regulatory T cells according to the time-dependent model of tolerance generation. Therefore, the presence of regulatory T cells is likely essential both at the initial phase and the maintenance phase of transplant tolerance. However, the generation of donor-specific regulatory T cells are not driven by the antibody therapies, rather, it occurs subsequently in response to the transplant itself. To take this concept further, while therapy-mediated sparing of regulatory T cells by the antibody therapies at the site of antigen exposure may contribute to early graft survival, the initial restrain for effector T cell response against the transplant is likely exerted primarily by the antibody therapies, while the later and continuous control of effector T cells during the maintenance of graft survival and tolerance is mediated by the regulatory T cells.

6.3.2.3 Stability of Transplantation Tolerance

Transplantation tolerance has been demonstrated in many models to be vulnerable. Many factors, from memory T cells to antibodies to toll-like receptor activation, have been shown to interfere with tolerance induction and accelerate transplant rejection (292, 297, 299–302). More importantly, bacterial infection in mice after established long-term transplant survival has recently been demonstrated to instigate transplant rejection (303). This phenomenon cannot be explained by the previously proposed models of tolerance generated by anti-LFA-1 or anti-CD154, which identify the therapeutic antibodies as the tolerance-inducing agent that causes immediate changes in T cell phenotypes towards a tolerogenic response. If this is the case, enduring transplantation tolerance should be established within the time frame during which the therapeutic antibodies are circulating the immune system, and graft survival should not be disrupted after this early tolerance-induction period. However, to be able to abrogate existing long-term graft survival and tolerance suggests that the immune response surrounding a "tolerant" transplant is not as stable as these models suggest.

In contrast, since my hypothesized model of transplant tolerance requires time-dependent and incremental evolution in the immune system after antibody therapies, this implies that tolerance can be incomplete and thus vulnerable long after initial transplantation and therapy administration. This would explain the ability of an *Listeria monocytogenes* infection to override an established tolerogenic immune response (303). Unfortunately, this also implies that there are potentially many factors that could tamper with transplantation tolerance throughout the life-span of a long-term surviving graft. For instance, reactivation of latent cytomegalovirus (CMV) is commonly reported in human solid transplant patients, and the transplantation procedure itself has been shown in mouse models to be sufficient in reactivating latent CMV (304). Furthermore, while the reactivated CMV did not appear to inhibit Treg generation, the type-I interferons associate with CMV infection was correlated to reduction of allograft acceptance (304). Therefore, reactivation of a chronic infection, such as CMV, during transplantation could conceivably disrupt the evolution of tolerance even when an active regulation mechanism has been partially established. Furthermore, since a

crucial goal of designing a tolerance-promoting, rather than immunosuppressive, therapy is to allow transplant recipients to retain their protective immunity, pathogen infections and subsequent immune response could break established tolerance. These observations combined with my hypothesized model of a gradual development of transplantation tolerance unfortunately suggest that the process of tolerance induction could be fragile and fraught with difficulties.

If the development of transplantation tolerance is not immediate and thorough and instead requires time and incremental changes to the immune system, how should we prevent disruptions to this potentially fragile process? The answer may be, once again, meticulous control of inflammation. I have described in the previous section how the presence of a pro-inflammatory immune response would block tolerance induction as indicated by abrogation of transplant tolerance in the presence of *L. monocytogenes* (292, 296). Interestingly, the pathogen shown to be able to override existing transplant tolerance is also the pro-inflammatory *L. monocytogenes* (303). Furthermore, reactivated latent CMV infection was shown to disrupt allograft acceptance in a mouse cardiac transplant model, and this phenomenon was correlated to the type-I interferons elicited by CMV (304). It seems that inflammation is a universal barrier to transplantation tolerance whether at the early or the late stage of the process. As such, the success of promoting donor-specific tolerance appears to require vigilant and consistent monitoring of the recipient immune responses after transplantation, and prevention of inflammation is especially crucial. Controlling peripheral pro-inflammatory responses therefore seems to be essential in ensuring stable graft survival during the process of generating transplantation tolerance.

6.4 FUTURE DIRECTIONS AND CONCLUSION

In this thesis, I have shown that transplantation tolerance-inducing therapeutic agents, anti-LFA-1 and anti-CD154, did not cause major changes in naïve T cell activation and differentiation during initial antigen exposure and therapy treatments. The antibodies did not induce clonal anergy, death and deletion, or alternate activation phenotype, the three primary paradigms currently

used in the field of transplantation immunology to explain the mechanism of tolerance by therapeutic antibodies. I have observed a large decrease in the magnitude of T cells in the draining lymph nodes likely due to a diverted T cell trafficking pattern and partial suppression in cytokine production by activated and proliferated T cells found in the nodes. Nevertheless, I have confirmed that, despite the lack of significant induction of T cell tolerance phenotypes, long-term surviving grafts after transient treatment with anti-LFA-1 and anti-CD154 were maintained by an active regulatory mechanism that controls the differentiation and function of naïve graft-specific T cells. Based upon my results, I hypothesize a model of transplantation tolerance that, instead of requiring the therapeutic agents to immediately tolerize the immune system, suggests the transplants itself as the tolerogen instead. In other words, to achieve transplantation tolerance, a therapeutic agent will down-regulate T cell immunity without completely suppressing it to allow initial graft survival, the surviving graft will subsequently drive the generation of T cell tolerance by activating graft-specific T cells in a relatively immunologically quiescent microenvironment, resulting in the gradual evolution of a donor-specific tolerance response.

The immediate next step to follow up these observations is to examine the validity of my proposed model of time-dependent tolerance induction. Specifically, the state of T cell reactivity and tolerance induction after the initial period of transplantation and therapy treatments need to be interrogated to support or refute my hypothesized model. The presence of the transplant as a requirement for generating tolerance can be tested by removing the transplant at various time points after initial transplantation and treatments with therapies. For instance, if my proposal of transplant as the tolerogen is correct, then removing the graft one month, perhaps even two, after transplantation would abrogate the generation of transplant tolerance. This procedure can subsequently be used to investigate how long the transplant needs to be present in order to promote transplantation tolerance. Secondly, similar adoptive transfer and T cell tracking methods can be used to interrogate the reactivity of T cells during the later stages of transplant survival. If tolerance is gradually generated throughout the time it takes a

transplant to be considered as achieving long-term survival, then tracking a specific population of T cells during this time frame can elucidate this process of evolving T cell tolerogenic responses. After all, many studies have shown the long-term consequence of transient antibody therapies as transplantation tolerance maintained by dominant and T cell-dependent regulatory mechanisms, and I have shown that the early stage of this process as decrease in T cell magnitude and partial suppression of effector function, it is curious as to when the regulatory mechanisms start to develop in between these two points in time.

Another question raised by my observations in this thesis is the role of trafficking as a proximal effect of anti-LFA-1. I have shown that anti-LFA-1 and combined anti-LFA-1/anti-CD154 resulted in striking decrease in the number of T cells in the draining lymph nodes without a significant induction of T cell apoptosis. Furthermore, I have shown altered T cell trafficking pattern as a consequence of anti-LFA-1 treatments. This suggests that a major impact of anti-LFA-1 on T cells is facilitating their emigration from lymph nodes into the circulation and the spleen. This offers a novel mechanism of therapeutic antibody-promoted early transplantation survival not through classical tolerance mechanisms such as anergy, deletion, and regulation, but rather through a kind of forced ignorance. If anti-LFA-1 prevents T cells from staying in the draining lymph nodes, then fewer T cells can become activated against transplant donor antigens. One way to test the necessity of trafficking as an element of anti-LFA-1-mediated transplant tolerance is by treating the recipients with FTY720 prior to transplantation and anti-LFA-1 treatments. FTY720 is an agonist for sphingosine-1-phosphate receptors and induces T cell trafficking out of the circulation and into the lymph nodes, which is the exact opposite consequence than anti-LFA-1 treatments (305). Therefore, FTY720 should abrogate anti-LFA-1's ability to alter T cell traffic, thus testing the requirement of trafficking as a mechanism of tolerance induction via anti-LFA-1 therapies.

In conclusion, results from my thesis have refuted the current paradigms of transplantation tolerance generation using therapeutic antibodies targeting T cell costimulation and/or adhesion molecules. The current paradigms propose

immediate generation of tolerance mediated by the direct interaction between the antibodies and the graft-specific T cells. In contrast, the model established by this thesis offers an alternative mechanism that suggests the role of the therapeutic antibodies not as direct inducers of T cell tolerance, but rather as facilitators of early transplant survival. Importantly, my proposed model gives the crucial role of inducing T cell tolerance to the transplant itself, and the requirement of time for gradual evolution of donor-specific transplantation tolerance. This model has many implications that change our current view of transplantation tolerance from methods to design a clinically applicable tolerance regimen to the stability and vulnerability of transplantation tolerance. Taken together, results from my thesis contribute to our existing knowledge of transplantation tolerance by modifying the existing paradigms of tolerance induction and offering alternative directions toward the understanding and development of a tolerance-promoting therapy.

6.5 FIGURES

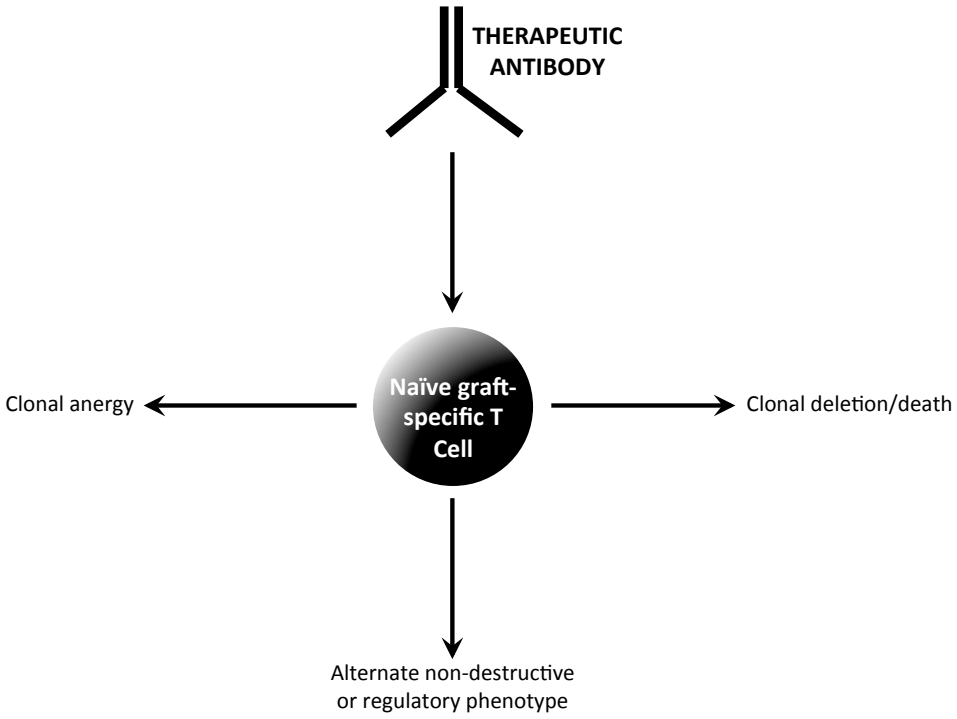

FIGURE 6-1. Current competing hypotheses of anti-LFA-1 and/or anti-CD154-induced T cell tolerance.

