Viral Abrogation of Stem Cell Transplantation Tolerance Causes Graft Rejection and Host Death by Different Mechanisms: A Dissertation

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VIRAL ABROGATION OF STEM CELL TRANSPLANTATION TOLERANCE CAUSES GRAFT REJECTION AND HOST DEATH BY DIFFERENT MECHANISMS

A Dissertation Presented

By

Daron Forman

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In partial fulfillment of the requirements for the degree of

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Program in Immunology and Virology
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ABSTRACT

Tolerance-based stem cell transplantation using sub-lethal conditioning is being considered for the treatment of human disease, but safety and efficacy remain to be established. In order to study these two issues, we first established that mouse bone marrow recipients treated with sub-lethal irradiation plus transient blockade of the CD40-CD154 costimulatory pathway develop permanent hematopoietic chimerism across allogeneic barriers. Our conditioning regimen of 6 Gy irradiation, a short course of anti-CD154 mAb and 25 million fully allogeneic BALB/c bone marrow cells consistently produced long-term, stable, and multilineage chimerism in C57BL/6 recipients. Furthermore, chimeric mice displayed donor-specific transplantation tolerance, as BALB/c skin allografts were permanently accepted while third-party CBA/JCr skin allografts were promptly rejected. We next determined both the safety and efficacy of this protocol by infecting chimeric mice with lymphocytic choriomeningitis virus (LCMV) either at the time of transplantation or at several time points afterwards. Infection with LCMV at the time of transplantation prevented engraftment of allogeneic, but not syngeneic, bone marrow in similarly treated mice. Surprisingly, infected allograft recipients also failed to clear the virus and died. Post-mortem study revealed hypoplastic bone marrow and spleens. Hypoplasia and death in these mice required the combination of 6 Gy irradiation, LCMV infection on the day of transplantation, and an allogeneic bone marrow transplant but did not require the presence of anti-CD154 mAb. Allochimeric mice infected with LCMV 15 days after transplantation were able to survive and maintain their bone marrow graft, indicating that the deleterious effects of
LCMV infection on host and graft survival are confined to a narrow window of time during the tolerization and transplantation process. The final section of this thesis studied the mechanisms of graft rejection and death in sublethally irradiated recipients of allogeneic bone marrow and infection with LCMV at the time of bone marrow transplantation. Infection of interferon-α/β receptor knockout mice at the time of transplantation prevented the engraftment of allogeneic bone marrow, but the mice survived. Therefore, IFN-αβ is involved in the development of marrow hypoplasia and death, whereas a second mechanism is involved in blocking the development of chimerism in these mice. Through the use of depleting mAb’s and knockout mice we demonstrate that three types of recipients survived and became chimeric after being given sublethal irradiation, anti-CD154 mAb, an allogeneic bone marrow transplant and a day 0 LCMV infection: mice depleted of CD8+ T cells, CD8 knockout mice, and TCR-αβ knockout mice. Our data indicate that the mediator of bone marrow allograft destruction in LCMV-infected mice treated with costimulatory blockade is a radioresistant CD8+ NK1.1− TCRαβ+ T cell. We conclude that a non-cytopathic viral infection at the time of transplantation can prevent engraftment of allogeneic bone marrow and result in the death of sub-lethally irradiated mice treated with costimulation blockade. The abrogation of allogeneic bone marrow engraftment is mediated by a population of CD8+ NK1.1− TCRαβ+ T cells and the mediator of hypoplasia and death is viral induction of IFN-αβ.
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ABBREVIATIONS

6-MP, 6-mercaptopurine

ADCC, antibody-dependent cell-mediated cytotoxicity

APCs, antigen-presenting cells

AZA, azathioprine

CsA, cyclosporine A

CMV, cytomegalovirus

CTL, cytotoxic T lymphocytes

DCs, dendritic cells

DTH, delayed-type hypersensitivity

DLA, dog leukocyte antigen

DST, donor-specific transfusion

EBV, Epstein-Barr virus

GM-CSF, granulocyte/macrophage colony stimulating factor

GVHD, graft versus host disease

HSV, herpes simplex virus

HLA, human leukocyte antigen

HSC, hematopoietic stem cell

IACUC, Institutional Animal Care and Use Committee
IFN-αβ, interferon-alpha/beta

IFN-αβR, interferon-alpha/beta receptor

i.p., intraperitoneally

i.v., intravenous

LCMV, lymphocytic choriomeningitis virus

MHC, the major histocompatibility complex

MST, median survival time

NK Cells, natural killer cells

PBMC, peripheral blood mononuclear cells

PFU, plaque forming units

s.d., standard deviation

TCR, T cell receptor

TI, thymic irradiation

TBI, total body irradiation

TNF, tumor necrosis factor

TNFR, tumor necrosis factor receptor

VZV, varicella zoster virus

VSV, vesicular stomatitis virus

WBI, whole body irradiation
INTRODUCTION

Human hematopoietic stem cell (HSC) transplantation has required lethal whole body irradiation and immunosuppression. Although these conditioning regimens have permitted stem cell engraftment while successfully lowering the incidence of rejection, there are a number of side effects associated with this therapy. The use of lethal conditioning and chronic immunosuppression leads to a state of severe neutropenia as well as a prolonged suppression of T and B cell function (1). As a result, patients are predisposed to a variety of bacterial, fungal and viral infections as well as neoplasia. Although immunosuppression can prevent acute graft-versus-host disease (GVHD), it cannot prevent chronic GVHD (1). Additionally, the requirement for lethal irradiation restricts this form of transplantation to those patients who suffer from lethal malignancies or hematological diseases. Finally, stem cell transplantation using lethal irradiation typically produces a state of full donor chimerism.

In order to overcome these problems, newer approaches to hematopoietic stem cell transplantation have focused on less toxic approaches, particularly sublethal conditioning regimens that do not rely on the use of chronic immunosuppression. These approaches have also focused on creating mixed hematopoietic chimeras, a state in which both donor and host cells co-exist in the recipient. The presence of a mixed chimera has several advantages over complete donor chimerism including improved immune reactivity (2) and a decrease in the likelihood of GVHD (3). Additionally, the use of sublethal conditioning could allow stem cell transplantation to be used as a realistic option for non-lethal malignancies, autoimmune disease and organ transplantation.
One approach to induce hematopoietic chimerism is the use of costimulation blockade. Costimulation blockade of T cell activation has been found to induce potent donor-specific non-responsiveness and, in the case of hematopoietic stem cell transplantation, facilitates the engraftment and establishment of allogeneic hematopoietic chimerism (4). This approach has been used to successfully establish hematopoietic chimeras in mice, while also significantly reducing the toxicity of the conditioning regimens (4).

Unfortunately, the safety and efficacy of protocols based on costimulation blockade and sublethal conditioning to create hematopoietic chimerism has not yet been well studied, particularly with respect to viral infection. Viral induction of inflammatory cytokines, T cell growth and differentiation factors as well as virus-specific CTL that react to allogeneic targets (5) could potentially compromise graft survival. Further, many viruses can enhance GVHD after bone marrow transplantation (6). Finally, patients treated with partial myeloablation combined with costimulation blockade could be less resistant to viral infection and its associated pathophysiological effects.

The goal of this thesis research was to determine the safety and efficacy of allogeneic hematopoietic stem cell transplantation based on costimulation blockade and sublethal irradiation. In particular, this thesis focused on the ability of LCMV infection to abrogate either the initiation or maintenance of hematopoietic chimerism and donor-specific transplantation tolerance.
1. History of Transplantation

A. Inconspicuous beginnings

Although Sir Peter Medewar is largely credited with establishing the field of modern transplantation biology in the 1960s, legends of successful transplantation can be traced as far back as 800 B.C. It was during this time that the Indian surgeon Susrata described methods for replacing amputated noses, a common punishment for crimes, with skin flaps from the criminals’ cheeks (7,8). Two other famous legends relating to transplantation occurred during the first few centuries. According to Chinese legend, the physician Pien Ch’iao exchanged the hearts of two men with opposite personalities in order to restore the Yin and Yang balance between them (9). A second legend called the “miracle of the black leg”, tells how the Saints Cosmas and Damian replaced the gangrenous leg of the Roman deacon Justinian with one taken from an Ethiopian Moor who had recently died (10). Justinian supposedly woke up and walked away with his healthy black leg the very next day.

In the 16th century Gaspare Tagliocozzi perfected the nose replacement techniques of Susrata. Instead of using cheek skin he typically transplanted skin from patients’ arms to re-create their noses (11). Interestingly, he was the first person to acknowledge the difficulties of transplanting between people and associated this problem to the “biochemical individuality” of people (11).

Many other cases of successful autografts (transplantation from one location on a patient’s body to another location on the same patient) were reported in the 17th and 18th centuries. However, as opposed to the success seen with autografts, transplantation from
one individual of a species to another individual of that species (allografts) universally failed. George Schone reviewed the status of transplantation research in the early 1900s and summarized the information in his "laws of transplantation" (12). These laws stated:

1. Transplantation into a foreign species invariably fails.
2. Transplantation into unrelated members of the same species usually fails.
3. Autografts almost invariably succeed.
4. There is a primary take and then delayed rejection of the first graft into an unrelated member of the same species.
5. There is accelerated rejection of a second graft in a recipient that had previously rejected a graft from the same donor, or of a first graft in a recipient that had been pre-immunized with material from the same donor.
6. The closer the "blood relationship" between donor and recipient, the more likely is graft success.

B. Groundbreaking discoveries

Four discoveries in the first half of the 20th century helped to elucidate the puzzle of allograft rejection and improve the techniques involved in transplantation. In 1900, Landsteiner noted that serum from certain individuals caused agglutination of red blood cells from other individuals (13). Twenty-five years later, this work led to the identification of the ABO blood group system, and the discovery that blood transfusions
within the same blood group succeeded whereas transfusions between different groups failed (14).

A second major discovery was made in 1912 by the French surgeon Alexis Carrel, who pioneered the method of joining blood vessels together, enabling surgeons to perform organ transplantation for the first time (15). The third discovery was made in 1948 by Gorer, Lyman and Snell who described a single dominant locus in mice that was responsible for either the acceptance or rejection of transplanted tissues (16). This locus would eventually be called the major histocompatibility complex (MHC) and is still one of the major determinants of allograft success.

The fourth key discovery was made by Thomas Gibson and Peter Medawar during their cumulative experience using skin allografts to treat burned aviators from World War II. In these studies they noted that autografts typically survived. In contrast, allografts universally failed within 15 days, and a repeat of a failed allograft typically rejected even faster (8 days) (17). Furthermore, they also showed that rejected grafts were invaded by white blood cells, confirming the immunological nature of graft rejection (17). For helping to jumpstart the field of transplantation biology, the Nobel Prize was awarded to Alexis Carrel in 1912, to Karl Landsteiner in 1930 and to Sir Peter Medawar in 1960. Together, these were the pioneers of modern day transplantation.

C. The dawn of immunosuppression

The pioneering work of Thomas Gibson and Peter Medawar showing that allograft rejection was the result of an active immune response led to the use of immunosuppressive drugs in transplantation, in the hope that this immune response could
be suppressed. The first form of immunosuppression was whole body irradiation (18,19). Although this form of immunosuppression led to allograft acceptance in a few cases, including the first successful kidney transplant performed by Murray and Merrill in 1954 (20), the majority of times it was unsuccessful (21). Furthermore, this non-specific form of immunosuppression proved to be highly toxic and was associated with a very high rate of mortality due to overwhelming infection (21).

The first drug to replace whole body irradiation was 6-mercaptopurine (6-MP). This drug, discovered in 1959 by Schwarz and Dameshek, tripled the survival time of skin allografts in rabbits (22). The British surgeon Sir Roy Calne popularized a similar form of immunosuppression, azathioprine (AZA), a precursor of 6-MP. Using AZA, Calne suppressed renal transplant rejection in dogs (23). Although 6-MP and AZA substantially reduced the risk of rejection, they both were associated with toxic side effects including predisposition to infection and anemia, as well as bone marrow suppression (1).

The key breakthrough in immunosuppression came in 1969 when Jean Borel discovered a fungus (Beauveria nivea) in soil samples from Wisconsin and the Hardanger Vidda fjord in Norway. Several years later he discovered the immunosuppressive properties of the metabolite produced by this fungus, cyclosporine A (CsA). More importantly, he discovered that CsA did not produce the severe side effects that were associated with 6-MP or AZA (24). In 1978, CsA was tested for the first time on humans. In a small cohort of 7 kidney transplant recipients, 5 patients successfully accepted their organ (25).
CsA was a major improvement over all other immunosuppressive drugs for several reasons. Previous immunosuppressants, such as 6-MP and AZA, suppress all immune cells, resulting in the inability of the immune system to mount a response to virtually any type of infection. CsA, on the other hand, selectively suppresses only T cells, leaving all other immune cells intact and capable of responding to infection. Second, unlike 6-MP and AZA, CsA is not associated with severe bone marrow suppression and anemia. The use of CsA dramatically improved 1-year survival rates of transplanted organs such as kidney, heart, liver and pancreas from 50-60% to over 80% (26,27). Unfortunately, it was eventually discovered that CsA had its own concerning side effects, including renal dysfunction, tremor and hypertension (28).

Since the discovery of CsA, many other immunosuppressive drugs have been identified. This long list includes a monoclonal antibody directed against CD3 (OKT3) (29), tacrolimus (FK-506) (30), rapamycin, mycophenolate mofetil and leflunomide (27,31). The use of CsA and newer immunosuppressive drugs has greatly improved graft survival, but major side effects still exist. Current immunosuppressive agents specifically target only T cells, instead of all immune cells, but they still induce a generalized immunosuppression that predisposes patients to infections and malignancies. The statistics associated with long-term use of immunosuppression is alarming: 10-45% of these patients develop a neoplasm after 10 years and 40-75% after 20 years (32,33). A second major concern of current immunosuppressive therapies is the lack of long-term organ survival due to the onset of chronic rejection. Whereas almost all kidney transplants are fully functional after 1 year, only 20% remain functional 10 years after
transplantation (34). Finally, immunosuppression is unable to prevent the recurrence of tissue-specific disease or autoimmunity that might damage the newly transplanted organ (35).

D. A future without immunosuppression

Currently, researchers are seeking alternatives to immunosuppression that will prevent both short-term and long-term graft rejection while remaining as safe as possible for the recipient. Many laboratories have focused on the induction of transplantation tolerance as a way to overcome the deficiencies associated with generalized immunosuppression. Transplantation tolerance is a state of non-responsiveness to one specific donor while all other immune responses remain intact. As the discovery of CsA reduced the target of immunosuppression from all immune cells to just T cells, transplantation tolerance would reduce the target of therapy from all T cells to just those T cells that recognize and destroy the allograft. In theory, this would leave all other T cells intact, considerably reducing the risks of infection and neoplasia for transplant recipients.
2. Transplantation Immunology

A. Immune response to allografts

In order to prevent the rejection of an allograft without globally blocking the immune response, one must first understand the nature of the immune system’s response to an allograft. Transplantation of an organ from one individual to another invokes many different facets of the immune response, and therefore requires knowledge of the various stages of the response, as well as of the different cell types that are involved.

The first host response to a newly transplanted allograft involves inflammation resulting from the transplantation process and the trauma associated with it. Removal of the allograft from the donor and transplantation into the host causes the induction of cytokines such as IL-1 and IL-6 and increased expression of adhesion molecules on the graft’s endothelium (36, 37). These two events combine to cause an early infiltration of the transplant by inflammatory cells, including macrophages (38). These early events occur in both syngeneic transplants (between identical members of the same species), as well as in allogeneic transplants (between different members of the same species), and typically do not result in graft rejection (39).