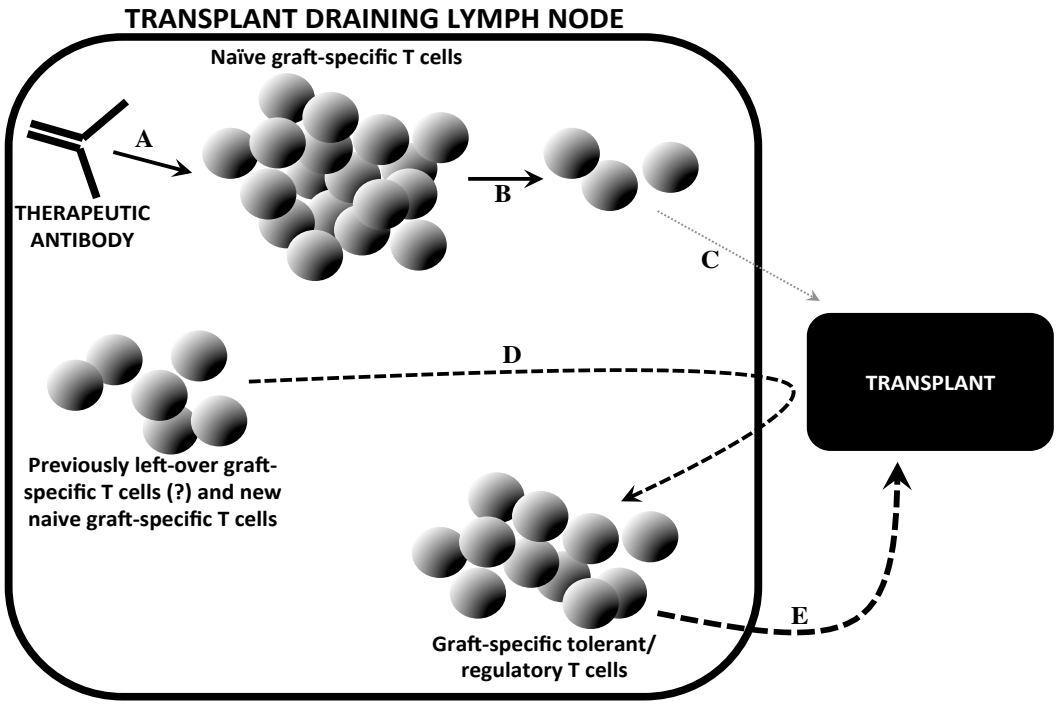

FIGURE 6-2. A proposed model of the generation of transplantation tolerance based on the observations in this thesis. *A.* Therapeutic antibodies (anti-LFA-1 and/or anti-CD154) reduce the magnitude and reactivity of naïve graft-specific T cells in the transplant draining lymph nodes. *B.* The reduction of graft-specific T cell number is likely due to altered trafficking and not deletion. *C.* Partially suppressed T cell magnitude and reactivity is not enough to induce sufficient graft damage and rejection. Graft survives in a microenvironment with dampened immune activity and inflammation. *D.* New naïve T cells are generated as the presence of antibodies wanes. They now respond to an immunologically quiescent graft and gradually develop into tolerance-promoting phenotypes. *E.* Long-term graft survival and donor-specific tolerance is eventually generated and maintained by these T cells.

CHAPTER SEVEN
BIBLIOGRAPHY

1. Goldman, M., A. L. Moine, M. Braun, V. Flamand, and D. Abramowicz. 2001. A role for eosinophils in transplant rejection. *Trends Immunol*. 22: 247–251.

2. Buonocore, S., M. Surquin, A. Le Moine, D. Abramowicz, V. Flamand, and M. Goldman. 2004. Amplification of T-cell responses by neutrophils: relevance to allograft immunity. *Immunol. Lett*. 94: 163–166.

3. Kroemer, A., K. Edtinger, and X. C. Li. 2008. The innate natural killer cells in transplant rejection and tolerance induction. *Curr. Opin. Organ Transplant*. 13: 339–343.

4. Yasunami, Y. 2005. V 14 NK T cell-triggered IFN- production by Gr-1+CD11b+ cells mediates early graft loss of syngeneic transplanted islets. *J. Exp. Med*. 202: 913–918.

5. Zarkhin, V., G. Chalasani, and M. M. Sarwal. 2010. The yin and yang of B cells in graft rejection and tolerance. *Transplant. Rev. Orlando Fla* 24: 67–78.

6. Issa, F., A. Schiopu, and K. J. Wood. 2010. Role of T cells in graft rejection and transplantation tolerance. *Expert Rev. Clin. Immunol*. 6: 155–169.

7. Rosenberg, A. S., T. Mizuochi, and A. Singer. 1986. Analysis of T-cell subsets in rejection of Kb mutant skin allografts differing at class I MHC. *Nature* 322: 829–831.

8. Rosenberg, A. S., T. Mizuochi, S. O. Sharrow, and A. Singer. 1987. Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. *J. Exp. Med*. 165: 1296–1315.

9. Hara, M., C. I. Kingsley, M. Niimi, S. Read, S. E. Turvey, A. R. Bushell, P. J. Morris, F. Powrie, and K. J. Wood. 2001. IL-10 Is Required for Regulatory T Cells to Mediate Tolerance to Alloantigens In Vivo. *J. Immunol*. 166: 3789–3796.

10. Lindahl, K. F., and D. B. Wilson. 1977. Histocompatibility antigen-activated cytotoxic T lymphocytes. I. Estimates of the absolute frequency of killer cells generated in vitro. *J. Exp. Med*. 145: 500–507.

11. Detours, V., and A. S. Perelson. 2000. The paradox of alloreactivity and self MHC restriction: Quantitative analysis and statistics. *Proc. Natl. Acad. Sci.* 97: 8479–8483.
12. Kishimoto, K., S. Sandner, J. Imitola, M. Sho, Y. Li, P. B. Langmuir, D. M. Rothstein, T. B. Strom, L. A. Turka, and M. H. Sayegh. 2002. Th1 cytokines, programmed cell death, and alloreactive T cell clone size in transplant tolerance. *J. Clin. Invest.* 109: 1471–1479.
13. Lindahl, K. F., and D. B. Wilson. 1977. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J. Exp. Med.* 145: 508–522.
14. Hinterberger, M., L. Klein, B. Kyewski, and G. Wirnsberger. 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat. Rev. Immunol.* 9: 833+.
15. Zinkernagel, R. M., and P. C. Doherty. 1974. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature* 251: 547–548.
16. Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 248: 701–702.
17. Wood, K. J., and R. Goto. 2012. Mechanisms of rejection: current perspectives. *Transplantation* 93: 1–10.
18. Herrera, O. B., D. Golshayan, R. Tibbott, F. Salcido Ochoa, M. J. James, F. M. Marelli-Berg, and R. I. Lechler. 2004. A novel pathway of alloantigen presentation by dendritic cells. *J. Immunol. Baltim. Md 1950* 173: 4828–4837.
19. Heeger, P. S. 2003. T-Cell Allorecognition and Transplant Rejection: A Summary and Update. *Am. J. Transplant.* 3: 525–533.
20. Bueno, V., and J. O. M. Pestana. 2002. The role of CD8+ T cells during allograft rejection. *Braz. J. Med. Biol. Res.* 35: 1247–1258.
21. Shen, Y., and S. Fujimoto. 1996. A Tumor-specific Th2 Clone Initiating Tumor Rejection via Primed CD8+ Cytotoxic T-Lymphocyte Activation in Mice. *Cancer Res.* 56: 5005–5011.

22. Schowengerdt, K. O., F. J. Fricker, K. S. Bahjat, and S. T. Kuntz. 2000. Increased expression of the lymphocyte early activation marker CD69 in peripheral blood correlates with histologic evidence of cardiac allograft rejection. *Transplantation* 69: 2102–2107.
23. Jones, N. D., A. Van Maurik, M. Hara, B. J. Gilot, P. J. Morris, and K. J. Wood. 1999. T-cell activation, proliferation, and memory after cardiac transplantation in vivo. *Ann. Surg.* 229: 570–578.
24. Diamond, A. S., and R. G. Gill. 2000. An essential contribution by IFN-gamma to CD8+ T cell-mediated rejection of pancreatic islet allografts. *J. Immunol. Baltim. Md 1950* 165: 247–255.
25. Jones, N. D., A. V. Maurik, M. Hara, B. M. Spriewald, O. Witzke, P. J. Morris, and K. J. Wood. 2000. CD40-CD40 Ligand-Independent Activation of CD8+ T Cells Can Trigger Allograft Rejection. *J. Immunol.* 165: 1111–1118.
26. Bishop, D. K., S. C. Wood, E. J. Eichwald, and C. G. Orosz. 2001. Immunobiology of Allograft Rejection in the Absence of IFN-γ: CD8+ Effector Cells Develop Independently of CD4+ Cells and CD40-CD40 Ligand Interactions. *J. Immunol.* 166: 3248–3255.
27. Valujskikh, A., O. Lantz, S. Celli, P. Matzinger, and P. S. Heeger. 2002. Cross-primed CD8+ T cells mediate graft rejection via a distinct effector pathway. *Nat. Immunol.* 3: 844–851.
28. Sleater, M., A. S. Diamond, and R. G. Gill. 2007. Islet allograft rejection by contact-dependent CD8+ T cells: perforin and FasL play alternate but obligatory roles. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* 7: 1927–1933.
29. Rocha, P. N., T. J. Plumb, S. D. Crowley, and T. M. Coffman. 2003. Effector mechanisms in transplant rejection. *Immunol. Rev.* 196: 51–64.
30. Choy, J. C. 2010. Granzymes and perforin in solid organ transplant rejection. *Cell Death Differ.* 17: 567–576.
31. Kown, M. H., T. Van der Steenhoven, F. G. Blankenberg, G. Hoyt, G. J. Berry, J. F. Tait, H. W. Strauss, and R. C. Robbins. 2000. Zinc-mediated reduction of apoptosis in cardiac allografts. *Circulation* 102: III228–232.