The next stage of the immune response to an allograft is the presentation of foreign antigen to host T cells. The single most important alloantigen presented to T cells is the major histocompatibility complex (MHC) (40). The presentation of this alloantigen can occur in one of two distinct ways. Donor antigen presenting cells (APCs), such as dendritic cells (DCs) can present donor MHC directly to host T cells in what is called direct antigen presentation. Conversely, allogeneic MHC antigens can be processed by
host APCs and presented in association with host-MHC in what is called indirect antigen presentation.

B. Mechanisms of graft rejection

Following the presentation of foreign antigen, T cells become activated, proliferate and initiate the rejection of the transplanted allograft through various humoral and cellular effector mechanisms. B cells can produce antibodies to the alloantigen and initiate antibody-dependent cell-mediated cytotoxicity (ADCC) (41) or they can bind complement and initiate complement-mediated lysis of the targeted graft (42). Natural killer (NK) cells can damage the allograft by the release of perforin or granzymes (43). Finally, activated T cells can cause graft rejection through a variety of effector mechanisms. T cells can activate macrophages to initiate delayed-type hypersensitivity (DTH) reactions or damage allografts through the release of reactive nitrogen, oxygen intermediates, and TNF-α (44). Alternatively, T cells can become cytotoxic T lymphocytes (CTL) and destroy targeted cells through the release of either TNF-α, perforin or granzyme, or via the activation of the Fas-FasL pathway (45).

Although several different effector mechanisms are involved in allograft rejection, it is believed that T cells play the most important role. This is supported by the discovery that both mice and rats that lack T cells are unable to reject allografts (46,47). It is for this reason that many laboratories are focusing on understanding T cell activation in hopes of being able to prevent allograft rejection as specifically as possible.
3. T Cell Activation

A. T cell activation: the two-signal hypothesis

Based on a two-signal model of B cell activation proposed by Bretscher and Cohn (48), Lafferty et al. proposed a similar two-signal model for the activation of T cells. According to his proposal, T cells require the interaction of their T cell receptor (TCR) with a specific antigen-MHC complex presented on antigen presenting cells (Signal 1) along with a different signal (Signal 2) provided by APCs (49). Supporting this model, it was found that T cells stimulated with Signal 1 alone did not become activated. Instead they became anergic, characterized by a state of unresponsiveness (50).

Currently, it is believed that Signal 2 (costimulation) is given by the interactions of CD154 with CD40 and by the interaction of CD80 and/or CD86 with CD28. Many additional molecules are able to modulate T cell activation and provide costimulation, but the costimulatory signals provided by these molecules appear to be less potent than those provided by the interaction of CD40 with CD154 and CD80/86 with CD28. These additional accessory molecules include a member of the integrin family (LFA-1 (51)), a member of the CD2 subfamily (CDw150 also called SLAM (52)), and members of the tumor necrosis factor receptor (TNFR) family (CD137 (53), CD134 (54), and CD27 (55)). Although these molecules do play a role in T cell activation they are considered minor players compared to the CD40-CD154 and CD80/CD86-CD28 pathways.

B. Costimulation: Role of CD40-CD154 interactions

CD40 is a type-1 integral membrane protein and a member of the TNFR superfamily (56). The protein has a molecular weight of 43-50 kDa, and is expressed on
many cell types including T and B cells, basophils, eosinophils, monocytes/macrophages, dendritic cells, epithelial and endothelial cells, smooth muscle cells, keratinocytes and fibroblasts (57). CD40 is constitutively expressed on all cell types, but its expression can be modified by various cytokines in both a positive and negative fashion. IL-1, IL-3, IL-4, TNF-α and granulocyte/macrophage colony stimulating factor (GM-CSF) all enhance the expression of CD40 (58-60), while TGF-β inhibits CD40 expression (61). The up-regulated expression of CD40 takes 6-12 hours to occur, peaks at 24 hours and is maintained on the cell surface for up to 72 hours (57).

CD154, the ligand for CD40, is a type-2 transmembrane protein with a molecular weight of ~39 kDa and is a member of the TNF gene superfamily (56). CD154 is expressed on a number of different cell types, many of which express both the ligand and its receptor. The expression of CD154 has been found on activated B and T cells, basophils, eosinophils, monocytes/macrophages, NK cells, platelets, mast cells, dendritic cells, endothelial cells, epithelial cells, and smooth muscle cells (57). CD154 expression, as opposed to its receptor, is non-constitutive. Expression can be induced by various mitogens (ionomycin, the combination of phytohemagglutinin along with concanavalin A, and PMA (62)), by several different proinflammatory cytokines (IL-1, TNF-α and IL-4) (63-65), or by CD40 ligation (66). The expression of CD154 on the cell surface is typically transient. In T cells, CD154 is expressed 5 minutes after activation (67), peaks at 6 hours and steadily declines to undetectable levels by 12-24 hours following activation (68).
The CD40-CD154 pathway was first observed to be critical for the development of humoral immunity to T cell-dependent antigen responses (69,70). This pathway has now been shown to be critical for the induction of many functions in both humoral and cellular immunity. Blocking the CD40-CD154 pathway prevents primary and secondary immune responses to T cell dependent antigens, the maturation of B cell memory, germinal center formation and immunoglobulin class switching (70,71). The CD40-CD154 pathway is also critical for the release of many pro-inflammatory cytokines (IL-1, IL-2, IL-4, IL-8, IL-10, IL-12, TNF-α and TGF-β), chemokines (IL-8, MIP-1α, MIP-1β, RANTES and MCF-1) and the upregulation of adhesion molecules (LFA-α, ICAM-1, VCAM-1, E-Selectin and VLA-4) (57).

C. Costimulation: Role of CD80/86-CD28/CTLA4 interactions

The second major set of molecules that provide costimulation for T cells are the ligands CD80 (B7-1) and CD86 (B7-2) and their receptors CD28 and CTLA-4 (72). CD80 and CD86 are members of the immunoglobulin gene superfamily. CD80/86 expression can be up-regulated on monocytes, dendritic cells, langerhans cells, B cells and T cells after activation by mitogens, MHC class II ligation, cell surface Ig cross-linking, the ligation of CD40 or a variety of cytokines (73,74). In addition, CD86 expression is also constitutive on resting monocytes and dendritic cells (74).

CD28, an Ig superfamily glycoprotein, is constitutively expressed on both resting and activated T cells, and its expression does not fluctuate significantly after T cell activation (75). CD28 ligation induces several changes in the T cell that stimulate both the magnitude and duration of the T cell response. These changes include upregulated
expression of the anti-apoptotic gene BCLXL and increased production of cytokines, such as IL-2 (76). However, CD28 signaling is not necessary for all T cell responses, as CD28 knockout mice can clear an LCMV infection and reject skin allografts (77-79).

A second receptor for the ligands CD80 and CD86 is CTLA-4 (72). CTLA-4 is a glycoprotein in the CD28 family, but has several different features than CD28. CTLA-4, unlike CD28, is not expressed constitutively but rather is upregulated after CD28 ligation (80). Once expressed, CTLA-4 has a much higher affinity and avidity for its ligands CD80 and CD86 than CD28 (81). Finally, whereas CD28 ligation gives a positive signal for T cell activation, CTLA-4 ligation appears to give a negative signal to the T cell and inhibits the synthesis of IL-2 (82). The role of CTLA-4 as a negative regulator of T cell activation is further supported by the finding that CTLA-4 deficient mice develop a fatal lymphoproliferative disorder (82).

CD80 and CD86 are the only two ligands known to interact with the two receptors CD28 and CTLA-4, and vice versa. Recently however, other novel members of both the B7 and CD28 families have been discovered. These include the receptors ICOS and PD-1 and their respective ligands B7H and B7H1 (83,84). Similar to CD28 and CTLA-4, ICOS has been shown to give a positive signal for T cell activation, while PD-1 inhibits T cell activation (72). Although these new ligands and receptors appear to be involved in T cell activation and inhibition, their exact roles are still being elucidated.

D. Redefining T cell activation: a three step process

Based on our current understanding of T cell activation, we now believe that there are 3 required steps for complete T cell activation (35). Initially, the TCR interacts with
its cognate antigen-MHC complex on the APC (Step 1). This interaction induces the upregulation of CD154 on the T cell surface (68). The newly expressed CD154 then interacts with its constitutively expressed receptor on the APC, CD40. The completion of the CD40-CD154 interaction (Step 2) induces B7-1 (CD80) and B7-2 (CD86) expression on the APC (85). CD80 and CD86 bind to the constitutively expressed CD28 receptor on the T cell (Step 3) allowing the T cell to become fully activated. Forty-eight to seventy-two hours after T cell activation, CTLA-4 becomes expressed on the T cell surface, which may represent a means of down regulating T cell activation (86). A schematic of these 3 interactions necessary for complete T cell activation is shown in Figure 1.
Figure 1: Summary of T cell activation
Legend to Figure 1: This figure schematically depicts the 3 steps of T cell activation. Specifically, it shows the activation of an alloreactive T cell by an APC. The first step of activation is the interaction of the TCR with the antigen/MHC complex on the APC. This interaction induces the expression of CD154 on the surface of the T cell. Newly expressed CD154 interacts with constitutively expressed CD40 found on the APC. The interaction of CD40 with CD154 (Step 2) induces the upregulation of B7-1 (CD80) and B7-2 (CD86) expression on the APC. Finally, CD80 and CD86 bind to the constitutively expressed CD28 molecules on the T cell (Step 3) allowing the alloreactive T cell to become fully activated.
4. Immunological Tolerance

A. Definition of tolerance

In order to overcome the deficiencies associated with generalized immunosuppression many laboratories have focused on the induction of transplantation tolerance. Transplantation tolerance is a state of non-responsiveness to one specific donor while all other immune responses remain intact. In theory, transplantation tolerance is the least toxic method for successful transplantation, as all other T cells will remain unaffected, thereby reducing the risks of infection and neoplasia associated with immunosuppression.

Although tolerance is defined as a state of non-responsiveness to antigen in the absence of immunosuppression, it can be further broken down into two distinct definitions, functional tolerance and immunological tolerance. Functional tolerance is based solely on graft outcome, and is defined as a graft that survives without the need for immunosuppression. In contrast, immunological tolerance is based solely on the immune response, and is defined as the lack of an immune response to a graft in the absence of immunosuppression (35). The distinction between these two definitions of tolerance was created because some forms of tolerance endure even in the presence of immune reactivity against the graft (87). Furthermore, the maintenance of tolerance is sometimes associated with graft infiltrates (88). In this thesis we use the functional definition of tolerance, as we focus on graft survival.
B. Discovery of central and peripheral tolerance

Owen first described tolerance in 1945, performing skin allografts on Freemartin cattle twins who share a placenta during gestation. He discovered that allografts between freemartin cattle twins survived without the use of immunosuppression, whereas skin allografts between non-twins failed (89). He hypothesized that tolerance was the result of alloantigen exposure to the immune system during early development (89).

These observations were extended by the work of Billingham and Burnet. Billingham observed that a state of tolerance could be experimentally induced when allogeneic cells were injected into neonatal recipients (90). Similarly, Burnet observed that neonatal mice infected with lymphocytic choriomeningitis virus (LCMV) became persistently infected with the virus (91). These observations led Burnet to develop a theory of clonal selection to describe how the immune system distinguishes self from non-self. His theory proposed that self-reactive cells were deleted in central lymphoid organs, leaving all non-self reactive cells intact (91). It is now known that the deletion of self-reactive T cells, called negative selection, takes place in the thymus. A second process in the thymus, called positive selection, selects for T cells that recognize foreign antigen in the context of self-MHC.

Burnet’s theory of clonal deletion is accurate, but does not explain the complete story of tolerance. Some antigens may never be expressed in the thymus, and therefore negative selection to these antigens would never occur. Furthermore, self-reactive cells have been detected in peripheral tissues, suggesting that some self-reactive cells can avoid the process of negative selection (92). In order for these self-reactive cells to be
controlled, the immune system has developed a second form of tolerance that takes place in the periphery, called peripheral tolerance.
5. Peripheral Tolerance

A. Mechanisms of induction and maintenance

Four major mechanisms have been proposed to maintain peripheral tolerance: anergy, ignorance, suppression and deletion. Anergy results from T cells receiving Signal 1 (TCR-MHC/Ag) in the absence of Signal 2 (costimulation) (50). Anergy is characterized by a state of unresponsiveness and is associated with decreased IL-2 production (93) and the down-regulation of TCR surface expression (94,95). This unresponsive state can usually be broken by the addition of exogenous IL-2 (96).

A second mechanism proposed to maintain peripheral tolerance is ignorance (97). Ignorance is characterized by a state of unresponsiveness, but, unlike anergy, these T cells are still capable of proliferation when incubated with their respective antigens ex vivo. It has been suggested that ignorance occurs because certain T cells are unable to contact their specific antigen due to an inability to migrate into the specific area where it is being presented (98).

A third form of peripheral tolerance, suppression, has been used to explain the presence of alloreactive T cells (99) and cellular infiltrates (100) that are sometimes found in healthy grafts. Further support for suppression comes from the observation that T cells from a tolerized animal can induce a similar state of unresponsiveness when adoptively transferred into untreated recipients, also known as infectious tolerance (101).

A fourth form of maintaining peripheral tolerance is deletion. Support for deletion comes from the observation that antigen-specific T cells are deleted following exposure to high doses of alloantigen (102), viral antigens (103) or superantigens (104). One
suggested mechanism accounting for deletion is the production of cytokines, as IL-4 and IL-10 were implicated in the deletion of allo-reactive cells in one study (105). A second mechanism that may be involved is the Fas-FasL pathway. Using transgenic mice specific for pigeon cytochrome C, Singer and Abbas observed that antigen-induced peripheral deletion occurred in wild-type mice but not in Fas\textsuperscript{lp}r/ Fas\textsuperscript{lp}r mice that expressed a defective Fas gene (106).

B. Methods of peripheral tolerance induction in vivo

To induce peripheral transplantation tolerance, many experimental protocols focus on the interference of T cell activation. These protocols typically involve blocking the interactions of molecules involved in Signal 1 (107) or Signal 2. Strategies that interfere with Signal 1, the interaction of TCR with the MHC-peptide complex, include a mAb specific for CD3 (OKT3) (108), or antibodies that mask MHC class I or II (109). Other strategies have focused on blocking costimulation (Signal 2). These strategies focus on blocking the various costimulatory interactions, including CD80/CD86 with CD28 and CTLA-4, CD40 with CD154, and LFA-1 with ICAM-1 (110-114).

C. Peripheral tolerance induced by DST and anti-CD154 mAb

Our laboratory has focused on the interaction of CD40 with CD154 to induce peripheral transplantation tolerance. Our approach, as diagrammed in Figure 2, uses a two-element protocol involving the combination of a single injection of allogeneic splenocytes as the donor-specific transfusion (DST) and a short course of anti-CD154 mAb. We originally hypothesized that this protocol led to the exposure of alloantigen to
Figure 2. Induction of Transplantation Tolerance: A two-element protocol

Anti-CD154 mAb (0.5 mg/dose, i.p.)

Day -7  Day -4  Day 0  Day +3

DST: BALB/c Splenocytes
BALB/c Skin Allograft

(i.v.)
Legend to Figure 2: This figure depicts our laboratory's two-element protocol to induce donor-specific peripheral tolerance. C57BL/6 recipient mice receive a single i.v. injection of BALB/c donor splenocytes on day -7 (7 days prior to skin transplantation) along with four (0.25 mg) injections of anti-CD154 mAb given on days -7, -3, 0 and +3 relative to receiving a BALB/c skin graft on day 0.
host T cells under conditions where CD154 could not interact with its receptor, CD40. The presence of Signal 1 in the absence of Signal 2 would render allo-specific T cells specific for cells and tissues that express the donor alloantigen anergic, while all other T cells would remain unaffected (115).

Initially, our laboratory's protocol was tested using pancreatic islet allografts (116). The combinatorial treatment of DST and anti-CD154 mAb induced permanent islet allograft survival in 96% of chemically induced diabetic mice (116). As a more rigorous test of transplantation tolerance, we next tested whether our two-element protocol could induce transplantation tolerance to skin allografts (115), as skin allografts represent a robust test of tolerance induction. The combination of DST and anti-CD154 mAb prolonged the survival of murine skin allografts to a median survival time of ~50 days, with graft survival reaching >100 days in 20% of treated mice (115).

The combination of a short treatment period along with a simple and minimal conditioning regimen led us to believe that this protocol could be translatable to the clinic. However, although our two-element protocol extended the survival of skin allografts, rejection still occurred roughly 50 days after treatment (115). In contrast, when thymectomized mice were used as recipients, permanent skin allograft survival was observed (117). This suggested that DST and anti-CD154 mAb might induce transplantation tolerance through the deletion of pre-existing peripheral alloreactive T cells. These results also suggested that newly exported alloreactive T cells from the thymus might be the cause of delayed skin allograft rejection in tolerized mice.
In order to test these hypotheses, we set up a Jenkins-like model system (118, 119). This assay involves the adoptive transfer of a small population of transgenic T cells into syngeneic non-transgenic hosts. This transfusion permits low level engraftment of the tracer transgenic population in a wild-type host unaltered by transgene expression.

Our modified version of the Jenkins assay involved the injection of a small tracer population of transgenic T cells (KB5 CD8+ T cells specific for H2b) into syngeneic wild-type hosts (CBA/JCr). The engraftment of KB5+CD8+ T cells, as well as their fate, was monitored with the use of a clonotypic mAb (DES) that recognizes specifically the transgenic CD8+ T cells. Following successful engraftment, mice were treated with our two-element protocol of DST (C57BL/6 (H2b) splenocytes) and anti-CD154 mAb and subsequently given a donor-specific skin allograft (C57BL/6). Confirming our previous hypotheses, we observed the deletion of KB5+CD8+ T cells 2-3 days following treatment with DST and anti-CD154 mAb (120).

In total, these results suggest that treatment of mice with DST and anti-CD154 mAb induce peripheral transplantation tolerance through the deletion of circulating alloreactive CD8+ T cells. Unfortunately, the results of our thymectomy study point out a major problem with inducing donor-specific peripheral tolerance through deletion. Namely, deleted cells can eventually be replaced with newly exported alloreactive T cells. Likewise, the induction of peripheral donor-specific tolerance through anergy, suppression or ignorance may also fail to permanently tolerize recipients due to cross-reactivity of T cells or virally induced proinflammatory stimuli (121). Because of these concerns, our laboratory searched for other means of inducing donor-specific tolerance
that would lead to permanent tolerance to all allografts in euthymic recipients. One attractive possibility was the induction of donor-specific central tolerance, and in particular, the creation of a hematopoietic chimera through successful bone marrow transplantation.
6. Central Tolerance

The injection of antigens directly into the thymus (122) and the creation of hematopoietic chimeras (123) are two of the most common means of inducing central transplantation tolerance. The focus of this thesis work is the induction of central tolerance by bone marrow transplantation. Therefore, the focus of this section will be on the induction of central tolerance by bone marrow transplantation.

A. History of bone marrow transplantation

The field of bone marrow transplantation can be traced as far back as 1951. In a landmark experiment, Lorenz et al. observed that mice given a normally lethal dose of irradiation survived if given bone marrow cell infusions immediately after irradiation (124). Four years later, Main and Prehn observed that successful bone marrow transplantation resulted in donor-specific tolerance. In their experiments, skin grafts permanently survived in lethally irradiated mice given bone marrow transplants when skin grafts were from the same donor as the bone marrow (125).

Based on these early studies in animals, E. Donnall Thomas and colleagues performed the first successful bone marrow transplant in humans in 1959 (126). His patients were two pairs of identical twins. In each pair, one of the twins suffered from acute lymphocytic leukemia. After treatment with irradiation and a bone marrow graft from the genetically identical healthy twin, both patients showed significant restoration in bone marrow function. Unfortunately, both patients had a recurrence in their leukemia and died within a few months (126).
The experiments of Thomas and colleagues led to great optimism for the use of bone marrow transplantation to successfully treat leukemia. Unfortunately, the vast majority of bone marrow transplants were unsuccessful unless bone marrow from an identical twin donor was used (126). During the 1950s and early 1960s over 200 allogeneic bone marrow transplants were performed in humans, without a single success (127).

Although the human equivalent of the mouse MHC (HLA-Human leukocyte antigen) was discovered in 1958 (128,129), it wasn’t until the late 1960s before the importance of the HLA system in bone marrow transplantation was fully understood. In a seminal experiment performed with dogs, bone marrow transplants between DLA-matched (dog leukocyte antigen) littermates were successful, while DLA-unmatched transplants failed (130). Based on these studies, the first human HLA-compatible allogeneic bone marrow transplants were successfully performed in 1968 and 1969 on patients with various immune deficiency diseases (131-133).

Although some allogeneic bone marrow transplants were successful, the majority of bone marrow transplants failed. The one exception to this rule was immunocompromised patients (134). As was true with organ transplantation, a greater understanding of the immune response to bone marrow transplantation was necessary before successful allogeneic bone marrow transplantation would be feasible.

B. Bone marrow transplantation: induction and maintenance

The use of bone marrow transplantation is the most popular means to induce central transplantation tolerance. The various stages of bone marrow transplantation that
occur in order to achieve tolerance are shown in Figure 3. When donor hematopoietic stem cells are injected into a recipient, they are capable of engrafting into the bone marrow and co-existing with host stem cells (135). If engraftment occurs, both donor and host stem cells mature and produce cells of all hematopoietic lineages. Additionally, certain bone marrow-derived cells called thymic dendritic cells travel to the thymus and engraft. These cells are particularly good mediators of intrathymic clonal deletion (136). As bone marrow-derived thymocytes are produced they travel to the thymus to mature and undergo positive and negative selection. Positive selection keeps thymocytes that recognize foreign antigens in the context of self-MHC alive, whereas negative selection deletes T cells that recognize self-antigens. The combination of these two processes ensure that only T cells that express self-MHC and recognize foreign antigens will survive and be exported into the periphery. The majority of cells (>95%) are not selected for and die in the thymus via apoptosis (121). After successful bone marrow engraftment, both donor and host thymic dendritic cells are present in the thymus, and as a result, both donor and host antigens are presented as self-antigens to maturing T cells. As a result of negative selection, both host-reactive and donor-reactive T cells are deleted in the thymus, ensuring a permanent state of donor-specific tolerance.

In order to achieve this state of donor-specific tolerance, successful bone marrow transplantation requires that two major issues be resolved. These issues include creating "space" to allow donor bone marrow to engraft and expand, and preventing the initial rejection of donor bone marrow.
Figure 3. Induction of central tolerance by bone marrow transplantation

Hematopoietic stem cells

Engraftment of cells in thymus

T cells tolerant to donor

Engraftment of stem cells into host bone marrow

Host

Donor
**Legend to Figure 3:** This figure depicts the induction of central transplantation tolerance by bone marrow transplantation. After injection, hematopoietic cells engraft into the bone marrow and coexist with host hematopoietic cells. Donor hematopoietic cells mature in the marrow and produce erythrocytes, B cells, granulocytes, and other hematopoietic derived cells. Immature donor thymocytes travel from the marrow to the thymus and engraft, where they help mediate negative selection of donor-reactive T cells and ensure permanent central transplantation tolerance is maintained. This schematic is a modified version of a figure originally published by M. Sykes (Transplantation. 68(4):459-467, 1999)
In order for donor bone marrow to engraft, it is believed that "space" needs to be created in the bone marrow compartment. This concept is supported by experiments in which 1.5-3.0 Gy of irradiation was necessary to produce long-term syngeneic bone marrow engraftment in C57BL/6 mice (137). Others have been able to obtain long-term bone marrow engraftment with high doses of donor bone marrow without the use of irradiation (138-142). As opposed to the idea that "space" needs to be created, these results suggest that host and donor bone marrow may compete for space to expand. This concept is supported by studies of allogeneic bone marrow transplantation in autoimmune mice, where the engraftment of donor allogeneic bone marrow was able to "out-compete" host autoimmune stem cells (143).

The second concern in bone marrow transplantation is preventing host T cells from rejecting donor bone marrow. In order to suppress these donor-reactive T cells, the use of lethal whole body irradiation was originally used. Lethal whole body irradiation would be desirable for treating hematological disease and leukemias, as it would also eliminate the tumor. In all other cases, however, the severe side effects and possibility of lethality due to unsuccessful engraftment make lethal whole body irradiation clinically unfeasible. In the attempt to create a less toxic and therefore more clinically relevant protocol for bone marrow transplantation, many laboratories are developing sub-lethal or non-myeloablative approaches to induce bone marrow engraftment. The various protocols that have been devised to induce allogeneic bone marrow engraftment under sublethal or non-myeloablative conditions, in both humans and mice, are discussed next.
C. Inducing central transplantation tolerance in humans

Currently, over 18,000 bone marrow transplants are performed in the United States each year and 40,000 worldwide (144). Bone marrow transplantation is used to treat aplastic anemia, immunodeficiency syndromes, congenital disorders of metabolism, and neoplastic diseases (144). Typical conditioning regimens for human bone marrow transplantation involve the use of total body irradiation (TBI) in either single or multiple doses. Total body irradiation is used to prevent graft rejection, to create "space" in the bone marrow compartment, and to treat the underlying malignancy (145). The alkylating agent busulfan is also used as a substitute for TBI (146). Along with TBI or busulfan, a myelosuppressive and/or immunosuppressive drug, such as cyclophosphamide, is typically added to the conditioning regimen to reduce the incidence of disease relapse (147). The combination of TBI or busulfan along with cyclophosphamide has decreased the incidence of graft rejection to <1% (146). In one published study following patients who underwent bone marrow transplantation for chronic myeloid leukemia, 10-year survival rates were 63-65% using either of these two conditioning regimens (148).

Current protocols have effectively decreased the occurrence of graft rejection and disease relapse in bone marrow transplant recipients. Unfortunately, these conditioning regimens are associated with many debilitating side effects; which include veno-occlusive disease, GVHD, and a high incidence of bacterial, fungal, and viral infections.

Veno-occlusive disease (VOD) is the third leading source of transplant-related mortality in allogeneic bone marrow transplant recipients (149). The disease results from loose connective tissue blocking intrahepatic veins in the liver (150). VOD manifests
within 20 days of transplantation (151) and causes unexplained weight gain, jaundice, and pain in the upper half of the body (152). VOD affects as many as 54% of bone marrow transplant recipients (153), with a reported mortality rate ranging as low as 3% and as high as 67% (150). Both chemotherapy and radiotherapy have been associated with VOD, especially the use of busulfan (154).

The second leading cause of death for bone marrow transplant recipients is GVHD (149). GVHD results from passenger T cells in donor bone marrow recognizing host antigens as foreign and initiating an immune response against them (152). By definition GVHD is broken down into either acute (occurs within 100 days of transplantation) or chronic forms (occurs after 100 days of transplantation).

Acute GVHD involves injury to the epithelial tissues of the liver, gastrointestinal tract and skin (155). This disease affects more than 30% of bone marrow transplant recipients, with a mortality rate of ~20% (152,156,157). Incidence of acute GVHD is associated with HLA disparity, and can reach as high as 80% in those patients who receive bone marrow from an unrelated donor (158).

Chronic GVHD manifests clinically as an autoimmune-like disease, typically affecting the skin, eyes, liver, intestines and esophagus (155). Incidence rates for chronic GVHD are greater than 30%, with a rate as high as 60% for patients who receive bone marrow from unrelated donors (159). Mortality rates for chronic GVHD, as in acute GVHD, approach 20% (156). Chronic GVHD is more common in older patients, patients who have already experienced acute GVHD, and in recipients of unrelated bone marrow (160).
The two most common treatments for preventing GVHD are the use of T cell depleted donor bone marrow and the use of immunosuppressive drugs such as CsA (152). T cell depletion of donor bone marrow correlates with much lower incidences of GVHD than cyclosporine (10% vs. 30-40% with cyclosporine) (145). However, it is associated with higher rates of graft failure (109) and increased rates of disease relapse (161). The existence of a facilitating CD8$^+$ T cell for stem cell engraftment in mice has been reported (162) and may be the cause of decreased engraftment using T cell depleted bone marrow.

The number one cause of mortality and morbidity in allogeneic bone marrow transplant recipients is infection (149). Infection can result from an infected organ or blood product, re-emergence of a latent viral infection, or as a result of radiation-induced damage to the mucosa or the prolonged neutropenia that follows radiotherapy (144). Clinicians use three separate risk periods for the infection of bone marrow transplant recipients: the pre-engraftment period, the early post-engraftment period and the late post-engraftment period (163).

The pre-engraftment period covers the first month after bone marrow transplantation. This time period is associated with severe neutropenia resulting from the use of irradiation or myeloablative drugs (144). Infections during this period include bacterial (Gram-negative and Gram-positive bacteria), fungal (Candida and Aspergillus), and viral (herpes simplex virus (HSV)) (164-166). Bacterial and fungal infections typically lead to neutropenic fever, although if untreated, infection can lead to organ failure and even death (144).
The early engraftment period covers the time period between 30 and 100 days after bone marrow transplantation. During this time period neutrophil numbers are recovering, but T and B cell functions are still suppressed and remain so for up to 18 months (163). Bacterial and viral infections are less frequent, but still occur in patients who receive unrelated bone marrow or are suffering from GVHD (167). Common viral infections during this period include cytomegalovirus (CMV), HSV, adenovirus and BK virus.

The third risk period for bone marrow transplant recipients is the late post-engraftment period. This period begins at 100 days after transplantation and terminates 18-36 months later, as patients discontinue the use of immunosuppressive drugs (163). Even at this late time point, cellular and humoral immunity remains suppressed, especially for those patients suffering from GVHD or recipients of unrelated donor bone marrow (144). As a result, patients are prone to viral infections such as varicella zoster virus (VZV), CMV and Epstein-Barr virus (EBV) (163).

Together, the incidence of VOD, GVHD and infection (fungal, bacterial and viral) poses a serious risk of morbidity and mortality to bone marrow transplant recipients. As a result, a new wave of bone marrow transplant conditioning regimens have recently been initiated (168-172). These less toxic regimens, dubbed “mini-transplants”, focus on sublethal or non-myeloablative conditioning (168). Mini-transplants hope to minimize post-transplant neutropenia, thereby lowering the high incidence of VOD and infection associated with bone marrow transplantation. Of equal importance, lowering the toxicity required for bone marrow transplantation would allow this therapy to be a viable option
for elderly patients, as well as patients with nonmalignant diseases (173). Another exciting application of sublethal mini-transplants is the possibility of simultaneous bone marrow and organ transplantation. In theory, donor-specific tolerance induced by bone marrow transplantation would eliminate the need for lifelong immunosuppression in solid organ transplantation. In one preliminary study the simultaneous transplantation of bone marrow and kidney was able to successfully cure a patient with multiple myeloma (174).

Mini-transplants have the potential to revolutionize the field of bone marrow transplantation (173). However, the ability of these new protocols to reduce the rates of infection and GVHD are still relatively unknown, as are the affects on long term survival and disease relapse. In one study looking at 12 patients who had undergone non-myeloablative peripheral stem cell transplantation, 17% of patients became infected with bacteria and 42% with CMV, suggesting that infection still poses a significant problem, at least with this particular regimen (175).