32. Bradley, J. A., A. M. Mowat, and E. M. Bolton. 1992. Processed MHC class I alloantigen as the stimulus for CD4+ T-cell dependent antibody-mediated graft rejection. *Immunol. Today* 13: 434–438.
33. Zelenika, D., E. Adams, A. Mellor, E. Simpson, P. Chandler, B. Stockinger, H. Waldmann, and S. P. Cobbold. 1998. Rejection of H-Y Disparate Skin Grafts by Monospecific CD4+ Th1 and Th2 Cells: No Requirement for CD8+ T Cells or B Cells. *J. Immunol*. 161: 1868–1874.
34. Zelenika, D., E. Adams, S. Humm, C.-Y. Lin, H. Waldmann, and S. P. Cobbold. 2001. The role of CD4+ T-cell subsets in determining transplantation rejection or tolerance. *Immunol. Rev*. 182: 164–179.
35. Dalloul, A. H., E. Chmouzis, K. Ngo, and W. P. Fung-Leung. 1996. Adoptively transferred CD4+ lymphocytes from CD8 -/- mice are sufficient to mediate the rejection of MHC class II or class I disparate skin grafts. *J. Immunol*. 156: 4114–4119.
36. Pietra, B. A., A. Wiseman, A. Bolwerk, M. Rizeq, and R. G. Gill. 2000. CD4 T cell-mediated cardiac allograft rejection requires donor but not host MHC class II. *J. Clin. Invest*. 106: 1003–1010.
37. Shizuru, J. A., A. K. Gregory, C. T. Chao, and C. G. Fathman. 1987. Islet allograft survival after a single course of treatment of recipient with antibody to L3T4. *Science* 237: 278–280.
38. Hao, L., Y. Wang, R. G. Gill, and K. J. Lafferty. 1988. CD4 T cells and allograft rejection. *Transplant. Proc*. 20: 56–60.
39. Simeonovic, C. J., R. Ceredig, and J. D. Wilson. 1990. Effect of GK1.5 monoclonal antibody dosage on survival of pig proislet xenografts in CD4+ T cell-depleted mice. *Transplantation* 49: 849–856.
40. Valujskikh, A., D. Matesic, A. Gilliam, D. Anthony, T. M. Haqqi, and P. S. Heeger. 1998. T cells reactive to a single immunodominant self-restricted allopeptide induce skin graft rejection in mice. *J. Clin. Invest*. 101: 1398–1407.
41. Grazia, T. J., R. J. Plenter, S. M. Weber, H. M. Lepper, F. Victorino, M. R. Zamora, B. A. Pietra, and R. G. Gill. 2010. Acute cardiac allograft rejection by

directly cytotoxic CD4 T cells: parallel requirements for Fas and perforin. *Transplantation* 89: 33–39.

42. Bickerstaff, A. A., J.-J. Wang, R. P. Pelletier, and C. G. Orosz. 2001. Murine Renal Allografts: Spontaneous Acceptance Is Associated with Regulated T Cell-Mediated Immunity. *J. Immunol*. 167: 4821–4827.

43. VanBuskirk, A. M., W. J. Burlingham, E. Jankowska-Gan, T. Chin, S. Kusaka, F. Geissler, R. P. Pelletier, and C. G. Orosz. 2000. Human allograft acceptance is associated with immune regulation. *J. Clin. Invest*. 106: 145–155.

44. VanBuskirk, A. M., M. E. Wakely, and C. G. Orosz. 1996. Transfusion of polarized TH2-like cell populations into SCID mouse cardiac allograft recipients results in acute allograft rejection. *Transplantation* 62: 229–238.

45. Jin, D., L. Zhang, J. Zheng, and Y. Zhao. 2008. The inflammatory Th 17 subset in immunity against self and non-self antigens. *Autoimmunity* 41: 154–162.

46. Stockinger, B., C. Bourgeois, and G. Kassiotis. 2006. CD4+ memory T cells: functional differentiation and homeostasis. *Immunol. Rev*. 211: 39–48.

47. Halloran, P. F. 2004. Immunosuppressive Drugs for Kidney Transplantation. *N. Engl. J. Med*. 351: 2715–2729.

48. Clipstone, N. A., and G. R. Crabtree. 1992. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* 357: 695–697.

49. Calne, R. Y., D. J. White, S. Thiru, D. B. Evans, P. McMaster, D. C. Dunn, G. N. Craddock, B. D. Pentlow, and K. Rolles. 1978. Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet* 2: 1323–1327.

50. Starzl, T. E., G. B. Klintmalm, K. A. Porter, S. Iwatsuki, and G. P. Schröter. 1981. Liver transplantation with use of cyclosporin a and prednisone. *N. Engl. J. Med*. 305: 266–269.

51. Kino, T., H. Hatanaka, S. Miyata, N. Inamura, M. Nishiyama, T. Yajima, T. Goto, M. Okuhara, M. Kohsaka, and H. Aoki. 1987. FK-506, a novel immunosuppressant isolated from a Streptomyces. II. Immunosuppressive effect of FK-506 in vitro. *J. Antibiot. (Tokyo)* 40: 1256–1265.

52. Wullschleger, S., R. Loewith, and M. N. Hall. 2006. TOR Signaling in Growth and Metabolism. *Cell* 124: 471–484.

53. Yang, Q., and K.-L. Guan. 2007. Expanding mTOR signaling. *Cell Res*. 17: 666–681.
54. Powell, J. D., and G. M. Delgoffe. 2010. The Mammalian Target of Rapamycin: Linking T Cell Differentiation, Function, and Metabolism. *Immunity* 33: 301–311.
55. Budde, K., and J. Gaedeke. 2012. Tuberous Sclerosis Complex–Associated Angiomyolipomas: Focus on mTOR Inhibition. *Am. J. Kidney Dis*. 59: 276–283.
56. Vézina, C., A. Kudelski, and S. N. Sehgal. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J. Antibiot. (Tokyo)* 28: 721–726.
57. Rizzo, M. T., G. Tricot, R. Hoffman, H. N. Jayaram, G. Weber, J. G. Garcia, and D. English. 1990. Inosine monophosphate dehydrogenase inhibitors. Probes for investigations of the functions of guanine nucleotide binding proteins in intact cells. *Cell. Signal*. 2: 509–519.
58. Zimmermann, A. G., J. J. Gu, J. Laliberté, and B. S. Mitchell. 1998. Inosine-5'-monophosphate dehydrogenase: regulation of expression and role in cellular proliferation and T lymphocyte activation. *Prog. Nucleic Acid Res. Mol. Biol*. 61: 181–209.
59. Mitsui, A., and S. Suzuki. 1969. Immunosuppressive effect of mycophenolic acid. *J. Antibiot. (Tokyo)* 22: 358–363.
60. Sollinger, H. W. 1995. Mycophenolate mofetil for the prevention of acute rejection in primary cadaveric renal allograft recipients. U.S. Renal Transplant Mycophenolate Mofetil Study Group. *Transplantation* 60: 225–232.
61. Halloran, P., T. Mathew, S. Tomlanovich, C. Groth, L. Hooftman, and C. Barker. 1997. Mycophenolate mofetil in renal allograft recipients: a pooled efficacy analysis of three randomized, double-blind, clinical studies in prevention of rejection. The International Mycophenolate Mofetil Renal Transplant Study Groups. *Transplantation* 63: 39–47.
62. Gabardi, S., S. T. Martin, K. L. Roberts, and M. Grafals. 2011. Induction immunosuppressive therapies in renal transplantation. *Am. J. Health. Syst. Pharm*. 68: 211–218.

63. Hardinger, K. L. 2006. Rabbit Antithymocyte Globulin Induction Therapy in Adult Renal Transplantation. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* 26: 1771–1783.
64. Mohty, M. 2007. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia* 21: 1387–1394.
65. Brennan, D. C., K. Flavin, J. A. Lowell, T. K. Howard, S. Shenoy, S. Burgess, S. Dolan, J. M. Kano, M. Mahon, M. A. Schnitzler, R. Woodward, W. Irish, and G. G. Singer. 1999. A randomized, double-blinded comparison of Thymoglobulin versus Atgam for induction immunosuppressive therapy in adult renal transplant recipients. *Transplantation* 67: 1011–1018.
66. Ferrajoli, A., S. O'Brien, and M. J. Keating. 2001. Alemtuzumab: a novel monoclonal antibody. *Expert Opin. Biol. Ther.* 1: 1059–1065.
67. Morris, P. J., and N. K. Russell. 2006. Alemtuzumab (Campath-1H): A Systematic Review in Organ Transplantation. *Transplantation* 81: 1361–1367.
68. Casey, M. J., and H.-U. Meier-Kriesche. 2011. Calcineurin inhibitors in kidney transplantation. *Curr. Opin. Nephrol. Hypertens.* 20: 610–615.
69. Prokai, A., A. Fekete, K. Pasti, K. Rusai, N. Banki, G. Reusz, and A. Szabo. 2012. The importance of different immunosuppressive regimens in the development of posttransplant diabetes mellitus. *Pediatr. Diabetes* 13: 81–91.
70. Fishman, J. A. 2011. Infections in immunocompromised hosts and organ transplant recipients: Essentials. *Liver Transpl.* 17: S34–S37.
71. Rama, I., and J. M. Grinyó. 2010. Malignancy after renal transplantation: the role of immunosuppression. *Nat. Rev. Nephrol.* 6: 511–519.
72. Lu, B. R., K. T. Park, M. Hurwitz, K. L. Cox, and W. E. Berquist. 2013. Impact of Immunosuppression on the Development of Epstein-Barr Virus (EBV) Viremia After Pediatric Liver Transplantation. *Transplant. Proc.* 45: 301–304.
73. Wilsdorf, N., B. Eiz-Vesper, C. Henke-Gendo, J. Diestelhorst, I. Oschlies, K. Hussein, L. Pape, U. Baumann, B. Tönshoff, M. Pohl, B. Höcker, A.-M. Wingen, W. Klapper, H. Kreipe, T. F. Schulz, C. Klein, and B. Maecker-Kolhoff. 2013. EBV-Specific T-Cell Immunity in Pediatric Solid Organ Graft Recipients With Posttransplantation Lymphoproliferative Disease. *Transplant. J.* 95: 247–255.