Before going in depth on our particular conditioning regimen, sublethal protocols for bone marrow transplantation in mice will be reviewed with an emphasis on those that utilize costimulation blockade.

D. Inducing central transplantation tolerance in mice: the future

For over 10 years, researchers have successfully transplanted allogeneic bone marrow in mice without the need for lethal whole body irradiation (176). These protocols, for the most part, are based on combinatorial regimens that include low levels of myeloablative drugs or sublethal irradiation in order to create “space” in the recipient marrow. Additionally, these regimens include either T cell depleting mAbs, thymic
irradiation (TI), antibodies that block costimulation (anti-CD80, anti-CD86, anti-CD154 and CTLA4-Ig), CsA, or a combination of these various reagents to prevent allograft rejection (4,177-185).

One of the original sublethal conditioning regimens consisted of depleting doses of anti-CD4 and anti-CD8 mAbs, along with 3 Gy of whole body irradiation (WBI) and 7 Gy of thymic irradiation (TI) (176). Further modification of this protocol showed that TI could be replaced by either additional injections of anti-CD4 and anti-CD8 mAbs (177), or by a single injection of CTLA4-Ig or anti-CD154 mAb (186). The use of anti-CD154 mAb led to a higher level and rate of chimerism than mice treated with CTLA4-Ig. Combined treatment of these reagents showed no improvement over treatment with anti-CD154 mAb alone.

All of these variations consistently induced mixed hematopoietic chimerism, as treated mice contained hematopoietic cells derived from both donor and host stem cells. Furthermore, donor-specific tolerance was established. Chimeric mice permanently accepted donor-specific skin grafts and rejected third party grafts. Although these sublethal regimens consistently led to hematopoietic chimerism and donor-specific tolerance, the use of anti-CD4 and anti-CD8 mAbs caused prolonged T cell depletion in recipient mice.

In contrast to the experiments above, some researchers have demonstrated that the combination of CTLA4-Ig and anti-CD154 mAb is better than the use of either reagent alone. Salam et al. have observed that either CTLA4-Ig or anti-CD154 mAb alone was insufficient for the engraftment of CBA/J bone marrow into BALB/c mice treated with 3
Gy (185). In contrast, the combination of both reagents allowed successful bone marrow engraftment at 3 Gy (185). Unfortunately, very low numbers of mice were used in this study (groups of 5-6 mice), and only 40% of mice became chimeric. Increasing the dose of CTLA4-Ig and anti-CD154 mAb, however, could increase this rate to 67%.

Taylor et al. have observed that irradiation requirements can be decreased when both the amount of bone marrow injected and the length of anti-CD154 mAb treatment are increased (184). Amazingly, chimerism was obtained with only 1 Gy of TBI when 40 million BALB/c bone marrow cells and a two-week course of anti-CD154 mAb were used (184). Although chimerism levels were relatively low (24%), they could be increased to 48% when 2 Gy of irradiation was used.

Another successful sublethal conditioning regimen utilized the injection of splenocytes and anti-CD154 mAb ten days prior to bone marrow transplantation (181). The pre-exposure of alloreactive T cells to donor antigen (splenocytes) and anti-CD154 mAb, has been suggested to lead to the deletion of alloreactive T cells prior to bone marrow transplantation (120). As a result of this deletion prior to transplantation, only 1 Gy of irradiation was required for stem cell engraftment.

The injection of donor splenocytes is also involved in a protocol utilizing splenocytes that have been irradiated and rendered apoptotic. Using 7 Gy of irradiation along with the injection of apoptotic splenocytes (187), stable and long-term engraftment of bone marrow was observed. Interestingly, the origin of apoptotic splenocytes was not restricted to the same origin as the bone marrow as third party or even human xenogeneic splenocytes were capable of facilitating bone marrow engraftment (187).
Recently, approaches have utilized the combination of anti-CD154 mAb and CTLA4-Ig to eliminate irradiation altogether. These protocols utilize immunosuppressive or myeloablative reagents such as sirolimus in combination with anti-lymphocyte serum (188) or busulfan (183) as a replacement for sublethal irradiation. Although irradiation is eliminated from these conditioning regimens, the use of myeloablative drugs or T cell depleting reagents may lead to a prolonged state of immune suppression similar to that of sublethal irradiation.

Conditioning regimens that can induce allogeneic bone marrow engraftment without the use of irradiation, immunosuppression or myeloablative drugs have recently been described (179). Wekerle et al. have obtained hematopoietic chimerism in C57BL/6 recipients with a single injection of 200 million B10.A bone marrow cells combined with anti-CD154 mAb and CTLA4-Ig (179,182). This protocol is by far the least toxic of all the protocols described, but the exceptionally high dose of bone marrow that was necessary for engraftment (200 million cells) is unlikely to be available from human bone marrow donors (189). A second drawback is the close genetic similarities between C57BL/6 and B10.A mice, which are similar in many of their non-MHC background genes, a situation not found in the clinic unless donors are closely related family members.

A second conditioning regimen that induces bone marrow engraftment in the absence of irradiation or myeloablation has been described by Durham et al (182). This protocol involves 8 injections of allogeneic bone marrow and anti-CD154 mAb over a 90-day period. This protocol induces chimerism in the majority of mice, but the levels of
chimerism are extremely low (average of 6% donor-derived cells in PBMC). Furthermore, the multiple injections over an extended time frame, would be a hindrance in converting a similar protocol to human clinical trials.

These sublethal/non-myeloablative protocols have been initiated with the hope of being less toxic to the recipient. Despite this, very little work has been performed to study the safety and efficacy of these new sublethal approaches, especially with respect to infection. Moreover, many of these protocols depend on the use of T cell depleting reagents, which may result in a prolonged deficiency of T cells. As T cells typically clear viral infections, the use of T cell-depleting antibodies could substantially increase the risks of viral infection.

The central focus of this thesis work was to understand the implications of viral infection during and after bone marrow transplantation. We therefore sought to determine the effect of viral infection on 1) the engraftment of allogeneic bone marrow, 2) the induction of donor-specific tolerance and 3) the ability of the host to clear the viral infection. To do so, we created a sublethal conditioning regimen that induced hematopoietic chimerism, based on costimulation blockade and sublethal irradiation.

E. Central tolerance induced by bone marrow, sublethal irradiation and anti-CD154 mAb

Our laboratory’s initial attempts to induce hematopoietic chimerism utilized sublethal irradiation and anti-CD154 mAb (190). This protocol involved two 0.5 mg injections of anti-CD154 mAb on days 0 (the day of bone marrow transplantation) and on day +3. On day 0, recipients received 4 Gy of irradiation followed by a single injection of twenty five million C57BL/6 allogeneic bone marrow cells (190). This protocol
successfully induced stable and long-term chimerism in all recipient mice, with >99% of the PBMC of donor-origin. All chimeric mice demonstrated donor-specific tolerance by accepting donor-origin skin allografts and promptly rejecting third-party skin allografts. Furthermore, no clinical evidence of GVHD was detected in any of the chimeric mice.

Interestingly, chimeric mice in these initial studies contained >99% donor-origin cells in their PBMC. The creation of a full chimera has several disadvantages over the creation of a mixed chimera including impaired immunocompetence (2,191) and an increase in the likelihood of GVHD (3,192). Based on these observations, one of the initial goals of this thesis was to develop a protocol using sublethal irradiation and anti-CD154 mAb capable of producing a state of mixed chimerism.

The second goal of this thesis was to determine whether viral infection could interfere with the induction or maintenance of hematopoietic chimerism and donor-specific tolerance. The species combination that we chose to use was BALB/c mice as donors and C57BL/6 mice as recipients. We chose this combination initially, as the converse donor-recipient combination led to the creation of full donor-origin chimeras. Furthermore, the use of C57BL/6 mice as recipients provides two major advantages. The first is the availability of various targeted mutations in C57BL/6 mice, allowing us to dissect the role of various cell populations and cytokines. Secondly, the immune response of C57BL/6 mice to LCMV infection is extremely well characterized.
7. LCMV

A. Introduction

LCMV has been intensely studied for over 65 years and is the prototype member of the arenaviridae family of viruses (193). The name Arenaviridae is derived from the Latin word *arenosus*, meaning sandy, due to the presence of host ribosomes inside the virion that appear as electron-dense granules when viewed in the electron microscope (194). This family of viruses contains Old World viruses derived from Africa and New World viruses derived mostly from South America. LCMV, Lassa, Mopeia and Mobala are examples of Old World viruses, whereas Junin, Machupo, Tacaribe, and Pichinde are members of New World viruses.

Several different strains of LCMV have been isolated including Armstrong, Traub and WE strains. Armstrong and Lillie first described the Armstrong strain of LCMV in 1934. After inoculating both monkeys and mice with cerebrospinal fluid from a patient diagnosed with St. Louis encephalitis, they observed the development of lymphocytic choriomeningitis (193). The Traub strain was discovered to produce a similar choriomeningitis in colonies of Swiss mice (195). The WE strain was discovered in 1936, when an employee at the Rockefeller University died from an apparent hemorrhagic fever after being exposed to a mouse infected with the Traub strain (196). Minor differences exist between strains, especially with regards to the site of replication. The Armstrong strain replicates mostly in the spleen and other lymphoid tissues, while the WE strain can also replicate in internal organs such as the liver and lung (197).
B. Immune Response to LCMV

A typical LCMV infection in mice results in the activation of many components of the immune system. Early after infection there is a potent induction of type 1 IFN (αβ) that peaks at day 2 post-infection (198). The release of IFN-αβ helps fight off viral infection by the production of 2'-5' oligoadenylate synthetase and RNAse L, which degrade viral mRNA, and the induction of the protein kinase PKR, which blocks the initiation of viral RNA translation (199). Type 1 IFN also induces the activation and proliferation of NK cells, monocytes/macrophages and cytotoxic T cells as well as the induction of MHC class 1 antigens (199-201).

During the first four days of infection, LCMV also induces the activation and accumulation of NK cells (201,202). Interestingly, depletion of NK cells does not increase LCMV viral titers, suggesting that NK cells play only a minor role in LCMV clearance (203). This may be related to the ability of type 1 IFN to upregulate the levels of class 1 MHC expression on virally infected cells, as NK cells are capable of killing cells that downregulate self MHC I expression (204,205).

During the early stage of LCMV infection, clonal expansion of T cells also begins. CD8+ T cells expand to a greater extent than CD4+ T cells, resulting in the change of CD4:CD8 T cell ratios from 2:1, to as much as 1:3 by day 8-9 post-infection (206,207). LCMV-specific CD8+ CTL become detectable by day 8-9 post-infection and are very proficient at clearing LCMV (197). LCMV-specific CTL lyse LCMV-infected targets through the release of perforin (208,209). These CTL develop without the need for any one particular adhesion molecule, costimulatory molecule or cytokine, as LCMV-
specific CTL are generated in knockout mice that lack CD4+ T cells, NK cells, B cells, CD2, CD54, CD11a, CD154, IL-2, IL-4, or IFN-γ (210-218).

At the peak of the T cell response to LCMV (day 8-9), numerous activated B cells and macrophages are also detectable (219,220). Mice depleted of B cells are able to clear an LCMV infection, indicating that B cells play a minor role in LCMV clearance (211). On the other hand, depletion of marginal zone macrophages led to an attenuated CTL response and viral persistence, indicating that macrophages play some role in the clearance of LCMV (221).

LCMV titers decrease 5 days after infection as LCMV-specific CD4 and CD8 T cells increase in numbers (197). Due mostly to the action of LCMV-specific CD8+ CTL, viral titers and antigen rapidly decline. Once LCMV is cleared, activated T cells undergo cell death, and T cell numbers and CD4:CD8 T cell ratios return to normal (222,223).

C. Three Outcomes of LCMV Infection

Three outcomes of LCMV infection can occur depending on the route of infection and the age of infected mice. Mice infected with LCMV either in utero or shortly after birth are unable to clear an LCMV infection, and as a result, become persistent carriers of the virus (224,225). Intracranial infection is associated with a fatal meningoencephalitis, which occurs 5-8 days after infection due to a massive infiltration of CD8+ T cells into the brain (226). In normal healthy adult mice, LCMV is a relatively noncytopathic virus (when infection occurs through a non-intracranial route). These mice are able to clear an LCMV infection within 7-10 days and thereafter maintain permanent immunity (227).
Viral persistence, however, can occur in immunocompromised mice and is thought to be the result of clonal exhaustion (103). According to this concept, there is a period of time allowed for the immune system to clear the virus in order to safeguard the host from virus-induced pathology. Healthy mice clear LCMV within this time frame. However, when viral replication is extremely high, the immune system may be unable to clear the virus in time, resulting in clonal exhaustion, the elimination of the T cell response and persistent viremia (197).

D. Safety and Efficacy: LCMV and Bone Marrow Transplantation

As mentioned above, viral infection is a major cause of human mortality and morbidity following allogeneic bone marrow transplantation (149). Viral infections have been implicated in allograft rejection, the enhancement of GVHD, and the loss of graft function (6,228-230).

Viral infections have the potential to disrupt bone marrow transplantation through several mechanisms. One mechanism is the induction of IFN-γ, a cytokine potently induced by LCMV and many other viruses (202). IFN-γ can directly suppress bone marrow growth (231). IFN- γ can also induce the activation and proliferation of NK cells, which are capable of rejecting allogeneic bone marrow grafts (202,232-234).

A second mechanism is the ability of viruses to induce T cell growth and differentiation factors. The increase in CD8+ T cell numbers over CD4+ T cells could have dramatic effects on either the induction or maintenance of tolerance. We have previously shown that CD4+ T cells are necessary for both the initiation and maintenance of tolerance induced by our two-element protocol of DST and anti-CD154 mAb (117).
Additionally, the production of cytokines such as IL-2 by the expanding T cell population could potentially break a state of tolerance through bystander activation (98,235).

A third mechanism is the ability of many viruses to induce virus-specific CTL that can lyse uninfected allogeneic targets (5,236,237). These cross-reactive CTL have the potential to lyse uninfected donor bone marrow cells, thus preventing bone marrow engraftment.

With respect to allogeneic bone marrow transplantation using costimulation blockade, there is one additional concern of viral infection in regard to safety and efficacy. Tolerance induction protocols based on costimulation blockade are based on the exposure of alloantigen under conditions where T cells receive signal 1 (TCR/MHC-Ag) without signal 2 (costimulation). The inability to receive signal 2 leads to the inactivation of alloreactive T cells and loss of donor-specific immunity. Similarly, a viral infection at the time of tolerance induction may induce tolerance to viral antigens, preventing an immune response against the invading virus.

Recently, we have tested whether acute LCMV infection can interfere with the induction of transplantation tolerance induced by our two-element protocol of DST and anti-CD154 mAb (238). Interestingly, the timing of LCMV infection relative to skin grafting was the major determinant of skin allograft survival. Combined treatment of DST and anti-CD154 mAb in uninfected thymectomized C57BL/6 recipients greatly enhanced the survival of donor skin allografts (MST >308 days). In contrast, similarly treated mice that were infected with LCMV 1 day after transplantation uniformly rejected their grafts (MST=26 days). Delaying LCMV infection had a beneficial effect on skin
allograft survival, as infection on day 15 (MST=78 days) and day 29 (MST=104 days) after transplantation showed progressively longer skin allograft survival. Mice infected with LCMV 51-57 days after transplantation had skin allograft survival (MST >237 days) similar to controls (MST >251 days) (238). Therefore, in this model of transplantation tolerance, LCMV was able to prevent both the initiation and maintenance of tolerance.

Based on these observations, we were concerned that an acute LCMV infection would interfere with the induction and maintenance of hematopoietic chimerism and transplantation tolerance induced by sublethal conditioning, costimulation blockade and allogeneic bone marrow. In order to examine the threat of viral infection, this thesis work had two initial goals. The first goal was to create a conditioning regimen capable of producing mixed allogeneic hematopoietic chimerism using sublethal irradiation and costimulation blockade. Our second goal was to determine whether LCMV infection could interfere with either the induction or maintenance of hematopoietic chimerism and donor-specific tolerance.
METHODS

1. Animals

Female C57BL/6 (H2<sup>b</sup>, Ly5.2), C57BL/6-Ly5.1, CBA/JCR (H2<sup>b</sup>) and BALB/c (H2<sup>d</sup>) mice were obtained from the National Cancer Institute, Frederick, MD. 129/Sv wild-type (H2<sup>b</sup>) and SV129 interferon-α/β receptor knockout mice (216) were obtained from a colony maintained at the University of Massachusetts Medical School. C57BL/6 mice in which the CD4, CD8, TCR-αβ or TCR-γδ gene was disrupted by homologous recombination were obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, LDH elevating virus, mouse poliovirus, Reo-3 virus, mouse adenovirus, LCMV, polyoma, *Mycoplasma pulmonis*, and *Encephalitozoon cuniculi*. All animals were housed in an SPF facility in microisolator cages and given *ad libitum* access to autoclaved food and acidified water. They were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School and recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

2. Antibodies and Flow Cytometry

Clone MR1 hamster anti-mouse CD154 mAb (70) was produced as ascites in *scid* mice and purified by Protein A affinity chromatography (Amersham Pharmacia Biotech)
AB, Uppsala, Sweden). Antibody concentration was determined by measurement of optical density and confirmed by ELISA as described previously (239). The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC) and was uniformly <10 units EU per mg of mAb. Mice received two 0.5 mg injections given intraperitoneally (i.p.). The first injection was made on the day of bone marrow transplantation (day 0) and the second injection was given 3 days later (day +3). Depleting antibodies were used according to the following schedules:

- Anti-NK1.1 mAb (PK-136 (240)) 1 mg i.p. on days -8, -1, +6;
- anti-CD122 mAb (TM-β1 (241-243)) 1 mg i.p. on day -1;
- anti-asialo-GM1 (ascites) 100 ul i.p. on days -1, +4;
- anti-CD4 mAb (GK1.5) and anti-CD8 mAb (2.43) 0.5 mg i.p. on days -3, -2 and -1.

FITC-conjugated anti-H2-K\textsuperscript{b} (AF6-88.5) and anti-Ly5.2 (104) mAbs, PE-conjugated anti-H2-K\textsuperscript{d} (SF1-1.1), anti-Ly5.1 (A20), anti-CD4 (L3T4), anti-CD8\textalpha{} (53-6.7), anti-TCR β chain (H7-597), anti-CD45R/B220 (RA3-6B2), anti-CD11b/Mac1 (M1/70), anti-NK1.1 (PK136) and anti-GR1 (RB6-8C5) mAbs, and biotinylated anti-H2-K\textsuperscript{d} (SF1-1.1) and anti-Ly5.1 (A20) mAbs were all obtained from Pharmingen (San Diego, CA). Flow cytometry was performed as described (190). Briefly, single cell suspensions were labeled with antibody, rinsed, washed, fixed in 1% paraformaldehyde, and analyzed on a FACScan\textsuperscript{®} (Becton Dickinson, Sunnyvale, CA). Forward angle and side scatter properties were used to distinguish lymphocytes, monocytes and granulocytes. Dead cells and erythrocytes were excluded by electronic gating. At least $10^4$ events were analyzed for each sample. The relative percentages of host- and donor-origin cells in the C57BL/6 (H2-K\textsuperscript{b}, Ly5.2) recipients of BALB/c (H2-K\textsuperscript{d}) or C57BL/6-
Ly5.1 (Ly5.1) bone marrow were determined by flow cytometry. In preliminary experiments, known mixtures of donor and host peripheral blood mononuclear cells were analyzed, and it was determined that the lower limit of sensitivity of the assay for detecting either donor (H2-K\textsuperscript{d} or Ly5.1) or host (H2-K\textsuperscript{b} or Ly5.2) cells was 0.5%. Because not all hematopoietic cells express MHC class I antigen, the relative percentage of donor-origin cells in chimeric mice recipients was calculated as follows: [\% donor cells ÷ (\% donor cells + \% host cells)] x 100 (190).

3. Cell Preparation and Bone Marrow Transplantation

Recipient mice were treated with 6 Gy whole body irradiation using a \textsuperscript{137}Cs source (Gammacell 40, Atomic Energy of Canada, Ottawa, ON, Canada). This dose was documented in preliminary experiments to be non-lethal for C57BL/6 mice. Within 1-3 hours of irradiation all recipients received a single intravenous (i.v.) injection of 18-25 x 10\textsuperscript{6} donor bone marrow cells in a volume of 0.5 ml via the lateral tail vein. Donor femora and tibiae (female BALB/c or C57BL/6-Ly5.1 mice at least 6 weeks of age) were flushed with RPMI medium using a syringe with a 24-gauge needle. Recovered cells were filtered through sterile nylon mesh (70 \(\mu\)m, Becton Dickinson, Franklin Lakes, NJ), counted, and re-suspended in RPMI. Recipient mice were females at least 6 weeks of age. To assess levels of chimerism, blood samples were obtained from mice that had received donor bone marrow 2 to 4 weeks earlier. Additional blood samples were obtained periodically as described in the Results. Hematopoietic chimerism was defined as the presence of >1.0% MHC class \(I^+\) donor-origin cells in the blood.
Single cell suspensions of spleen cells were filtered through sterile nylon mesh (70 μm), centrifuged, and erythrocytes were lysed with hypotonic NH₄Cl. The cells were then resuspended in RPMI and counted in the presence of crystal violet using a hemocytometer.

4. LCMV Infection and Assay for Infectious Units of LCMV

Mice were inoculated i.p. with 5 x 10⁴ plaque forming units (PFU) of LCMV, strain Armstrong, propagated in baby hamster kidney cells (238). Mice were inoculated with LCMV immediately after bone marrow injection, or 2 or 7 weeks post-transplantation as described in the text.

LCMV viral titers were measured by LCMV viral plaque assays (244). Results are expressed as geometric mean titers, i.e. the arithmetic mean of the log₁₀ values.

5. Circulating Levels of IFN-αβ

NCTC-929 cells were incubated overnight in 96-well plates with 2-fold dilutions of peripheral blood. These mixtures were then infected with vesicular stomatitis virus (VSV), incubated for 3 days and examined in order to determine the last dilution of peripheral blood that prevented VSV-induced cytotoxicity. Estimates of units/mL were based on the addition of known quantities of purified IFN-αβ to NCTC-929 cells, prior to infection with VSV.

6. Skin Transplantation

Full thickness skin grafts ~1 cm in diameter were obtained from shaved donors, scraped to remove muscle, and grafted without suturing onto prepared sites on the flanks of anesthetized recipients. Skin grafts were dressed with Vaseline®-impregnated gauze
and an adhesive bandage for the first 7 days after surgery. Thereafter, skin grafts were assessed 3 times weekly, and rejection was defined as the first day on which the entire graft surface appeared necrotic (245). Because we have previously determined that fully allogeneic skin grafts placed on untreated recipients typically survive for 10-12 days (246), grafts that were adherent to the bandage or fully necrotic on day 7 were deemed technical failures and were excluded from analysis.

7. Histology

Samples of transplanted skin, host skin, small intestine, large intestine, femur, spleen, and liver were recovered from selected experimental mice, fixed and stored in 10% buffered formalin, embedded in paraffin and processed for light microscopy. Sections for routine light microscopy were stained with hematoxylin and eosin. A qualified pathologist, who was unaware of the treatment status of specimen donors, performed histological analyses.

8. Statistical Analysis

Parametric data are presented as the arithmetic mean ± one standard deviation. Average duration of graft survival is presented as the median survival time (MST). Graft survival among groups was compared using the method of Kaplan and Meier (247); the equality of allograft survival distributions for animals in different treatment groups was tested using the log rank statistic (248). P values <0.05 were considered statistically significant. Comparisons of proportions in 2 x 2 tables used the Fisher exact statistic (249).
CHAPTER I

SUBLETHAL IRRADIATION AND ANTI-CD154 ANTIBODY LEADS TO LONG-TERM AND STABLE MIXED HEMATOPOIETIC CHIMERISM AND DONOR-SPECIFIC TRANSPLANTATION TOLERANCE

INTRODUCTION CHAPTER I

Human hematopoietic stem cell (HSC) transplantation has required lethal whole body irradiation and immunosuppression. These conditioning regimens have permitted stem cell engraftment and lowered the incidence of rejection, but there are a number of severe side effects associated with this therapy. These negative side effects include the induction of severe neutropenia that leads to a high rate of infections, the need for chronic immunosuppression, the failure of this chronic immunosuppression to prevent chronic GVHD, and the high incidence of neoplasia due to the chronic immunosuppression (1). These side effects restrict this therapy to patients who suffer from lethal malignancies or hematological diseases. Furthermore, stem cell transplantation using lethal irradiation typically produces a state of full donor chimerism, which is associated with decreased immune reactivity (2) and an increase in GVHD (3) compared to a state of mixed chimerism.

In order to overcome these problems, newer approaches to hematopoietic stem cell transplantation have focused on less toxic approaches, particularly sublethal conditioning regimens that do not rely on the use of chronic immunosuppression. These conditioning regimens hope to reduce the length and severity of neutropenia associated
with stem cell transplantation, thereby reducing the risk of infection. The lack of need for chronic immunosuppression would also decrease the risk of developing neoplasia. Another goal of sublethal conditioning regimens is to allow this therapy to be used for non-lethal malignancies, autoimmune disease and organ transplantation.

One approach to induce hematopoietic chimerism is the use of costimulation blockade. Costimulation blockade of T cell activation has been found to facilitate the engraftment and establishment of allogeneic hematopoietic chimerism (4). This approach has been used to successfully establish mixed hematopoietic chimeras in mice, while also significantly reducing the toxicity of the conditioning regimens (4). The goal of this chapter is to utilize costimulation blockade and sublethal irradiation in order to achieve stable mixed hematopoietic chimerism in the absence of GVHD and with minimal conditioning to the host.
RESULTS CHAPTER I

1. Stable engraftment of BALB/c bone marrow into C57BL/6 recipients requires 6 Gy of irradiation

We first determined how much irradiation was necessary to induce allogeneic hematopoietic chimerism in C57BL/6 recipient mice. Mice were injected intravenously with a single dose of 18-25 million allogeneic BALB/c bone marrow cells immediately after receiving 3-7 Gy of irradiation, as depicted in Figure 4. Experimental mice were next split into two groups, one of which received two 0.5 mg injections of anti-CD154 mAb. The first of these injections was given on day 0, the day of transplantation, whereas the second injection was given on day +3, three days after bone marrow transplantation. The second group of experimental mice received no further treatment. The amount of bone marrow cells used in these experiments as well as the timing and dose of anti-CD154 mAb was previously optimized in our laboratory (190). Flow cytometry was performed at 2-4 weeks on peripheral blood mononuclear cells (PBMC) in order to determine the level of donor and host-derived cells using MHC class I markers (H2\textsuperscript{b} vs. H2\textsuperscript{d}).

The results, as shown in Table 1, document that at least 5 Gy of irradiation was necessary for allogeneic bone marrow engraftment. Mice that received 3-5 Gy of irradiation contained undetectable levels of donor-origin cells in their PBMC, irregardless of whether they received anti-CD154 mAb or not. The one exception was a single chimeric mouse (28% donor-derived cells in PBMC) in the group treated with 5 Gy and anti-CD154 mAb. Mice that received 6 Gy of irradiation required anti-CD154 mAb for
engraftment. The majority of mice (67%, N=9) treated with anti-CD154 mAb became chimeric with an average of 68% donor-origin cells in their PBMC, whereas none of the 5 mice that did not receive anti-CD154 mAb became chimeric. Finally, all mice that received 7 Gy of irradiation (N=5) became chimeric regardless of whether or not they received anti-CD154 mAb. However, mice treated with 7 Gy without anti-CD154 mAb expressed low levels of chimerism (27%) which disappeared within 7 weeks of transplantation. In contrast, mice that received 7 Gy and anti-CD154 mAb contained much higher levels of donor-origin cells (90%) and chimerism was stable throughout the 7 weeks of observation. Given that our goal was to minimize the conditioning of our mice while maintaining a high percentage of chimeric mice, all future experiments were conducted with 6 Gy of irradiation and anti-CD154 mAb.
Figure 4: Induction of Bone Marrow Chimeras Using $\alpha$-CD154 mAb

Anti-CD154 monoclonal Ab (0.5 mg/dose, i.p.)(2 doses)

Days 0 → 3

C57BL/6

3-7 Gy irradiation

Bone marrow cells ($25 \times 10^6$) i.v.

Allo-chimeric C57BL/6 mouse

BALB/c
Legend to Figure 4: This figure schematically depicts our protocol for bone marrow transplantation using sublethal irradiation and costimulation blockade. C57BL/6 mice were injected intravenously with a single dose of 18-25 million allogeneic BALB/c bone marrow cells immediately after receiving 3-7 Gy of irradiation. Experimental mice also received two 0.5 mg injections of anti-CD154 mAb. The first of these injections was given on day 0, the day of transplantation, and the second injection was given on day +3, three days after bone marrow transplantation.
Table 1. Percentage of donor-origin peripheral blood mononuclear cells in C57BL/6 recipients of allogeneic BALB/c bone marrow and 3-7 Gy of irradiation with or without anti-CD154 mAb

<table>
<thead>
<tr>
<th>Irradiation (Gy)</th>
<th>Anti-CD154 mAb</th>
<th>Frequency of Chimerism at 2-4 wk</th>
<th>Percentage of Donor-Origin PBMC in Chimeric Mice at 2-4 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Gy</td>
<td>No</td>
<td>0/5</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>3 Gy</td>
<td>Yes</td>
<td>0/5</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>4 Gy</td>
<td>No</td>
<td>0/5</td>
<td>&lt;1%</td>
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</tr>
<tr>
<td>5 Gy</td>
<td>No</td>
<td>0/5</td>
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</tr>
<tr>
<td>5 Gy</td>
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<td>1/5</td>
<td>28%</td>
</tr>
<tr>
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<td>7 Gy</td>
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<td>27±37%</td>
</tr>
<tr>
<td>7 Gy</td>
<td>Yes</td>
<td>5/5</td>
<td>90 ±5%</td>
</tr>
</tbody>
</table>
Legend to Table 1: C57BL/6 mice were irradiated with 3-7 Gy. Within 1-3 hours of irradiation all recipients received a single intravenous injection of 18-25 x 10^6 BALB/c donor bone marrow cells in a volume of 0.5 ml via the lateral tail vein. As indicated, anti-CD154 mAb (0.5 mg) was injected intraperitoneally on the day of irradiation and on day +3. The percentage of H2d donor-origin peripheral blood mononuclear cells (PBMC) was determined by flow cytometry 2-4 weeks after irradiation. Chimerism was defined as the presence of >1% MHC class I^+ donor-origin cells two weeks after transplantation. Only mice that remained chimeric were used to determine the percentage of donor-origin cells. Mice that contained undetectable levels of donor-derived cells are listed as having <1%, as the lowest level of chimerism detectable by our system was 0.5%. Each data point represents the arithmetic mean ± 1 standard deviation (s.d.).
2. Stable and long-term engraftment of BALB/c bone marrow into C57BL/6 recipients treated with 6 Gy requires anti-CD154 mAb

We next sought to determine the durability of hematopoietic chimerism induced by 6 Gy of irradiation, and whether the engraftment of allogeneic bone marrow differed from syngeneic bone marrow at this irradiation dose. C57BL/6 (H2b) mice received 6 Gy of irradiation and were injected intravenously with 18-25 million bone marrow cells from either syngeneic C57BL/6-Ly5.1 or fully allogeneic BALB/c donors. These mice were then split into groups that either received two 0.5 mg injections of anti-CD154 mAb or received no further treatment.