74. Nashan, B., and F. Citterio. 2012. Wound Healing Complications and the Use of Mammalian Target of Rapamycin Inhibitors in Kidney Transplantation. *Transplant. J.* 94: 547–561.
75. Borrows, R., G. Chusney, M. Loucaidou, A. James, J. Lee, J. v. Tromp, J. Owen, T. Cairns, M. Griffith, N. Hakim, A. McLean, A. Palmer, V. Papalois, and D. Taube. 2006. Mycophenolic Acid 12-h Trough Level Monitoring in Renal Transplantation: Association with Acute Rejection and Toxicity. *Am. J. Transplant.* 6: 121–128.
76. Jeong, H., and B. Kaplan. 2007. Therapeutic Monitoring of Mycophenolate Mofetil. *Clin. J. Am. Soc. Nephrol.* 2: 184–191.
77. Wieland, E., M. Shipkova, U. Schellhaas, E. Schütz, P. D. Niedmann, V. W. Armstrong, and M. Oellerich. 2000. Induction of cytokine release by the acyl glucuronide of mycophenolic acid: a link to side effects? *Clin. Biochem.* 33: 107–113.
78. Jones, J. L., C.-L. Phuah, A. L. Cox, S. A. Thompson, M. Ban, J. Shawcross, A. Walton, S. J. Sawcer, A. Compston, and A. J. Coles. 2009. IL-21 drives secondary autoimmunity in patients with multiple sclerosis, following therapeutic lymphocyte depletion with alemtuzumab (Campath-1H). *J. Clin. Invest.* 119: 2052–2061.
79. Owen, R. D. 1945. Immunogenetic Consequences of Vascular Anastomoses between Bovine Twins. *Science* 102: 400–401.
80. Burnet, F. M., and F. Fenner. 1948. Genetics and immunology. *Heredity (Edinb)* 2: 289–324.
81. Billingham, R. E., L. Brent, and P. B. Medawar. 1953. Actively acquired tolerance of foreign cells. *Nature* 172: 603–606.
82. Hasek, M., and T. Hraba. 1955. Immunological effects of experimental embryonal parabiosis. *Nature* 175: 764–765.
83. Mintz, B., and W. K. Silvers. 1970. Histocompatibility antigens on melanoblasts and hair follicle cells. Cell-localized homograft rejection in allophenic skin grafts. *Transplantation* 9: 497–505.
84. Lederberg, J. 1959. Genes and antibodies. *Science* 129: 1649–1653.

85. Frey, J. R., H. Geleick, and A. Deweck. 1964. Immunological tolerance induced in animals previously sensitized to simple chemical compounds. *Science* 144: 853–854.
86. Leskowitz, S. 1967. Production of hapten-specific unresponsiveness in adult guinea-pigs by prior injection of monovalent conjugates. *Immunology* 13: 9–17.
87. Thorbecke, G. J., and B. Benacerraf. 1967. Tolerance in adult rabbits by repeated non-immunogenic doses of bovine serum albumin. *Immunology* 13: 141–145.
88. Moloney, P. J., and A. E. Tirpak. 1969. Immunotolerance to ox insulin induced in the adult guinea pig. *Can. Med. Assoc. J.* 100: 573–575.
89. Kawaguchi, S. 1970. Studies on the induction of immunological paralysis to bovine ?-globulin in adult mice. *Immunology* 19: 277–289.
90. Rowley, D. A., and F. W. Fitch. 1965. The mechanism of tolerance produced in rats to sheep erythrocytes. *J. Exp. Med.* 121: 671–681.
91. Martinez, C., J. M. Smith, M. Blaese, and R. A. Good. 1963. Production of immunological tolerance in mice after repeated injections of disrupted spleen cells. *J. Exp. Med.* 118: 743–758.
92. Miller, J. F. a. P., and G. F. Mitchell. 1967. The Thymus and the Precursors of Antigen Reactive Cells. *Nature* 216: 659–663.
93. Miller, J. F. a. P., and G. F. Mitchell. 1968. Cell to Cell Interaction in the Immune Response I. Hemolysin-Forming Cells in Neonatally Thymectomized Mice Reconstituted with Thymus or Thoracic Duct Lymphocytes. *J. Exp. Med.* 128: 801–820.
94. Mitchell, G. F., and J. F. Miller. 1968. Immunological activity of thymus and thoracic-duct lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* 59: 296–303.
95. Miller, J. F. a. P. 2002. The discovery of thymus function and of thymus-derived lymphocytes. *Immunol. Rev.* 185: 7–14.
96. Good, M. F., K. W. Pyke, and G. J. Nossal. 1983. Functional clonal deletion of cytotoxic T-lymphocyte precursors in chimeric thymus produced in vitro from embryonic Anlagen. *Proc. Natl. Acad. Sci. U. S. A.* 80: 3045–3049.

97. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49: 273–280.
98. Kisielow, P., H. Blüthmann, U. D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. *Nature* 333: 742–746.
99. Derbinski, J., A. Schulte, B. Kyewski, and L. Klein. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* 2: 1032–1039.
100. Scott, H. S., M. Heino, P. Peterson, L. Mittaz, M. D. Lalioti, C. Betterle, A. Cohen, M. Seri, M. Lerone, G. Romeo, P. Collin, M. Salo, R. Metcalfe, A. Weetman, M. P. Papasavvas, C. Rossier, K. Nagamine, J. Kudoh, N. Shimizu, K. J. Krohn, and S. E. Antonarakis. 1998. Common mutations in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients of different origins. *Mol. Endocrinol. Baltim. Md* 12: 1112–1119.
101. Anderson, M. S., and M. A. Su. 2011. Aire and T cell development. *Curr. Opin. Immunol.* 23: 198–206.
102. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298: 1395–1401.
103. Derbinski, J., J. Gabler, B. Brors, S. Tierling, S. Jonnakuty, M. Hergenhahn, L. Peltonen, J. Walter, and B. Kyewski. 2005. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J. Exp. Med.* 202: 33–45.
104. Liston, A., S. Lesage, J. Wilson, L. Peltonen, and C. C. Goodnow. 2003. Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* 4: 350–354.
105. Liston, A., D. H. D. Gray, S. Lesage, A. L. Fletcher, J. Wilson, K. E. Webster, H. S. Scott, R. L. Boyd, L. Peltonen, and C. C. Goodnow. 2004. Gene Dosage-limiting Role of Aire in Thymic Expression, Clonal Deletion, and Organ-specific Autoimmunity. *J. Exp. Med.* 200: 1015–1026.

106. Xing, Y., and K. A. Hogquist. 2012. T-Cell Tolerance: Central and Peripheral. *Cold Spring Harb. Perspect. Biol.* 4.
107. Vaitaitis, G. M., M. Poulin, R. J. Sanderson, K. Haskins, and D. H. Wagner. 2003. Cutting Edge: CD40-Induced Expression of Recombination Activating Gene (RAG) 1 and RAG2: A Mechanism for the Generation of Autoaggressive T Cells in the Periphery. *J. Immunol.* 170: 3455–3459.
108. Kurts, C., H. Kosaka, F. R. Carbone, J. F. A. P. Miller, and W. R. Heath. 1997. Class I–restricted Cross-Presentation of Exogenous Self-Antigens Leads to Deletion of Autoreactive CD8+ T Cells. *J. Exp. Med.* 186: 239–245.
109. Triplett, E. L. 1962. On the Mechanism of Immunologic Self Recognition. *J. Immunol.* 89: 505–510.
110. McCullagh, P. 1989. Interception of the development of self tolerance in fetal lambs. *Eur. J. Immunol.* 19: 1387–1392.
111. Garza, K. M., S. S. Agersborg, E. Baker, and K. S. K. Tung. 2000. Persistence of Physiological Self Antigen Is Required for the Regulation of Self Tolerance. *J. Immunol.* 164: 3982–3989.
112. Schwartz, R. H. 2003. T Cell Anergy*. *Annu. Rev. Immunol.* 21: 305–334.
113. Schwartz, R. H. 1990. A Cell Culture Model for T Lymphocyte Clonal Anergy. *Science* 248: 1349–1356.
114. 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. *J. Exp. Med.* 165: 302–319.
115. Quill, H., and R. H. Schwartz. 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J. Immunol.* 138: 3704–3712.
116. Vendetti, S., J.-G. Chai, J. Dyson, E. Simpson, G. Lombardi, and R. Lechler. 2000. Anergic T Cells Inhibit the Antigen-Presenting Function of Dendritic Cells. *J. Immunol.* 165: 1175–1181.
117. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* 12: 991–1045.

118. Kawabe, Y., and A. Ochi. 1991. Programmed cell death and extrathymic reduction of Vβ8+CD4+ T cells in mice tolerant to Staphylococcus aureus enterotoxin B. *Nature* 349: 245–248.
119. Cohen, P. L., and R. A. Eisenberg. 1991. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9: 243–269.
120. Priyadharshini, B., D. L. Greiner, and M. A. Brehm. 2012. T-cell activation and transplantation tolerance. *Transplant. Rev. Orlando Fla* 26: 212–222.
121. Bach, J.-F., and J. François Bach. 2003. Regulatory T cells under scrutiny. *Nat. Rev. Immunol.* 3: 189–198.
122. Nishizuka, Y., and T. Sakakura. 1969. Thymus and Reproduction: Sex-Linked Dysgenesia of the Gonad after Neonatal Thymectomy in Mice. *Science* 166: 753–755.
123. Mordes, J. P., D. L. Gallina, E. S. Handler, D. L. Greiner, N. Nakamura, A. Pelletier, and A. A. Rossini. 1987. Transfusions enriched for W3/25+ helper/inducer T lymphocytes prevent spontaneous diabetes in the BB/W rat. *Diabetologia* 30: 22–26.
124. Penhale, W. J., A. Farmer, and W. J. Irvine. 1975. Thyroiditis in T cell-depleted rats. Influence of strain, radiation dose, adjuvants and antilymphocyte serum. *Clin. Exp. Immunol.* 21: 362–375.
125. 1993. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. *J. Exp. Med.* 177: 627–636.
126. Seddon, B., and D. Mason. 1999. Peripheral Autoantigen Induces Regulatory T Cells that Prevent Autoimmunity. *J. Exp. Med.* 189: 877–882.
127. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155: 1151–1164.

128. Godfrey, V. L., J. E. Wilkinson, and L. B. Russell. 1991. X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am. J. Pathol.* 138: 1379–1387.
129. Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paeper, L. B. Clark, S.-A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27: 68–73.
130. Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* 3: 253–257.
131. Curotto de Lafaille, M. A., and J. J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30: 626–635.
132. Shevach, E. M. 2009. Mechanisms of Foxp3+ T Regulatory Cell-Mediated Suppression. *Immunity* 30: 636–645.
133. Powrie, F., J. Carlino, M. W. Leach, S. Mauze, and R. L. Coffman. 1996. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J. Exp. Med.* 183: 2669–2674.
134. Garín, M. I., C.-C. Chu, D. Golshayan, E. Cernuda-Morollón, R. Wait, and R. I. Lechler. 2007. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* 109: 2058–2065.
135. Pandiyan, P., L. Zheng, S. Ishihara, J. Reed, and M. J. Lenardo. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation–mediated apoptosis of effector CD4+ T cells. *Nat. Immunol.* 8: 1353–1362.
136. Grossman, W. J., J. W. Verbsky, W. Barchet, M. Colonna, J. P. Atkinson, and T. J. Ley. 2004. Human T Regulatory Cells Can Use the Perforin Pathway to Cause Autologous Target Cell Death. *Immunity* 21: 589–601.
137. Serra, P., A. Amrani, J. Yamanouchi, B. Han, S. Thiessen, T. Utsugi, J. Verdaguer, and P. Santamaria. 2003. CD40 Ligation Releases Immature Dendritic Cells from the Control of Regulatory CD4+CD25+ T Cells. *Immunity* 19: 877–889.