As shown in Table 2, the addition of anti-CD154 mAb treatment was not required for the generation of syngeneic hematopoietic chimerism and had no effect on the percentage of syngeneic donor-origin cells that engrafted. In contrast, mice that received allogeneic BALB/c bone marrow cells plus 6 Gy of irradiation once again failed to become chimeric in the absence of anti-CD154 mAb. However, when two 0.5 mg doses of anti-CD154 mAb were added to the conditioning regimen, the majority (9 of 14 recipient mice) developed mixed hematopoietic chimerism 2 weeks after transplantation.

In order to determine both the stability and duration of chimerism in these mice, flow cytometry was performed at intervals up to 27 weeks after transplantation. Two allochimeric mice lost chimerism within 7 weeks of transplantation and 2 additional allochimeric mice were removed from the study for histological analysis. In the remaining 5 allochimeric mice, the percentage of donor-origin cells remained stable and high throughout the 27 weeks of observation. All 15 of the mice (100%) that received
syngeneic bone marrow, regardless of whether they received anti-CD154 mAb or not, became chimeric and remained chimeric throughout the 27 week period of observation.
Table 2. Percentage of donor-origin PBMC in C57BL/6 recipients of syngeneic or allogeneic bone marrow and 6 Gy of irradiation with or without anti-CD154 mAb

<table>
<thead>
<tr>
<th>Bone Marrow</th>
<th>Anti-CD154</th>
<th>Frequency of Chimerism on Day 14</th>
<th>Percentage of Donor-Origin Peripheral Blood Mononuclear Cells in Chimeric Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 14</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>No</td>
<td>5/5</td>
<td>50±27%</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>Yes</td>
<td>10/10</td>
<td>84±4%</td>
</tr>
<tr>
<td>BALB/c</td>
<td>No</td>
<td>0/14</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Yes</td>
<td>9/14</td>
<td>84±12% (N=9) (^a)</td>
</tr>
</tbody>
</table>
Legend to Table 2: C57BL/6 mice were irradiated with 6 Gy. Within 1-3 hours of irradiation all recipients received a single intravenous injection of $18-25 \times 10^6$ donor bone marrow cells in a volume of 0.5 ml via the lateral tail vein. Anti-CD154 mAb (0.5 mg) was injected intraperitoneally on the day of irradiation and on day +3. The percentage of H2^d or Ly5.1 donor-origin PBMC was determined by flow cytometry 2-9 weeks after irradiation. Chimerism was defined as the presence of $>1.0\%$ MHC class I^+ donor-origin cells two weeks after transplantation. All mice with $<1.0\%$ donor-origin cells at the two week time point had $<1.0\%$ donor cells at all other time points. Only mice that remained chimeric were used to determine the percentage of donor-origin cells. Each data point represents the arithmetic mean $\pm$ 1 s.d. In cases where mice were removed from the experiment for histological analysis, the number of animals tested at each time point is given in parentheses. a: one mouse was killed at this time point for histology; b: 2 mice were no longer chimeric at this time point, and one additional chimeric mouse was killed at this time point for histology.
3. Blockade of the CD140-CD154 pathway prevents GVHD in allogeneic hematopoietic chimeras

Graft versus host disease (GVHD) is a problematic side effect associated with bone marrow transplantation. In order to determine whether our protocol of sublethal irradiation and anti-CD154 mAb treatment prevented the development of GVHD, we electively killed 1 synchimeric mouse 7 weeks post transplantation and 2 allochimeric mice 2 and 7 weeks post transplantation. Histological examination of skin, liver, as well as the small and large intestine in all 3 of these mice revealed no evidence of GVHD. Furthermore, there was no histological evidence of GVHD at the time mice were electively killed, up to 340 days after bone marrow transplantation.
4. Allogeneic and syngeneic hematopoietic chimeras display multi-lineage chimerism

The 5 stable allochimeric mice shown in Table 2 were also analyzed by flow cytometry to determine the phenotype of donor cells that exist in the PBMC of our chimeric mice. To do so, the percentage of donor-origin T cells, B cells, macrophages, and granulocytes present in their peripheral blood was determined. As shown in Figure 5A, donor-origin cells representing each of these 4 lineages were present in allochimeric C57BL/6 recipients throughout the 9-week period of observation. The percentage of donor-origin T and B cells rose over time, whereas the percentage of donor-origin granulocytes was maximal 2 weeks after transplantation and declined thereafter. As expected, donor-origin cells representing all 4 lineages were also present in syngeneic chimeric recipients (Figure 5B) and exhibited the same temporal changes in percentage as their allogeneic counterparts.
Figure 5. Multi-lineage analysis of hematopoietic chimeras

a. Allogeneic Chimeras

b. Syngeneic Chimeras
**Legend to Figure 5:** The 5 stable allochimeric mice and 10 synchimeric mice shown in Table 2 (lines 4 and 2 respectively) were analyzed by flow cytometry at 2, 4, 7 and 9 weeks post transplantation, as described in Methods. The percentage of donor-origin T cells, B cells, macrophages, and granulocytes present in their peripheral blood was determined by using appropriate fluorescent-labeled markers. Each data point represents the mean ± 1 s.d.
5. Donor-specific skin grafts survive indefinitely on allogeneic and syngeneic mixed chimeras

Having generated stable and long-term mixed hematopoietic chimerism in the absence of GVHD, we next determined whether donor-specific tolerance had been induced in our chimeric mice. To do so, a total of 20 C57BL/6 mice received BALB/c skin grafts 8 to 17 weeks after transplantation of BALB/c bone marrow. Among these bone marrow recipients, 9 had been conditioned with sub-lethal irradiation without anti-CD154 mAb treatment and were non-chimeric. The median survival time (MST) of donor skin grafts in these non-chimeric mice was short (Figure 6B, MST=12 d, range 10-12 d). The remaining 11 mice had been conditioned with sub-lethal irradiation, as well as anti-CD154 mAb as described above, and at the time of skin grafting all were chimeric. The percentage of donor-origin PBMC in these recipients was 58 to 96%. All 11 skin grafts were still intact at the time these allochimeric animals were electively killed 72 to 251 days after transplantation. This skin graft survival was significantly longer than that which was seen for the 9 mice that did not receive anti-CD154 mAb and did not become chimeric (p<0.001 vs. allochimeric recipients).

Histological analysis of transplanted skin was performed on a subset of 2 chimeric mice with healed-in BALB/c skin grafts that had survived intact for 205 and 212 days. In neither instance was there evidence of inflammation suggestive of graft rejection.

As expected, the donor-specific (C57BL/6-Ly5.1) skin grafts on chimeric C57BL/6 recipients of syngeneic C57BL/6-Ly5.1 bone marrow survived indefinitely (Figure 6A). This was true both for recipients conditioned with irradiation alone
(MST=138, Range 32-164 days, N=5) and for recipients conditioned with both irradiation and anti-CD154 mAb (MST=156, Range 60-297 days, N=23).
Figure 6.
Skin graft survival on hematopoietic bone marrow chimeras

A. Syngeneic Chimeras

B. Allogeneic Chimeras
Legend to Figure 6: C57BL/6 mice received 6 Gy of irradiation and 2 injections of anti-CD154 mAb (days 0 and +3). Mice also received 25 million bone marrow cells from either syngeneic (C57BL/6-Ly5.1) or allogeneic (BALB/c) donors. Eight to 17 weeks after transplantation mice were given a donor-specific or third party skin graft as described in Methods. Mice that received BALB/c bone marrow were transplanted with BALB/c and CBA/JCr skin grafts, whereas mice that received C57BL/6-Ly5.1 bone marrow were transplanted with C57BL/6-Ly5.1 and CBA/JCr skin grafts. Skin graft survival was measured and quantified using median survival time (MST) and presented using the method of Kaplan and Meier.
6. Third party skin grafts are promptly rejected on allogeneic chimeras

After documenting that our chimeric mice were tolerant to donor-specific skin grafts, we next determined whether this tolerance was donor-specific by transplanting third party skin grafts onto our chimeric mice. Three allochimeric mice with healed-in donor-specific (H2^d) skin grafts that had been in place for 30 days were selected at random and given a third party CBA/JCR (H2^k) skin graft on the contralateral flank. Survival of the CBA/JCR skin allografts was brief (Figure 6, MST=11 days, range 10-11 days). The BALB/c skin grafts on these mice were still intact at the conclusion of the experiment; 127 days after the CBA/JCR skin allografts had been rejected. One additional allochimeric mouse received only a CBA/JCR skin allograft, and survival of that graft was brief (MST=11 days). These data demonstrate that T cell function was present in our allochimeric mice and that they were specifically tolerant to H2^d-expressing cells.
7. Chapter I Summary

The data described in this chapter documents a model system characterized by mixed hematopoietic chimerism and donor-specific central tolerance. Chimeric mice, both allogeneic and syngeneic, displayed stable and long-term multi-lineage chimerism. They also permanently accepted donor-specific skin grafts while maintaining the ability to promptly destroy third party skin grafts. Mixed hematopoietic chimerism was obtained in the absence of GVHD and with minimal preparative risk to the recipient. Because this system of inducing hematopoietic chimerism accurately models an approach that could be used in the clinic, it was deemed appropriate for use in analyses of safety and durability in the presence of viral infection, a common complication of clinical bone marrow transplantation.
CHAPTER II

LCMV INFECTION AT THE TIME OF ALLOGENEIC BONE MARROW TRANSPLANTATION LEADS TO GRAFT REJECTION AND HOST DEATH

INTRODUCTION CHAPTER II

Having established stable mixed hematopoietic chimerism using sublethal irradiation and costimulation blockade, we next sought to determine whether viral infection could interfere with either the induction or maintenance of hematopoietic chimerism and donor-specific tolerance in our model system. Many viral infections can induce inflammatory cytokines (232), T cell growth and differentiation factors and virus-specific CTL that react to allogeneic targets (5), any of which could potentially compromise graft survival and overcome tolerance. Furthermore, recipient mice treated with partial myeloablation combined with costimulation blockade could be less resistant to viral infection and its associated pathophysiological effects. Therefore, the experiments in this chapter focus on the safety and efficacy of our conditioning regimen with regard to viral infection. LCMV was chosen as the model virus for these studies, as it induces all of the attributes of viral infection listed above. Additionally, LCMV is one of the most well studied viral pathogens, particularly in the C57BL/6 mice.
RESULTS CHAPTER II

1. LCMV infection does not interfere with the induction or maintenance of syngeneic hematopoietic chimerism

In order to determine whether viral infection could interfere with either the induction or maintenance of hematopoietic chimerism in our model system, C57BL/6 mice were randomized into two groups and treated with irradiation, anti-CD154 mAb, and either syngeneic C57BL/6-Ly5.1 or allogeneic BALB/c bone marrow, as shown in Figure 7. The transplanted mice in both groups were then randomly assigned to one of four sub-groups. The first sub-group received no further treatment. Mice in the remaining three sub-groups were given an intraperitoneal injection of LCMV, strain Armstrong, on the same day as transplantation, or on day 15 or day 50 after transplantation.

As shown in Table 3, there was no effect of LCMV infection at any time point on the recipients of syngeneic C57BL/6-Ly5.1 bone marrow with respect to the number of mice becoming chimeric or the percentage of donor-origin cells present 2-9 weeks after transplantation. The percentage of donor-origin cells at each time point was comparable to that observed in the uninfected control mice (Table 2, line 2). None of the mice in any of the 4 syngeneic groups appeared sick or died during the period of observation.
Figure 7: Bone Marrow Transplantation in LCMV Infected Mice

Anti-CD154 mAb (0.5 mg/dose, i.p.) (2 doses)

Days 0

+3

??

C57BL/6

LCMV

5 x 10^4 PFU

Day 0, 15 or 50 post BM injection

6 Gy irradiation

Bone marrow cells (25 x 10^6) i.v.

BALB/c

Allo-chimeric C57BL/6 mouse
Legend to Figure 7: This figure schematically depicts our protocol for transplanting allogeneic bone marrow into LCMV-infected mice, using sublethal irradiation and costimulation blockade. C57BL/6 mice were injected intravenously with a single dose of 18-25 million allogeneic BALB/c bone marrow cells immediately after receiving 6 Gy of irradiation. Experimental mice also received two 0.5 mg injections of anti-CD154 mAb. The first of these injections was given on day 0, the day of transplantation, and the second injection was given on day +3, three days after bone marrow transplantation. Finally, mice received an i.p. injection of LCMV, strain Armstrong, either on the day of transplantation or 15 or 50 days after transplantation.
Table 3. Percentage of donor-origin PBMC in C57BL/6 recipients of syngeneic or allogeneic bone marrow infected with LCMV at various times

<table>
<thead>
<tr>
<th>Bone Marrow</th>
<th>LCMV</th>
<th>N</th>
<th>Frequency of Chimerism on Days 12-13</th>
<th>Percentage of Donor-Origin Peripheral Blood Mononuclear Cells in Chimeric Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor Infection Day</td>
<td></td>
<td></td>
<td>Day 12-13</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>Day 0</td>
<td>10</td>
<td>10/10</td>
<td>97±2</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>Day 15</td>
<td>10</td>
<td>10/10</td>
<td>77±4</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>Day 50</td>
<td>10</td>
<td>10/10</td>
<td>81±8</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Day 0</td>
<td>15</td>
<td>0/12*</td>
<td>&lt;1</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Day 15</td>
<td>10</td>
<td>9/9</td>
<td>59±25</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Day 50</td>
<td>10</td>
<td>9/10</td>
<td>80±15</td>
</tr>
</tbody>
</table>
Legend to Table 3: C57BL/6 mice were irradiated with 6 Gy. Within 1-3 hours of irradiation all recipients received a single intravenous injection of 18-25 x 10^6 donor bone marrow cells in a volume of 0.5 ml via the lateral tail vein. Anti-CD154 mAb (0.5 mg) was injected intraperitoneally into all mice on the day of irradiation and on day +3. Mice were injected on the days indicated with 5 x 10^4 PFU of LCMV, strain Armstrong, as described in Methods. The percentage of H2^d or Ly5.1 donor-origin peripheral blood mononuclear cells was determined by flow cytometry 2-9 weeks after bone marrow transplantation. Chimerism was defined as the presence of >1% MHC class I^+ donor-origin cells two weeks after transplantation. The one mouse with <1% donor-origin cells at the two week time point had <1% donor cells at all other time points. All mice chimeric at week 2 remained chimeric throughout the duration of the experiment. Each data point represents the mean ± 1 s.d. *Three additional mice in this cohort died on day 12, before they could be tested for chimerism.
2. **LCMV infection at the time of transplantation abrogates the induction of allogeneic hematopoietic chimerism and is fatal**

In contrast to the results seen with syngeneic bone marrow, all C57BL/6 recipients of allogeneic BALB/c bone marrow that were infected with LCMV immediately after transplantation died 13-18 days after transplantation and infection (N=15). Among these 15 mice, 12 survived long enough to be tested for chimerism on days 12-13. No peripheral donor-origin cells were detectable in any of these mice (Table 3), indicating that a day 0 LCMV infection can block the development of allogeneic hematopoietic chimerism.