138. Misra, N., J. Bayry, S. Lacroix-Desmazes, M. D. Kazatchkine, and S. V. Kaveri. 2004. Cutting Edge: Human CD4+CD25+ T Cells Restrain the Maturation and Antigen-Presenting Function of Dendritic Cells. *J. Immunol*. 172: 4676–4680.
139. Liang, B., C. Workman, J. Lee, C. Chew, B. M. Dale, L. Colonna, M. Flores, N. Li, E. Schweighoffer, S. Greenberg, V. Tybulewicz, D. Vignali, and R. Clynes. 2008. Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II. *J. Immunol*. 180: 5916–5926.
140. Borsellino, G., M. Kleinewietfeld, D. D. Mitri, A. Sternjak, A. Diamantini, R. Giometto, S. Höpner, D. Centonze, G. Bernardi, M. L. Dell'Acqua, P. M. Rossini, L. Battistini, O. Rötzschke, and K. Falk. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110: 1225–1232.
141. Starzl, T. E., and R. M. Zinkernagel. 2001. Transplantation tolerance from a historical perspective. *Nat. Rev. Immunol*. 1: 233–239.
142. Billingham, R. E., L. Brent, and P. B. Medawar. 1956. Quantitative Studies on Tissue Transplantation Immunity. III. Actively Acquired Tolerance. *Philos. Trans. R. Soc. Lond. B. Biol. Sci*. 239: 357–414.
143. Ildstad, S. T., S. M. Wren, S. O. Sharrow, D. Stephany, and D. H. Sachs. 1984. In vivo and in vitro characterization of specific hyporeactivity to skin xenografts in mixed xenogeneically reconstituted mice (B10 + F344 rat----B10). *J. Exp. Med*. 160: 1820–1835.
144. Schwartz, R., and W. Dameshek. 1959. Drug-induced Immunological Tolerance. *Nature* 183: 1682–1683.
145. Staples, P. J., I. Gery, and B. H. Waksman. 1966. Role of the thymus in tolerance. *J. Exp. Med*. 124: 127–139.
146. 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: 1293–1295.
147. 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. *Int. Immunol.* 10: 1637–1646.
148. Fabre, J. W., and P. J. Morris. 1972. The effect of donor strain blood pretreatment on renal allograft rejection in rats. *Transplantation* 14: 608–617.
149. Dallman, M. J., O. Shiho, T. H. Page, K. J. Wood, and P. J. Morris. 1991. Peripheral tolerance to alloantigen results from altered regulation of the interleukin 2 pathway. *J. Exp. Med.* 173: 79–87.
150. Baldwin, T. A., K. A. Hogquist, and S. C. Jameson. 2004. The Fourth Way? Harnessing Aggressive Tendencies in the Thymus. *J. Immunol.* 173: 6515–6520.
151. Suchin, E. J., P. B. Langmuir, E. Palmer, M. H. Sayegh, A. D. Wells, and L. A. Turka. 2001. Quantifying the Frequency of Alloreactive T Cells In Vivo: New Answers to an Old Question. *J. Immunol.* 166: 973–981.
152. Benichou, G., P. A. Takizawa, C. A. Olson, M. McMillan, and E. E. Sercarz. 1992. Donor major histocompatibility complex (MHC) peptides are presented by recipient MHC molecules during graft rejection. *J. Exp. Med.* 175: 305–308.
153. Braun, M. Y., I. Grandjean, P. Feunou, L. Duban, R. Kiss, M. Goldman, and O. Lantz. 2001. Acute Rejection in the Absence of Cognate Recognition of Allograft by T Cells. *J. Immunol.* 166: 4879–4883.
154. Lafferty, K. J., and A. J. Cunningham. 1975. A new analysis of allogeneic interactions. *Immunol. Cell Biol.* 53: 27–42.
155. Gross, J. A., E. Callas, and J. P. Allison. 1992. Identification and distribution of the costimulatory receptor CD28 in the mouse. *J. Immunol.* 149: 380–388.
156. Jenkins, M. K., P. S. Taylor, S. D. Norton, and K. B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* 147: 2461–2466.
157. Viola, A., and A. Lanzavecchia. 1996. T Cell Activation Determined by T Cell Receptor Number and Tunable Thresholds. *Science* 273: 104–106.
158. Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 2010. Pillars Article: CD28 Costimulation Can Promote T Cell Survival by Enhancing the Expression of Bcl-xL. Immunity. 1995. 3: 87–98. *J. Immunol.* 185: 3788–3799.

159. Lucas, P. J., I. Negishi, K. Nakayama, L. E. Fields, and D. Y. Loh. 1995. Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. *J. Immunol.* 154: 5757–5768.
160. Sharpe, A. H., and G. J. Freeman. 2002. The B7–CD28 superfamily. *Nat. Rev. Immunol.* 2: 116–126.
161. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1: 405–413.
162. Walunas, T. L., C. Y. Bakker, and J. A. Bluestone. 1996. CTLA-4 ligation blocks CD28-dependent T cell activation. *J. Exp. Med.* 183: 2541–2550.
163. Linsley, P. S., W. Brady, M. Urnes, L. S. Grosmaire, N. K. Damle, and J. A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 174: 561–569.
164. Brunner, M. C., C. A. Chambers, F. K.-M. Chan, J. Hanke, A. Winoto, and J. P. Allison. 1999. CTLA-4-Mediated Inhibition of Early Events of T Cell Proliferation. *J. Immunol.* 162: 5813–5820.
165. Greenwald, R. J., V. A. Boussiotis, R. B. Lorsbach, A. K. Abbas, and A. H. Sharpe. 2001. CTLA-4 Regulates Induction of Anergy In Vivo. *Immunity* 14: 145–155.
166. Krummel, M. F., and J. P. Allison. 2011. Pillars Article: CD28 and CTLA-4 Have Opposing Effects on the Response of T Cells to Stimulation. The Journal of Experimental Medicine. 1995. 182: 459–465. *J. Immunol.* 187: 3459–3465.
167. Clarkson, M. R., and M. H. Sayegh. 2005. T-cell costimulatory pathways in allograft rejection and tolerance. *Transplantation* 80: 555–563.
168. Larsen, C. P., and T. C. Pearson. 1997. The CD40 pathway in allograft rejection, acceptance, and tolerance. *Curr. Opin. Immunol.* 9: 641–647.
169. 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: 747–752.

170. Grewal, I. S., and R. A. Flavell. 1996. The role of CD40 ligand in costimulation and T-cell activation. *Immunol. Rev*. 153: 85–106.
171. Schoenberger, S. P., R. E. M. Toes, E. I. H. van der Voort, R. Offringa, and C. J. M. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature* 393: 480–483.
172. Bennett, S. R. M., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. A. P. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393: 478–480.
173. Dustin, M. L., S. K. Bromley, Z. Kan, D. A. Peterson, and E. R. Unanue. 1997. Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A*. 94: 3909–3913.
174. Monks, C. R. F., B. A. Freiberg, H. Kupfer, N. Sciaky, and A. Kupfer. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395: 82–86.
175. Friedl, P., A. T. den Boer, and M. Gunzer. 2005. Tuning immune responses: diversity and adaptation of the immunological synapse. *Nat. Rev. Immunol*. 5: 532–545.
176. Mempel, T. R., S. E. Henrickson, and U. H. von Andrian. 2004. T-cell priming by dendriticcells in lymph nodes occurs in three distinct phases. *Nature* 427: 154–159.
177. Egen, J. G., and J. P. Allison. 2002. Cytotoxic T Lymphocyte Antigen-4 Accumulation in the Immunological Synapse Is Regulated by TCR Signal Strength. *Immunity* 16: 23–35.
178. Andres, P. G., K. C. Howland, D. Dresnek, S. Edmondson, A. K. Abbas, and M. F. Krummel. 2004. CD28 Signals in the Immature Immunological Synapse. *J. Immunol*. 172: 5880–5886.
179. Maldonado, R. A., D. J. Irvine, R. Schreiber, and L. H. Glimcher. 2004. A role for the immunological synapse in lineage commitment of CD4 lymphocytes. *Nature* 431: 527–532.
180. Purtic, B., L. A. Pitcher, N. S. C. van Oers, and C. Wulfing. 2005. T cell receptor (TCR) clustering in the immunological synapse integrates TCR and

costimulatory signaling in selected T cells. *Proc. Natl. Acad. Sci. U. S. A.* 102: 2904–2909.

181. Dedrick, R. L., S. Bodary, and M. R. Garovoy. 2003. Adhesion molecules as therapeutic targets for autoimmune diseases and transplant rejection. *Expert Opin. Biol. Ther.* 3: 85–95.

182. Woska, J. R., Jr, M. M. Morelock, D. D. Jeanfavre, and B. J. Bormann. 1996. Characterization of molecular interactions between intercellular adhesion molecule-1 and leukocyte function- associated antigen-1. *J. Immunol. Baltim. Md 1950* 156: 4680–4685.

183. Hogg, N., M. Laschinger, K. Giles, and A. McDowall. 2003. T-cell integrins: more than just sticking points. *J. Cell Sci.* 116: 4695–4705.

184. Van Seventer, G. A., Y. Shimizu, K. J. Horgan, and S. Shaw. 1990. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *J. Immunol. Baltim. Md 1950* 144: 4579–4586.

185. Cai, Z., A. Brunmark, M. R. Jackson, D. Loh, P. A. Peterson, and J. Sprent. 1996. Transfected Drosophila cells as a probe for defining the minimal requirements for stimulating unprimed CD8+ T cells. *Proc. Natl. Acad. Sci. U. S. A.* 93: 14736–14741.

186. Zuckerman, L. A., L. Pullen, and J. Miller. 1998. Functional Consequences of Costimulation by ICAM-1 on IL-2 Gene Expression and T Cell Activation. *J. Immunol.* 160: 3259–3268.

187. Ni, H. T., M. J. Deeths, W. Li, D. L. Mueller, and M. F. Mescher. 1999. Signaling pathways activated by leukocyte function-associated Ag-1-dependent costimulation. *J. Immunol. Baltim. Md 1950* 162: 5183–5189.

188. Abraham, C., J. Griffith, and J. Miller. 1999. The dependence for leukocyte function-associated antigen-1/ICAM-1 interactions in T cell activation cannot be overcome by expression of high density TCR ligand. *J. Immunol. Baltim. Md 1950* 162: 4399–4405.

189. Verma, N. K., E. Dempsey, A. Long, A. Davies, S. P. Barry, P. G. Fallon, Y. Volkov, and D. Kelleher. 2012. Leukocyte function-associated antigen-

1/intercellular adhesion molecule-1 interaction induces a novel genetic signature resulting in T-cells refractory to transforming growth factor-β signaling. *J. Biol. Chem.* 287: 27204–27216.
190. Lenschow, D. J., Y. Zeng, K. S. Hathcock, L. A. Zuckerman, G. Freeman, J. R. Thistlethwaite, G. S. Gray, R. J. Hodes, and J. A. Bluestone. 1995. Inhibition of transplant rejection following treatment with anti-B7-2 and anti-B7-1 antibodies. *Transplantation* 60: 1171–1178.
191. Kirk, A. D., D. K. Tadaki, A. Celniker, D. Scott Batty, J. D. Berning, J. O. Colonna, F. Cruzata, E. A. Elster, G. S. Gray, and R. L. Kampen. 2001. Induction therapy with monoclonal antibodies specific for cd80 and cd86 delays the onset of acute renal allograft rejection in non-human primates1. *Transplantation* 72: 377.
192. Hausen, B., J. Klupp, U. Christians, J. P. Higgins, R. E. Baumgartner, L. E. Hook, S. Friedrich, A. Celnicker, and R. E. Morris. 2001. Coadministration of either cyclosporine or steroids with humanized monoclonal antibodies against CD80 and CD86 successfully prolong allograft survival after life supporting renal transplantation in cynomolgus monkeys. *Transplantation* 72: 1128–1137.
193. Birsan, T., B. Hausen, J. P. Higgins, R. W. Hubble, J. Klupp, M. Stalder, A. Celniker, S. Friedrich, R. M. O???Hara, and R. E. Morris. 2003. Treatment with humanized monoclonal antibodies against CD80 and CD86 combined with sirolimus prolongs renal allograft survival in cynomolgus monkeys1. *Transplantation* 75: 2106–2113.
194. Laskowski, I. A., J. Pratschke, M. J. Wilhelm, V. M. Dong, F. Beato, M. Taal, M. Gasser, W. W. Hancock, M. H. Sayegh, and N. L. Tilney. 2002. Anti-CD28 Monoclonal Antibody Therapy Prevents Chronic Rejection of Renal Allografts in Rats. *J. Am. Soc. Nephrol.* 13: 519–527.
195. Haspot, F., C. Séveno, A.-S. Dugast, F. Coulon, K. Renaudin, C. Usal, M. Hill, I. Anegon, M. Heslan, R. Josien, S. Brouard, J.-P. Soulillou, and B. Vanhove. 2005. Anti-CD28 Antibody-Induced Kidney Allograft Tolerance Related to Tryptophan Degradation and TCR– Class II– B7+ Regulatory Cells. *Am. J. Transplant.* 5: 2339–2348.

196. Albert, M. H., X.-Z. Yu, P. J. Martin, and C. Anasetti. 2005. Prevention of lethal acute GVHD with an agonistic CD28 antibody and rapamycin. *Blood* 105: 1355–1361.
197. Yu, X.-Z., P. J. Martin, and C. Anasetti. 2003. CD28 Signal Enhances Apoptosis of CD8 T Cells After Strong TCR Ligation. *J. Immunol*. 170: 3002–3006.
198. Yu, X.-Z., M. H. Albert, P. J. Martin, and C. Anasetti. 2004. CD28 ligation induces transplantation tolerance by IFN-?-dependent depletion of T cells that recognize alloantigens. *J. Clin. Invest*. 113: 1624–1630.
199. Rodríguez-Palmero, M., T. Hara, A. Thumbs, and T. Hünig. 1999. Triggering of T cell proliferation through CD28 induces GATA-3 and promotes T helper type 2 differentiation in vitro and in vivo. *Eur. J. Immunol*. 29: 3914–3924.
200. Beyersdorf, N., S. Gaupp, K. Balbach, J. Schmidt, K. V. Toyka, C.-H. Lin, T. Hanke, T. Hunig, T. Kerkau, and R. Gold. 2005. Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. *J. Exp. Med*. 202: 445–455.
201. Suntharalingam, G., M. R. Perry, S. Ward, S. J. Brett, A. Castello-Cortes, M. D. Brunner, and N. Panoskaltsis. 2006. Cytokine Storm in a Phase 1 Trial of the Anti-CD28 Monoclonal Antibody TGN1412. *N. Engl. J. Med*. 355: 1018–1028.
202. Stebbings, R., L. Findlay, C. Edwards, D. Eastwood, C. Bird, D. North, Y. Mistry, P. Dilger, E. Liefooghe, I. Cludts, B. Fox, G. Tarrant, J. Robinson, T. Meager, C. Dolman, S. J. Thorpe, A. Bristow, M. Wadhwa, R. Thorpe, and S. Poole. 2007. "Cytokine Storm" in the Phase I Trial of Monoclonal Antibody TGN1412: Better Understanding the Causes to Improve PreClinical Testing of Immunotherapeutics. *J. Immunol*. 179: 3325–3331.
203. Ford, M. L., and C. P. Larsen. 2009. Translating costimulation blockade to the clinic: lessons learned from three pathways. *Immunol. Rev*. 229: 294–306.
204. Baliga, P., K. D. Chavin, L. Qin, J. Woodward, J. Lin, P. S. Linsley, and J. S. Bromberg. 1994. CTLA4Ig prolongs allograft survival while suppressing cell-mediated immunity. *Transplantation* 58: 1082–1090.

205. Lin, H., S. F. Bolling, P. S. Linsley, R. Q. Wei, D. Gordon, C. B. Thompson, and L. A. Turka. 1993. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J. Exp. Med.* 178: 1801–1806.
206. 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: 1701–1706.
207. Sayegh, M. H., E. Akalin, W. W. Hancock, M. E. Russell, C. B. Carpenter, P. S. Linsley, and L. A. Turka. 1995. CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. *J. Exp. Med.* 181: 1869–1874.
208. Tran, H. M., P. W. Nickerson, A. C. Restifo, M. A. Ivis-Woodward, A. Patel, R. D. Allen, T. B. Strom, and P. J. O'Connell. 1997. Distinct mechanisms for the induction and maintenance of allograft tolerance with CTLA4-Fc treatment. *J. Immunol. Baltim. Md 1950* 159: 2232–2239.
209. Wekerle, T., J. Kurtz, M. H. Sayegh, H. Ito, A. D. Wells, S. Bensinger, J. Shaffer, L. A. Turka, and M. Sykes. 2001. Peripheral Deletion After Bone Marrow Transplantation with Costimulatory Blockade Has Features of Both Activation-Induced Cell Death and Passive Cell Death. *J. Immunol.* 166: 2311–2316.
210. 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: 9560–9564.
211. Markees, T. G., N. E. Phillips, R. J. Noelle, L. D. Shultz, J. P. Mordes, D. L. Greiner, and A. A. Rossini. 1997. Prolonged survival of mouse skin allografts in recipients treated with donor splenocytes and antibody to CD40 ligand. *Transplantation* 64: 329–335.
212. 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. *J. Clin. Invest.* 101: 2446–2455.

213. Larsen, C. P., D. Z. Alexander, D. Hollenbaugh, E. T. Elwood, S. C. Ritchie, A. Aruffo, R. Hendrix, and T. C. Pearson. 1996. CD40-gp39 interactions play a critical role during allograft rejection. Suppression of allograft rejection by blockade of the CD40-gp39 pathway. *Transplantation* 61: 4–9.

214. Zheng, X. X., T. G. Markees, W. W. Hancock, Y. Li, D. L. Greiner, X. C. Li, J. P. Mordes, M. H. Sayegh, A. A. Rossini, and T. B. Strom. 1999. CTLA4 signals are required to optimally induce allograft tolerance with combined donor-specific transfusion and anti-CD154 monoclonal antibody treatment. *J. Immunol. Baltim. Md 1950* 162: 4983–4990.

215. Li, Y., X. C. Li, X. X. Zheng, A. D. Wells, L. A. Turka, and T. B. Strom. 1999. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat. Med.* 5: 1298–1302.

216. Honey, K., S. P. Cobbold, and H. Waldmann. 1999. CD40 ligand blockade induces CD4+ T cell tolerance and linked suppression. *J. Immunol. Baltim. Md 1950* 163: 4805–4810.

217. Rothstein, D. M., M. F. A. Livak, K. Kishimoto, C. Ariyan, H.-Y. Qian, S. Fecteau, M. Sho, S. Deng, X. X. Zheng, M. H. Sayegh, and G. P. Basadonna. 2001. Targeting Signal 1 Through CD45RB Synergizes with CD40 Ligand Blockade and Promotes Long Term Engraftment and Tolerance in Stringent Transplant Models. *J. Immunol.* 166: 322–329.

218. Nicolls, M. R., M. Coulombe, J. Beilke, H. C. Gelhaus, and R. G. Gill. 2002. CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J. Immunol. Baltim. Md 1950* 169: 4831–4839.

219. Rayat, G. R., and R. G. Gill. 2005. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. *Diabetes* 54: 443–451.

220. Kirk, A. D., L. C. Burkly, D. S. Batty, R. E. Baumgartner, J. D. Berning, K. Buchanan, J. H. Fechner Jr, R. L. Germond, R. L. Kampen, N. B. Patterson, S. J. Swanson, D. K. Tadaki, C. N. TenHoor, L. White, S. J. Knechtle, and D. M. Harlan. 1999. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat. Med.* 5: 686–693.

221. 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: 8132–8137.

222. Elster, E. A., H. Xu, D. K. Tadaki, S. Montgomery, L. C. Burkly, J. D. Berning, R. E. Baumgartner, F. Cruzata, R. Marx, D. M. Harlan, and A. D. Kirk. 2001. Treatment with the humanized CD154-specific monoclonal antibody, hu5C8, prevents acute rejection of primary skin allografts in nonhuman primates. *Transplantation* 72: 1473–1478.

223. Preston, E. H., H. Xu, K. K. Dhanireddy, J. P. Pearl, F. V. Leopardi, M. F. Starost, D. A. Hale, and A. D. Kirk. 2005. IDEC-131 (anti-CD154), sirolimus and donor-specific transfusion facilitate operational tolerance in non-human primates. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* 5: 1032–1041.

224. Pearl, J. P., H. Xu, F. Leopardi, E. Preston, and A. D. Kirk. 2007. CD154 blockade, sirolimus, and donor-specific transfusion prevents renal allograft rejection in cynomolgus monkeys despite homeostatic T-cell activation. *Transplantation* 83: 1219–1225.

225. Graca, L., K. Honey, E. Adams, S. P. Cobbold, and H. Waldmann. 2000. Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J. Immunol. Baltim. Md 1950* 165: 4783–4786.