In a separate experiment, C57BL/6 mice treated with 6 Gy, anti-CD154 mAb and given an allogeneic bone marrow transplant were tested for chimerism at time points immediately after bone marrow transplantation and LCMV infection. No donor-origin cells were found in the PBMC of LCMV-infected mice on days 1, 4 or 7 post-transplantation, suggesting that injected allogeneic bone marrow cells never engraft as opposed to donor cells initially engrafting and then being destroyed.
3. LCMV infection does not interfere with the maintenance of allogeneic hematopoietic chimerism

We next determined the durability of our chimeric state by delaying LCMV infection until 2 or 7 weeks after the establishment of mixed allogeneic hematopoietic chimerism. Among the 10 mice randomized to receive LCMV 15 days after transplantation, one died before infection. The remaining 9 mice were all chimeric on the day before infection. Subsequent to infection, the percentage of donor-origin PBMC declined by 17% on day 28 and remained at approximately this same level on days 49 and 63. None of these mice appeared ill and none died. At each time point after infection, the percentage of donor-origin PBMC in the LCMV-infected mice (Table 3) was somewhat lower than in the uninfected controls (Table 2, Line 4). These results suggest that the deleterious effects of LCMV infection on host and graft survival are confined to a narrow window of time immediately after the tolerization and transplantation process.

Among the 10 mice randomized to receive LCMV 50 days after transplantation, 9 were chimeric on the day before infection. Subsequent to infection, the percentage of donor-origin PBMC in the chimeric mice declined by ~11% on day 63. None of the 10 mice appeared ill and none died. At corresponding time points after infection, the percentage of donor-origin PBMC in the LCMV-infected mice (Table 3) was once again lower than in the uninfected controls (Table 2, Line 4).
4. Clearance kinetics of LCMV in infected hematopoietic chimeras

We next hypothesized that the differential survival of syngeneic vs. allogeneic bone marrow recipients, infected with LCMV at the time of transplantation, was due to differential ability to clear the LCMV virus. To test this hypothesis we measured LCMV titers in the PBMC of infected chimeric mice at different times after transplantation. As shown in Table 4, all recipients of allogeneic or syngeneic bone marrow infected on the day of transplantation failed to clear the LCMV virus during the first two weeks after infection. Two weeks after transplantation and infection, viral titers were similar between these two groups, suggesting that neither viral load *per se* nor ability to completely clear virus was the determinant of differential survival.

Syngeneic chimeras infected 15 days after transplantation were able to clear the LCMV infection, whereas allogeneic chimeras infected at day 15 became persistent carriers of virus. Both allogeneic and syngeneic chimeras infected with LCMV 50 days after successful bone marrow transplantation cleared the LCMV infection within two weeks.
Table 4. Viral titers in the serum of allogeneic and syngeneic hematopoietic chimeras infected with LCMV

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Virus Infection</th>
<th>N</th>
<th>2 wks</th>
<th>5 wks</th>
<th>7 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>C57BL/6</td>
<td>Day 0</td>
<td>6</td>
<td>3.5±0.3</td>
<td>3.8±0.4</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>Day 0</td>
<td>12</td>
<td>3.3±0.8</td>
<td>dead</td>
<td>—</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>Day 15</td>
<td>9</td>
<td>2.8±0.4</td>
<td>4.2±0.4</td>
<td>4.3±0.5</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>Day 50</td>
<td>8</td>
<td>&lt;2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>C57BL/6</td>
<td>Day 0</td>
<td>10</td>
<td>3.4±0.3</td>
<td>3.3±0.4</td>
<td>3.8±0.6</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>C57BL/6</td>
<td>Day 15</td>
<td>10</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>C57BL/6-Ly5.1</td>
<td>C57BL/6</td>
<td>Day 50</td>
<td>9</td>
<td>&lt;2</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
Legend to Table 4: Serum from mice presented in Table 3 were assayed for LCMV titers as described in Materials at 2-7 weeks after LCMV infection. Results are expressed as geometric mean titers, i.e. the arithmetic mean of the log$_{10}$ values.
5. LCMV infection does not interfere with the maintenance of donor-specific tolerance

After discovering that acute LCMV infection prevented the induction of allogeneic chimerism, we next sought to determine whether LCMV infection could interfere with the maintenance of donor-specific tolerance. Groups of C57BL/6 mice were treated with 6 Gy of irradiation, anti-CD154 mAb and given either allogeneic BALB/c or syngeneic C57BL/6-Ly5.1 bone marrow. After testing for chimerism several times to ensure that chimerism was durable, these mice were given donor-origin skin grafts 89-109 days after bone marrow transplantation. Immediately after skin grafting, these mice were given an LCMV infection. Prior to skin grafting, the 8 allochimeric mice contained a range of 63-90% donor cells, with the exception of a single mouse which contained only 6%. Median survival time of the BALB/c skin grafts in these 9 mice was greatly prolonged (MST=164 d, range 131-232 d), with the exception of the mouse that contained only 6% donor cells. This mouse quickly rejected its skin graft (MST=14 d). Unfortunately, chimerism was tested in this mouse two weeks prior to skin grafting but not afterwards. Therefore, it is unknown whether this mouse had lost chimerism prior to skin grafting.

Six stable syngeneic chimeras, with a range of 94-99% donor cells, were also given an LCMV infection immediately after being transplanted with donor-origin skin grafts (C57BL/6-Ly5.1). Similar to the results seen in allogeneic chimeras, skin grafts on these syngeneic chimeras had prolonged survival (MST=196 days, range 120-353 d).
Overall, these data indicate that LCMV infection does not interfere with the maintenance of donor-specific tolerance in established hematopoietic bone marrow chimeras.
6. Chapter II summary

In this chapter we have shown that in mice treated with irradiation, anti-CD154 mAb and given an allogeneic bone marrow transplant, LCMV infection can have a dramatic effect on the survival of both the bone marrow transplant and the recipient mouse. C57BL/6 mice given a day 0 LCMV infection not only fail to become chimeric, but also die shortly after bone marrow transplantation. This result is all the more unusual and interesting as it occurs after mice receive a sublethal dose of irradiation, and a viral infection that is normally non-cytopathic. The lethal dose of irradiation in healthy C57BL/6 mice is typically 10-11 Gy of irradiation (personal observations). Therefore, the 6 Gy of irradiation used in these experiments is well below the lethal dose for C57BL/6 mice. Moreover, the Armstrong strain of LCMV that was used in these studies is normally non-lethal when given as an i.p. injection, even when infection occurs in immunocompromised C57BL/6 mice. Somehow, the combination of irradiation and a day 0 LCMV infection triggers the rejection of the allogeneic bone marrow transplant and the death of the recipient. Fortunately, both mortality and the destruction of the bone marrow transplant are confined to a narrow window of time immediately following transplantation.
CHAPTER III

VIRAL ABROGATION OF STEM CELL TRANSPLANTATION TOLERANCE CAUSES GRAFT REJECTION AND HOST DEATH BY DIFFERENT MECHANISMS: A RADIORESISTANT CD8<sup>+</sup> TCR-<alpha><beta><sup>+</sup> NK1.1<sup>−</sup> T CELL POPULATION AND VIRUS-INDUCED INTERFERON-<alpha>/beta>

INTRODUCTION CHAPTER III

We have shown in chapter II that LCMV infection, at the time of transplantation, had severe consequences for the survival of the allograft and the host. Mice that received 6 Gy of irradiation, anti-CD154 mAb and an LCMV infection at the time of allogeneic bone marrow transplantation failed to engraft BALB/c bone marrow and died 2-3 weeks after transplantation. The inability of these mice to clear the LCMV virus was not associated with allograft rejection or death. Therefore, we focused on the various cell populations that are activated and the cytokines that are induced following LCMV infection. Both NK cells and CD8<sup>+</sup> T cells dramatically increase shortly after LCMV infection (197). Furthermore, both cell populations have been implicated in allograft rejection (180, 232). Additionally, cytokines such as IFN-<gamma> and IFN-<alpha><beta> are induced by LCMV infection and have been associated with hematopoietic stem cell suppression (197, 250, 251). Based on these reports, this chapter analyzed the role of NK and T cells, as well as IFN-<gamma> and IFN-<alpha><beta> in allograft rejection and death in our recipient mice.
RESULTS CHAPTER III

1. Mouse fatality is dependent on the presence of allogeneic bone marrow

Our initial goal was to determine the mechanism(s) responsible for the mortality of C57BL/6 mice that received allogeneic bone marrow, 6 Gy of irradiation, anti-CD154 mAb, and a day 0 LCMV infection. Initially, we asked why mice that received our conditioning regimen along with syngeneic bone marrow survived whereas recipients of allogeneic bone marrow died. In order to answer this question, we tested whether mortality was due to a deleterious effect of allogeneic bone marrow or whether our conditioning regimen itself was lethal to C57BL/6 recipients.

Groups of C57BL/6 mice were treated with one or more of the following parts of our conditioning regimen: 6 Gy of irradiation, two 0.5 mg injections of anti-CD154 mAb, an allogeneic bone marrow transplant or an LCMV infection. The results, shown in Table 5, indicate that 6 Gy of irradiation by itself (Group 1, 100% survival at 4 weeks, N=5) or in combination with anti-CD154 mAb (Group 2, 100%, N=5) does not cause mortality in C57BL/6 mice. When 6 Gy of irradiation was combined with an LCMV infection, the majority of mice once again survived (Group 3, 89%, N=9). Mice that received irradiation, anti-CD154 mAb and an LCMV infection had a slightly lower rate of survival, as only 13 of 19 mice (Group 4, 68%) survived. In contrast to all other groups, the vast majority of mice died when given an allogeneic bone marrow transplant, irradiation, and an LCMV infection. This was true irrespective of whether mice were additionally treated with anti-CD154 mAb (Group 5, 20% survival, N=5) or not (Group 6, 0% survival, N=15). Combined, these data suggest that mouse mortality is dependent
on the combination of three treatments: 6 Gy of irradiation, a day 0 LCMV infection and an allogeneic bone marrow transplant.
Table 5. Survival of C57BL/6 mice treated with various conditioning regimens

<table>
<thead>
<tr>
<th>Group</th>
<th>LCMV</th>
<th>Anti-CD154 mAb</th>
<th>Allogeneic</th>
<th>N</th>
<th>Survival at 4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>1</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>5</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>5</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>9</td>
<td>89%</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>19</td>
<td>68%</td>
</tr>
<tr>
<td>5</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>5</td>
<td>20%</td>
</tr>
<tr>
<td>6</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>15</td>
<td>0%</td>
</tr>
</tbody>
</table>
Legend to Table 5: C57BL/6 mice were treated with 6 Gy of irradiation. When indicated, recipients were also treated with one or more of the following: two 0.5 mg injections of anti-CD154 mAb given on day 0 and day +3, LCMV infection on day 0, or injection with 25 million BALB/c allogeneic bone marrow cells. Mice were observed during a 4-week period following the initiation of the various treatments.
2. LCMV infection prevents allogeneic bone marrow engraftment via an allogeneic-specific response

We next sought to determine whether the presence of allogeneic bone marrow in our LCMV-infected recipients invoked a non-specific destruction of injected bone marrow cells or whether they induced a specific anti-allogeneic response. In order to distinguish between these two possibilities, irradiated recipients were injected with syngeneic and allogeneic bone marrow simultaneously and than infected with LCMV. The results are shown in Table 6. Once again, allogeneic bone marrow engrafted into the majority of uninfected irradiated recipients (9/14) and mice survived (14/14), whereas mice infected with LCMV failed to become chimeric (0/14) and died (0/14). Only 5 of the 13 uninjected mice (38%) that received both 25 million syngeneic bone marrow cells and 25 million allogeneic cells had allogeneic engraftment, whereas syngeneic bone marrow engraftment occurred in all 13 mice (100%). Similar results have been reported previously, as several groups have demonstrated that injection of up to a 10:1 ratio of allogeneic to syngeneic bone marrow is required to engraft allogeneic cells when co-injected with syngeneic cells (252,253).

Interestingly, when conditioned recipients of both allogeneic and syngeneic cells were infected with LCMV on the day of transplantation there was a complete lack of allogeneic engraftment. Of 17 LCMV-infected recipients of syngeneic and allogeneic bone marrow, 2 mice died before being tested for chimerism. None of the remaining mice had detectable levels of allogeneic donor cells (0/15), whereas all of these mice contained high levels of syngeneic cells (15/15). Moreover, 14 of the 17 (82%) total mice survived,
suggesting that syngeneic engraftment saved these mice from death. Overall, these data suggest that allogeneic bone marrow, in LCMV-infected recipients treated with anti-CD154 mAb and sublethal irradiation, invokes a specific anti-allogeneic response. Further they also suggest that syngeneic bone marrow engraftment can prevent death in mice treated with 6 Gy, anti-CD154 mAb and given an LCMV infection at the time of allogeneic bone marrow transplantation.
Table 6. Percentage of donor-origin PBMC in uninfected and LCMV-infected C57BL/6 recipients of both syngeneic and allogeneic bone marrow

<table>
<thead>
<tr>
<th>Bone Marrow</th>
<th>LCMV</th>
<th>N</th>
<th>Engraftment at 2-4 weeks</th>
<th>Survival at 4 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Syn</td>
<td>Allo</td>
</tr>
<tr>
<td>Allogeneic</td>
<td>None</td>
<td>14</td>
<td>9/14</td>
<td>14/14</td>
</tr>
<tr>
<td>Allogeneic</td>
<td>Yes</td>
<td>14</td>
<td>0/14</td>
<td>0/14</td>
</tr>
<tr>
<td>Syn and Allo</td>
<td>None</td>
<td>13</td>
<td>13/13</td>
<td>5/13</td>
</tr>
<tr>
<td>Syn and Allo</td>
<td>Yes</td>
<td>17</td>
<td>15/15</td>
<td>0/15</td>
</tr>
</tbody>
</table>


Legend to Table 6: C57BL/6 mice were irradiated with 6 Gy. Within 1-3 hours of irradiation all recipients received a single intravenous injection of either $25 \times 10^6$ allogeneic cells, or $25 \times 10^6$ allogeneic plus $25 \times 10^6$ syngeneic bone marrow cells. Anti-CD154 mAb (0.5 mg) was injected intraperitoneally into all mice on the day of irradiation and on day +3. Mice were injected on day 0, immediately after the bone marrow transplant, with LCMV. The percentage of H2d or Ly5.1 donor-origin PBMC was determined by flow cytometry 2-4 weeks after bone marrow transplantation. Chimerism was defined as the presence of >1% MHC class I donor-origin cells two weeks after transplantation.
3. Lack of allogeneic bone marrow engraftment can be overcome when high numbers of donor bone marrow cells are injected into LCMV-infected recipients

We next tested whether increasing the injected dose of allogeneic bone marrow cells into our LCMV-infected recipients could overwhelm their resistance to allogeneic bone marrow engraftment. C57BL/6 mice were treated with our usual conditioning regimen of 6 Gy irradiation and anti-CD154 mAb. These mice were then split into various groups and injected with increasing doses of allogeneic BALB/c bone marrow cells ranging from 25 to 200 million, followed immediately by an LCMV infection. The results, shown in Table 7, once again document that uninfected recipients injected with 25 million allogeneic bone marrow cells readily become chimeric, whereas similarly treated LCMV-infected mice fail to become chimeric and die. When 50 or 100 million allogeneic bone marrow cells were injected into LCMV-infected mice, there was still a complete lack of detectable donor cell engraftment and all mice died. In contrast, 2 of the 3 mice injected with 200 million allogeneic bone marrow cells became chimeric and survived, suggesting that the failure of allogeneic bone marrow to engraft in LCMV-infected mice can be overcome by increasing the amount of allogeneic bone marrow cells injected.
Table 7. Increasing the dose of allogeneic cells can induce hematopoietic chimerism in LCMV-infected recipients

<table>
<thead>
<tr>
<th>Bone Marrow Cells Injected</th>
<th>LCMV Infection</th>
<th>% Chimeric Mice at 5 Weeks</th>
<th>% Survival at 5 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 Million</td>
<td>No</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>25 Million</td>
<td>Yes</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>50 Million</td>
<td>Yes</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>100 Million</td>
<td>Yes</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>200 Million</td>
<td>Yes</td>
<td>2/3</td>
<td>2/3</td>
</tr>
</tbody>
</table>
Legend to Table 7: C57BL/6 mice were irradiated with 6 Gy. Within 1-3 hours of irradiation all recipients received a single intravenous injection of allogeneic bone marrow ranging from 25 to 200 million cells. Anti-CD154 mAb (0.5 mg) was injected intraperitoneally into all mice on the day of irradiation and on day +3. When indicated, mice were infected with LCMV immediately after transplantation. The percentage of H2d donor-origin PBMC was determined by flow cytometry 5 weeks after bone marrow transplantation. Chimerism was defined as the presence of >1% MHC class I+ donor-origin cells two weeks after transplantation.
4. The bone marrow and lymphoid compartments in LCMV-infected recipients given irradiation, anti-CD154 mAb, and allogeneic bone marrow are markedly hypoplastic

To determine the cause of death in allogeneic bone marrow recipients infected with LCMV on the day of bone marrow transplantation, cohorts of control and infected bone marrow recipients were killed 7 or 14 days after transplantation. Light microscopic analysis of sections of femurs of infected mice (N=8) revealed severe reductions in the number of all hematopoietic populations (Figure 8). Reductions in marrow cellularity averaged 86±12% (range 60-97%) compared to uninfected controls. Histological examination of the spleens of LCMV-infected mice revealed lymphoid depletion in all cases, and, with the exception of a single splenic nodule in one mouse that showed regenerative activity, there was no evidence of extramedullary hematopoiesis.