226. Iwakoshi, N. N., J. P. Mordes, T. G. Markees, N. E. Phillips, A. A. Rossini, and D. L. Greiner. 2000. Treatment of allograft recipients with donor-specific transfusion and anti-CD154 antibody leads to deletion of alloreactive CD8+ T

cells and prolonged graft survival in a CTLA4-dependent manner. *J. Immunol. Baltim. Md 1950* 164: 512–521.

227. Quezada, S. A., K. Bennett, B. R. Blazar, A. Y. Rudensky, S. Sakaguchi, and R. J. Noelle. 2005. Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J. Immunol. Baltim. Md 1950* 175: 771–779.

228. Rigby, M. R., A. M. Trexler, T. C. Pearson, and C. P. Larsen. 2008. CD28/CD154 Blockade Prevents Autoimmune Diabetes by Inducing Nondeletional Tolerance After Effector T-Cell Inhibition and Regulatory T-Cell Expansion. *Diabetes* 57: 2672–2683.

229. Taylor, P. A., R. J. Noelle, and B. R. Blazar. 2001. Cd4+Cd25+ Immune Regulatory Cells Are Required for Induction of Tolerance to Alloantigen via Costimulatory Blockade. *J. Exp. Med.* 193: 1311–1318.

230. Ferrer, I. R., M. E. Wagener, M. Song, A. D. Kirk, C. P. Larsen, and M. L. Ford. 2011. Antigen-specific induced Foxp3+ regulatory T cells are generated following CD40/CD154 blockade. *Proc. Natl. Acad. Sci. U. S. A.* 108: 20701–20706.

231. Hancock, W. W., M. H. Sayegh, X. G. Zheng, R. Peach, P. S. Linsley, and L. A. Turka. 1996. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. *Proc. Natl. Acad. Sci. U. S. A.* 93: 13967–13972.

232. Ensminger, S. M., B. M. Spriewald, O. Witzke, K. Morrison, A. van Maurik, P. J. Morris, M. L. Rose, and K. J. Wood. 2000. Intragraft interleukin-4 mRNA expression after short-term CD154 blockade may trigger delayed development of transplant arteriosclerosis in the absence of CD8+ T cells. *Transplantation* 70: 955–963.

233. Ferrer, I. R., D. Liu, D. F. Pinelli, B. H. Koehn, L. L. Stempora, and M. L. Ford. 2012. CD40/CD154 blockade inhibits dendritic cell expression of inflammatory cytokines but not costimulatory molecules. *J. Immunol. Baltim. Md 1950* 189: 4387–4395.

234. Kawai, T., D. Andrews, R. B. Colvin, D. H. Sachs, and A. B. Cosimi. 2000. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat. Med.* 6: 114–114.
235. Haanstra, K. G., J. Ringers, E. A. Sick, S. Ramdien-Murli, E.-M. Kuhn, L. Boon, and M. Jonker. 2003. Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates. *Transplantation* 75: 637–643.
236. Adams, A. B., N. Shirasugi, T. R. Jones, M. M. Durham, E. A. Strobert, S. Cowan, P. Rees, R. Hendrix, K. Price, N. S. Kenyon, D. Hagerty, R. Townsend, D. Hollenbaugh, T. C. Pearson, and C. P. Larsen. 2005. Development of a Chimeric Anti-CD40 Monoclonal Antibody That Synergizes with LEA29Y to Prolong Islet Allograft Survival. *J. Immunol.* 174: 542–550.
237. Gilson, C. R., Z. Milas, S. Gangappa, D. Hollenbaugh, T. C. Pearson, M. L. Ford, and C. P. Larsen. 2009. Anti-CD40 Monoclonal Antibody Synergizes with CTLA4-Ig in Promoting Long-Term Graft Survival in Murine Models of Transplantation. *J. Immunol.* 183: 1625–1635.
238. Thompson, P., K. Cardona, M. Russell, I. R. Badell, V. Shaffer, G. Korbutt, G. R. Rayat, J. Cano, M. Song, W. Jiang, E. Strobert, R. Rajotte, T. Pearson, A. D. Kirk, and C. P. Larsen. 2011. CD40-Specific Costimulation Blockade Enhances Neonatal Porcine Islet Survival in Nonhuman Primates. *Am. J. Transplant.* 11: 947–957.
239. Isobe, M., H. Yagita, K. Okumura, and A. Ihara. 1992. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science* 255: 1125–1127.
240. Nishihara, M., M. Gotoh, H. Ohzato, Y. Ohta, Z. Luo, K. Dono, K. Umeshita, M. Sakon, M. Monden, H. Yagita, K. Okumura, and M. Miyasaka. 1997. Awareness of donor alloantigens in antiadhesion therapy induces antigen-specific unresponsiveness to islet allografts. *Transplantation* 64: 965–970.
241. Nicolls, M. R., M. Coulombe, H. Yang, A. Bolwerk, and R. G. Gill. 2000. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. *J. Immunol. Baltim. Md 1950* 164: 3627–3634.

242. Nicolls, M. R., and R. G. Gill. 2006. LFA-1 (CD11a) as a therapeutic target. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* 6: 27–36.
243. Corbascio, M., H. Mahanty, C. Osterholm, Z. Qi, T. C. Pearson, C. P. Larsen, C. E. Freise, and H. Ekberg. 2002. Anti-lymphocyte function-associated antigen-1 monoclonal antibody inhibits CD40 ligand-independent immune responses and prevents chronic vasculopathy in CD40 ligand-deficient mice. *Transplantation* 74: 35–41.
244. Shao, W., G. Yan, Y. Lin, J. Chen, H. Dai, F. Wang, Y. Xi, H. Thorlacius, and Z. Qi. 2011. CD44/CD70 blockade and anti-CD154/LFA-1 treatment synergistically suppress accelerated rejection and prolong cardiac allograft survival in mice. *Scand. J. Immunol.* 74: 430–437.
245. Reisman, N. M., T. L. Floyd, M. E. Wagener, A. D. Kirk, C. P. Larsen, and M. L. Ford. 2011. LFA-1 blockade induces effector and regulatory T-cell enrichment in lymph nodes and synergizes with CTLA-4Ig to inhibit effector function. *Blood* 118: 5851–5861.
246. Arefanian, H., E. B. Tredget, R. V. Rajotte, R. G. Gill, G. S. Korbutt, and G. R. Rayat. 2010. Short-term administrations of a combination of anti-LFA-1 and anti-CD154 monoclonal antibodies induce tolerance to neonatal porcine islet xenografts in mice. *Diabetes* 59: 958–966.
247. Badell, I. R., M. C. Russell, P. W. Thompson, A. P. Turner, T. A. Weaver, J. M. Robertson, J. G. Avila, J. A. Cano, B. E. Johnson, M. Song, F. V. Leopardi, S. Swygert, E. A. Strobert, M. L. Ford, A. D. Kirk, and C. P. Larsen. 2010. LFA-1-specific therapy prolongs allograft survival in rhesus macaques. *J. Clin. Invest.* 120: 4520–4531.
248. Grazia, T. J., R. G. Gill, H. C. Gelhaus Jr, A. N. Doan, M. L. Sleater, and B. A. Pietra. 2005. Perturbation of leukocyte function-associated antigen-1/intercellular adhesion molecule-1 results in differential outcomes in cardiac vs islet allograft survival. *J. Heart Lung Transplant. Off. Publ. Int. Soc. Heart Transplant.* 24: 1410–1414.

249. Sterry, W., M. Bagot, C. Ferrandiz, K. Kragballe, K. Papp, and G. Stingl. 2009. Immunosuppressive therapy in dermatology and PML. *J. Dtsch. Dermatol. Ges.* 7: 5.
250. Schwab, N., J. C. Ulzheimer, R. J. Fox, T. Schneider-Hohendorf, B. C. Kieseier, C. M. Monoranu, S. M. Staugaitis, W. Welch, S. Jilek, R. A. Du Pasquier, W. Bruck, K. V. Toyka, R. M. Ransohoff, and H. Wiendl. 2012. Fatal PML associated with efalizumab therapy. *Neurology* 78: 458–467.
251. Vincenti, F., R. Mendez, M. Pescovitz, P. R. Rajagopalan, A. H. Wilkinson, K. Butt, D. Laskow, D. P. Slakey, M. I. Lorber, J. P. Garg, and M. Garovoy. 2007. A phase I/II randomized open-label multicenter trial of efalizumab, a humanized anti-CD11a, anti-LFA-1 in renal transplantation. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* 7: 1770–1777.
252. Posselt, A. M., G. L. Szot, L. A. Frassetto, U. Masharani, M. Tavakol, R. Amin, J. McElroy, M. D. Ramos, R. K. Kerlan, L. Fong, F. Vincenti, J. A. Bluestone, and P. G. Stock. 2010. Islet transplantation in type 1 diabetic patients using calcineurin inhibitor-free immunosuppressive protocols based on T-cell adhesion or costimulation blockade. *Transplantation* 90: 1595–1601.
253. Turgeon, N. A., J. G. Avila, J. A. Cano, J. J. Hutchinson, I. R. Badell, A. J. Page, A. B. Adams, M. H. Sears, P. H. Bowen, A. D. Kirk, T. C. Pearson, and C. P. Larsen. 2010. Experience with a novel efalizumab-based immunosuppressive regimen to facilitate single donor islet cell transplantation. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* 10: 2082–2091.
254. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34–40.
255. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68: 869–877.

256. Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med.* 184: 923–930.
257. Ehst, B. D., E. Ingulli, and M. K. Jenkins. 2003. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transpl. Surg.* 3: 1355–1362.
258. Moon, J. J., H. H. Chu, J. Hataye, A. J. Pagán, M. Pepper, J. B. McLachlan, T. Zell, and M. K. Jenkins. 2009. Tracking epitope-specific T cells. *Nat. Protoc.* 4: 565–581.
259. Ford, M. L., B. H. Koehn, M. E. Wagener, W. Jiang, S. Gangappa, T. C. Pearson, and C. P. Larsen. 2007. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J. Exp. Med.* 204: 299–309.
260. Ford, M. L., M. E. Wagener, S. S. Hanna, T. C. Pearson, A. D. Kirk, and C. P. Larsen. 2008. A Critical Precursor Frequency of Donor-Reactive CD4+ T Cell Help Is Required for CD8+ T Cell-Mediated CD28/CD154-Independent Rejection. *J. Immunol.* 180: 7203–7211.
261. Page, E. K., W. A. Dar, and S. J. Knechtle. 2012. Biologics in organ transplantation. *Transpl. Int. Off. J. Eur. Soc. Organ Transplant.* 25: 707–719.
262. 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: 434–438.
263. Iwakoshi, N. N., T. G. Markees, N. Turgeon, T. Thornley, A. Cuthbert, J. Leif, N. E. Phillips, J. P. Mordes, D. L. Greiner, and A. A. Rossini. 2001. Skin allograft maintenance in a new synchimeric model system of tolerance. *J. Immunol. Baltim. Md 1950* 167: 6623–6630.
264. Wells, A. D., X. C. Li, Y. Li, M. C. Walsh, X. X. Zheng, Z. Wu, G. Nuñez, A. Tang, M. Sayegh, W. W. Hancock, T. B. Strom, and L. A. Turka. 1999.

Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat. Med.* 5: 1303–1307.

265. Tubo, N. J., A. J. Pagán, J. J. Taylor, R. W. Nelson, J. L. Linehan, J. M. Ertelt, E. S. Huseby, S. S. Way, and M. K. Jenkins. 2013. Single Naive CD4+ T Cells from a Diverse Repertoire Produce Different Effector Cell Types during Infection. *Cell* 153: 785–796.

266. Appelt, U., A. Sheriff, U. S. Gaipl, J. R. Kalden, R. E. Voll, and M. Herrmann. 2004. Viable, apoptotic and necrotic monocytes expose phosphatidylserine: cooperative binding of the ligand Annexin V to dying but not viable cells and implications for PS-dependent clearance. *Cell Death Differ.* 12: 194–196.

267. Dillon, S. R., M. Mancini, A. Rosen, and M. S. Schlissel. 2000. Annexin V Binds to Viable B Cells and Colocalizes with a Marker of Lipid Rafts upon B Cell Receptor Activation. *J. Immunol.* 164: 1322–1332.

268. Dillon, S. R., A. Constantinescu, and M. S. Schlissel. 2001. Annexin V Binds to Positively Selected B Cells. *J. Immunol.* 166: 58–71.

269. McComb, S., R. Mulligan, and S. Sad. 2010. Caspase-3 Is Transiently Activated without Cell Death during Early Antigen Driven Expansion of CD8+ T Cells In Vivo. *PLoS ONE* 5: e15328.

270. Segawa, K., J. Suzuki, and S. Nagata. 2011. Constitutive exposure of phosphatidylserine on viable cells. *Proc. Natl. Acad. Sci.* 108: 19246–19251.

271. Kurtz, J., J. Shaffer, A. Lie, N. Anosova, G. Benichou, and M. Sykes. 2004. Mechanisms of early peripheral CD4 T-cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic bone marrow transplantation: evidence for anergy and deletion but not regulatory cells. *Blood* 103: 4336–4343.

272. García-Sáez, A. J. 2012. The secrets of the Bcl-2 family. *Cell Death Differ.* 19: 1733–1740.

273. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2: 933–944.

274. Zhou, L., M. M. W. Chong, and D. R. Littman. 2009. Plasticity of CD4+ T Cell Lineage Differentiation. *Immunity* 30: 646–655.

275. Matisz, C. E., J. J. McDougall, K. A. Sharkey, and D. M. McKay. 2011. Helminth Parasites and the Modulation of Joint Inflammation. *J. Parasitol. Res.* 2011.
276. Wang, L. J., Y. Cao, and H. N. Shi. 2008. Helminth infections and intestinal inflammation. *World J. Gastroenterol. WJG* 14: 5125–5132.
277. Weinstock, J. V., R. W. Summers, and D. E. Elliott. 2005. Role of helminths in regulating mucosal inflammation. *Springer Semin. Immunopathol.* 27: 249–271.
278. Whelan, R. A. K., S. Hartmann, and S. Rausch. 2012. Nematode modulation of inflammatory bowel disease. *Protoplasma* 249: 871–886.
279. Zaccone, P., and A. Cooke. 2013. Vaccine against autoimmune disease: can helminths or their products provide a therapy? *Curr. Opin. Immunol.* .
280. Van Maurik, A., B. Fazekas de St Groth, K. J. Wood, and N. D. Jones. 2004. Dependency of direct pathway CD4+ T cells on CD40-CD154 costimulation is determined by nature and microenvironment of primary contact with alloantigen. *J. Immunol. Baltim. Md 1950* 172: 2163–2170.
281. Coulombe, M., and R. G. Gill. 1996. T lymphocyte indifference to extrathymic islet allografts. *J. Immunol. Baltim. Md 1950* 156: 1998–2003.
282. Coulombe, M., and R. G. Gill. 1994. Tolerance induction to cultured islet allografts. I. Characterization of the tolerant state. *Transplantation* 57: 1195–1200.
283. Coulombe, M., H. Yang, L. A. Wolf, and R. G. Gill. 1999. Tolerance to Antigen-Presenting Cell-Depleted Islet Allografts Is CD4 T Cell Dependent. *J. Immunol.* 162: 2503–2510.
284. Donohoe, J. A., L. Andrus, K. M. Bowen, C. Simeonovic, S. J. Prowse, and K. J. Lafferty. 1983. Cultured thyroid allografts induce a state of partial tolerance in adult recipient mice. *Transplantation* 35: 62–67.
285. La Rosa, F. G., D. Smilek, D. W. Talmage, K. J. Lafferty, P. Bauling, and T. J. Ammons. 1992. Evidence that tolerance to cultured thyroid allografts is an active immunological process. Protection of third-party grafts bearing new antigens when associated with tolerogenic antigens. *Transplantation* 53: 903–913.

286. Bingaman, A. W., J. Ha, S.-Y. Waitze, M. M. Durham, H. R. Cho, C. Tucker-Burden, R. Hendrix, S. R. Cowan, T. C. Pearson, and C. P. Larsen. 2000. Vigorous Allograft Rejection in the Absence of Danger. *J. Immunol*. 164: 3065–3071.

287. Chan, W. F., A. Perez-Diez, H. Razavy, and C. C. Anderson. 2007. The ability of natural tolerance to be applied to allogeneic tissue: determinants and limits. *Biol. Direct* 2: 10.

288. Baan, C. C., B. J. van der Mast, M. Klepper, W. M. Mol, A. M. A. Peeters, S. S. Korevaar, A. H. M. M. Balk, and W. Weimar. 2005. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation* 80: 110–117.

289. Blaha, P., S. Bigenzahn, Z. Koporc, M. Schmid, F. Langer, E. Selzer, H. Bergmeister, F. Wrba, J. Kurtz, C. Kiss, E. Roth, F. Muehlbacher, M. Sykes, and T. Wekerle. 2003. The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. *Blood* 101: 2886–2893.

290. Demirkiran, A., V. D. K. D. Sewgobind, J. van der Weijde, A. Kok, C. C. Baan, J. Kwekkeboom, H. W. Tilanus, H. J. Metselaar, and L. J. W. van der Laan. 2009. Conversion From Calcineurin Inhibitor to Mycophenolate Mofetil-Based Immunosuppression Changes the Frequency and Phenotype of CD4+FOXP3+ Regulatory T Cells. *Transplantation* 87: 1062–1068.

291. Noris, M., F. Casiraghi, M. Todeschini, P. Cravedi, D. Cugini, G. Monteferrante, S. Aiello, L. Cassis, E. Gotti, F. Gaspari, D. Cattaneo, N. Perico, and G. Remuzzi. 2007. Regulatory T Cells and T Cell Depletion: Role of Immunosuppressive Drugs. *J. Am. Soc. Nephrol*. 18: 1007–1018.

292. 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. *Am. J. Transplant*. 11: 936–946.

293. Høiby, N., H. Krogh Johansen, C. Moser, Z. Song, O. Ciofu, and A. Kharazmi. 2001. Pseudomonas aeruginosa and the in vitroand in vivo biofilm mode of growth. *Microbes Infect*. 3: 23–35.

294. Song, Z., H. Wu, O. Ciofu, K.-F. Kong, N. Høiby, J. Rygaard, A. Kharazmi, and K. Mathee. 2003. Pseudomonas aeruginosa alginate is refractory to Th1 immune response and impedes host immune clearance in a mouse model of acute lung infection. *J. Med. Microbiol.* 52: 731–740.
295. Pamer, E. G. 2004. Immune responses to Listeria monocytogenes. *Nat. Rev. Immunol.* 4: 812–823.
296. Wang, T., L. Chen, E. Ahmed, L. Ma, D. Yin, P. Zhou, J. Shen, H. Xu, C.-R. Wang$, M.-L. Alegre, and A. S. Chong. 2008. Prevention of allograft tolerance by bacterial infection with Listeria monocytogenes. *J. Immunol. Baltim. Md 1950* 180: 5991–5999.
297. LaRosa, D. F., A. H. Rahman, and L. A. Turka. 2007. The Innate Immune System in Allograft Rejection and Tolerance. *J. Immunol.* 178: 7503–7509.
298. Kendal, A. R., Y. Chen, F. S. Regateiro, J. Ma, E. Adams, S. P. Cobbold, S. Hori, and H. Waldmann. 2011. Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. *J. Exp. Med.* 208: 2043–2053.
299. Alegre, M.-L., and A. S. Chong. 2012. The impact of infection and tissue damage in solid-organ transplantation. *Nat. Rev. Immunol.* 12: 459+.
300. Alegre, M.-L., J. Leemans, A. Le Moine, S. Florquin, V. De Wilde, A. Chong, and M. Goldman. 2008. The Multiple Facets of Toll-Like Receptors in Transplantation Biology. *Transplantation* 86: 1–9.
301. Burns, A. M., and A. S. Chong. 2011. Alloantibodies Prevent the Induction of Transplantation Tolerance by Enhancing Alloreactive T Cell Priming. *J. Immunol.* 186: 214–221.
302. Valujskikh, A., and F. G. Lakkis. 2003. In remembrance of things past: memory T cells and transplant rejection. *Immunol. Rev.* 196: 65–74.
303. Wang, T., E. B. Ahmed, L. Chen, J. Xu, J. Tao, C.-R. Wang, M.-L. Alegre, and A. S. Chong. 2010. Infection with the Intracellular Bacterium, Listeria monocytogenes, Overrides Established Tolerance in a Mouse Cardiac Allograft Model. *Am. J. Transplant.* 10: 1524–1533.

304. Cook, C. H., A. A. Bickerstaff, J.-J. Wang, P. D. Zimmerman, M. R. Forster, T. Nadasdy, R. B. Colvin, G. A. Hadley, and C. G. Orosz. 2009. Disruption of Murine Cardiac Allograft Acceptance by Latent Cytomegalovirus. *Am. J. Transplant*. 9: 42–53.
305. Xie, J. H., N. Nomura, S. L. Koprak, E. J. Quackenbush, M. J. Forrest, and H. Rosen. 2003. Sphingosine-1-Phosphate Receptor Agonism Impairs the Efficiency of the Local Immune Response by Altering Trafficking of Naive and Antigen-Activated CD4+ T Cells. *J. Immunol*. 170: 3662–3670.