In additional studies, total cell counts were performed on both spleen and femoral bone marrow recovered 4 to 35 days after transplantation as shown in Figure 9. Femoral and splenic cell counts were severely depressed immediately after irradiation and transplantation for syngeneic chimeras, irrespective of whether recipients were infected with LCMV at the time of transplantation. In both uninfected and infected synchimeric femurs, cell counts were similar to untreated age-matched control mice 10 days after transplantation. Splenic cell counts in uninfected syngeneic chimeras returned to untreated control levels 2 weeks after transplantation, but remained severely depressed 7 weeks after transplantation in LCMV-infected synchimeras.
Uninfected allogeneic chimeras also had depressed femoral and splenic cell counts immediately after transplantation. As was the case with synchimeric mice, recovery was much faster in the bone marrow compartment than in the spleen. Femoral cellularity was back to control levels 2 weeks after transplantation, whereas spleen cellularity was still depressed 7 weeks after transplantation. In contrast to all other groups, however, cell counts in the recipients of allogeneic bone marrow infected with LCMV on the day of transplantation never recovered, and all mice died before day 21. Splenic and femoral cell counts in these mice were approximately 1 million cells at all time points tested, compared to the 100 million spleen cells and 25 million femoral cells found in age-matched untreated wild type C57BL/6 mice. This cellularity data combined with the histological analyses suggest that recipients given an allogeneic bone marrow transplant, our conditioning regimen of 6 Gy and anti-CD154 mAb, and an LCMV infection at the time of transplantation die as a result of a marked hypoplasia in both the spleen and bone marrow.
Figure 8. Histology of femoral bone marrow
Legend to Figure 8: Sections of bone marrow from C57BL/6 wild type recipients on day fourteen after injection of (A) Ly5 congenic bone marrow, (B) Ly5 congenic bone marrow and LCMV, (C) allogeneic BALB/c bone marrow, or (D) allogeneic BALB/c bone marrow plus LCMV. Panel E shows bone marrow from a C57BL/6 IFN-αβ-receptor knock out mouse and panel F shows bone marrow from a LCMV-infected C57BL/6 IFN-αβ receptor knock out mouse on day 13 after bone marrow injection. All mice were given 6 Gy radiation and two doses of anti-CD154 mAb on day 0 and +3 relative to bone marrow cell injection on day 0. LCMV was given on day 0. The marrow from the mouse that received allogeneic BALB/c bone marrow (D) shows severe marrow hypoplasia. The other marrows are unremarkable. (Hematoxylin and Eosin, magnification =131).
Figure 9. Cell counts in the femoral and splenic compartments of uninfected and LCMV-infected recipients of allogeneic or syngeneic bone marrow.
Legend to Figure 9: C57BL/6 mice were irradiated with 6 Gy. Within 1-3 hours of irradiation all recipients received a single intravenous injection of $25 \times 10^6$ donor bone marrow cells. Anti-CD154 mAb (0.5 mg) was injected intraperitoneally on the day of irradiation and on day +3. LCMV was given on the day of transplantation to mice as indicated. At the various time points, femora from the right leg and spleens were removed from mice and prepared for cell counts as described in Methods. Cell counts are shown as the mean ± 1 s.d. The dotted horizontal lines indicate the mean splenic and femoral bone marrow cell numbers in 3 untreated 12-week-old C57BL/6 mice.
5. Host IFN-αβ receptor expression is required for LCMV-induced hypoplasia and death in allogeneic bone marrow recipients given anti-CD154 mAb

Having discovered severe hypoplasia restricted to recipients of allogeneic bone marrow and LCMV infection, we next sought to determine its cause. We first hypothesized that the cause was related to cytokine release. Reversible depression of hematopoiesis is known to occur early in the course of LCMV infection and has been reported to be a direct effect of IFN-αβ (251). To determine the role of IFN-αβ in our model system, we repeated our experiments using 129/Sv IFN-αβ receptor (IFN-αβR) knockout mice (216). 129/Sv+/+ and 129/Sv IFN-αβR knockout mice were irradiated (6 Gy), given 25 million BALB/c bone marrow cells and two injections of anti-CD154 mAb on days 0 and +3. Half of the mice were also infected with LCMV on the day of transplantation. As shown in Table 8, uninfected 129/Sv+/+ and 129/Sv IFN-αβR knockout recipients readily accepted BALB/c bone marrow and became chimeric. As expected, LCMV-infection of control 129/Sv+/+ recipients led to failure of engraftment and death. Therefore, the ability of LCMV to induce death in recipients treated with 6 Gy of irradiation, anti-CD154 mAb and infected with LCMV on the day of transplantation is not strain-specific as two different mice strains (C57BL/6 and 129/Sv) died shortly after receiving this conditioning regimen.

In contrast to the 129/Sv+/+ recipients, LCMV infection of 129/Sv IFN-αβR knockout recipients also led to failure of the bone marrow allograft, but all of the mice survived. As was the case for LCMV-infected C57BL/6 recipient mice (Figure 8), histological study of LCMV-infected SV129+/+ recipients revealed bone marrow
hypoplasia and splenic lymphopenia. In contrast, the spleen and bone marrow of SV129 IFN-αβR knockout mice treated in a similar way showed normal cellularity 14 days after transplantation (Figure 8). These data suggest that LCMV-induced death was the consequence of a Type 1 interferon-mediated process, but rejection of the allogeneic marrow graft was due to a different mechanism.
Table 8. Chimerism and survival in uninfected and LCMV-infected 129/Sv wild-type and 129/Sv IFN-αβ receptor knockout mice

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Virus</th>
<th>N</th>
<th>Chimerism at 2 Weeks</th>
<th>Survival at 7 Weeks</th>
<th>2 wks</th>
<th>4-7 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>129/Sv +/+</td>
<td>None</td>
<td>15</td>
<td>15/15</td>
<td>15/15</td>
<td>65 ± 23</td>
<td>75 ± 20</td>
</tr>
<tr>
<td>129/Sv +/+</td>
<td>Day 0</td>
<td>15</td>
<td>0/2*</td>
<td>0/15</td>
<td>&lt;1</td>
<td>Dead</td>
</tr>
<tr>
<td>129/Sv IFN-αβR KO</td>
<td>None</td>
<td>15</td>
<td>14/15</td>
<td>15/15</td>
<td>52 ± 23</td>
<td>63 ± 23</td>
</tr>
<tr>
<td>129/Sv IFN-αβR KO</td>
<td>Day 0</td>
<td>15</td>
<td>0/15</td>
<td>15/15</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
Legend to Table 8: Groups of 129/Sv wild type and 129/Sv IFN-αβ receptor knockout mice were treated with irradiation, 25 million BALB/c bone marrow cells and anti-CD154 mAb. Immediately after transplantation, mice in each of these two groups were randomized, and half of each group was injected with LCMV. The percentage of donor-origin PBMC was determined by flow cytometry at various time points after transplantation. Each data point represents the mean ± 1 s.d. *Thirteen mice in this cohort died before they could be tested for chimerism.
6. Circulating levels of IFN-αβ are similar in LCMV-infected recipients of syngeneic and allogeneic bone marrow

After discovering that host IFN-αβ receptor expression is critical for the mortality of mice that received allogeneic bone marrow, irradiation, and an LCMV infection, we hypothesized that these mice would have higher circulating levels of IFN-αβ than similarly treated mice that received syngeneic bone marrow. In order to test this hypothesis, C57BL/6 mice were treated with irradiation and anti-CD154 mAb as previously described. Recipient mice were then split into two groups. The first group received an allogeneic bone marrow transplant, while the second group received a syngeneic bone marrow transplant. Following the bone marrow transplant both groups were immediately infected with LCMV. At selected time points (2, 4 and 14 days post-transplantation) mice were tested for levels of circulating IFN-αβ in serum. As seen in Table 9, there were no significant differences in the circulating levels of IFN-αβ between LCMV-infected recipients of allogeneic or syngeneic bone marrow at all 3 time points tested. Early after infection, high levels of IFN-αβ were detected in both syngeneic and allogeneic recipients. As expected, levels of circulating IFN-αβ dropped dramatically by day 14 post-infection.

There are several possible explanations for our inability to find a difference in the circulating levels of IFN-αβ between LCMV-infected recipients of syngeneic or allogeneic bone marrow. One possibility is that increased levels of IFN-αβ in LCMV-infected allogeneic bone marrow recipients are only found at the site of its damage, the
bone marrow. A second possibility is that LCMV-induced IFN-αβ interacts with other cytokines such as IFN-γ or TNF-α. Both of these cytokines can inhibit hematopoietic progenitor cell growth (254), and therefore could combine with LCMV-induced IFN-αβ to produce recipient death.

In a preliminary experiment, we pretreated C57BL/6 mice with anti-IFN-γ mAb before giving them our normal conditioning regimen of sublethal irradiation, anti-CD154 mAb, and an allogeneic bone marrow transplant. All of the uninfected C57BL/6 mice treated with anti-IFN-γ mAb survived (5/5) and became chimeric (5/5, average of 79% donor-origin cells in PBMC). Similarly, 4 of 5 LCMV-infected mice treated with anti-IFN-γ mAb survived, but none of the 4 surviving mice became chimeric. This suggests that IFN-γ may be involved, along with IFN-αβ, in the death of mice that receive allogeneic bone marrow, sublethal irradiation, anti-CD154 mAb and a day 0 LCMV infection. Unfortunately, the role of TNF-α has yet to be determined in our system.
Table 9. Circulating levels of IFN-αβ in LCMV-infected recipients of syngeneic or allogeneic bone marrow

<table>
<thead>
<tr>
<th>Bone Marrow</th>
<th>N</th>
<th>Day 2 Post-infection</th>
<th>Day 4 Post-infection</th>
<th>Day 14 Post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Log2 U/mL (10^4)</td>
<td>Log2 U/mL (10^4)</td>
<td>Log2 U/mL (10^4)</td>
</tr>
<tr>
<td>Syngeneic</td>
<td>6</td>
<td>11.3</td>
<td>5.3</td>
<td>10.3</td>
</tr>
<tr>
<td>Allogeneic</td>
<td>6</td>
<td>11.0</td>
<td>12.3</td>
<td>10.3</td>
</tr>
</tbody>
</table>
Legend to Table 9. C57BL/6 mice were irradiated with 6 Gy. Within 1-3 hours of irradiation all recipients received a single intravenous injection of $25 \times 10^6$ syngeneic (C57BL/6-Ly5.1) or allogeneic (BALB/c) bone marrow cells. Anti-CD154 mAb (0.5 mg) was injected intraperitoneally on the day of irradiation and on day +3. LCMV was given on day 0, the day of transplantation. On the day indicated, NCTC-929 cells were incubated in 96-well plates with 2-fold dilutions of peripheral blood overnight. These mixtures were then infected with VSV, incubated for 3 days and then examined to determine the last dilution of peripheral blood that prevented VSV-induced cytotoxicity. Data are presented as Log$_2$ of the last dilution that prevented cytotoxicity, and as an estimated units/mL based on infected mixtures of NCTC-929 cells with known quantities of purified IFN-αβ.
7. Poly I:C treatment is unable to induce hematopoietic hypoplasia and death but does prevent allogeneic bone marrow engraftment

We have shown that host IFN-αβ receptors play a pivotal role in the mortality of mice infected with LCMV immediately after receiving both irradiation and an allogeneic bone marrow transplant. However, we were unable to detect a difference in the levels of circulating IFN-αβ between LCMV-infected recipients of syngeneic bone marrow (which become chimeric and survive) and allogeneic bone marrow (which fail to become chimeric and die). In order to further our understanding of the role of IFN-αβ, we tested whether a Poly I:C injection, a potent inducer of type 1 interferons in mice, could simulate an LCMV infection and produce mortality in irradiated recipients of allogeneic bone marrow.

Groups of C57BL/6 mice were treated with 6 Gy of irradiation, anti-CD154 mAb, and given a BALB/c allogeneic bone marrow transplant. Mice were then randomized into three separate groups. The first group received no further treatment. The second group received a Poly I:C injection of 0.5 mg immediately after transplantation and the third group received two 0.5 mg injections of Poly I:C on the day of transplantation and 5 days later.

Surprisingly, as shown in Table 10, Poly I:C treatment did not result in the death of any of the recipient mice. This was true for mice given either 1 or 2 injections of Poly I:C. Interestingly, although Poly I:C treatment did not prevent death, it did prevent the engraftment of allogeneic bone marrow, implying that a Poly I:C-induced response (either cytokines or a cellular component) is capable of preventing the engraftment of
allogeneic bone marrow in recipients treated with sublethal irradiation and anti-CD154 mAb.
Table 10. Poly I:C infection prevents allogeneic bone marrow engraftment but does not cause death in recipient mice treated with irradiation and anti-CD154 mAb

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Poly I:C</th>
<th>% Chimerism at 2 weeks</th>
<th>% Survival at 2 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>None</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Day 0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>Day 0 + Day 5</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
Legend to Table 10: C57BL/6 mice were irradiated with 6 Gy. Within 1-3 hours of irradiation all recipients received a single intravenous injection of $25 \times 10^6$ allogeneic (BALB/c) bone marrow cells. Anti-CD154 mAb (0.5 mg) was injected intraperitoneally on the day of irradiation and on day +3. Group 1 received no further treatment. Group 2 was given an intravenous injection of Poly I:C (0.5 mg) on the day of transplantation. Group 3 was treated with two 0.5 mg injections of Poly I:C, on day 0 and day +5. The percent chimerism was determined by flow cytometry analyses of peripheral blood taken 2 weeks post-transplantation.
8. NK cells are not involved in preventing allogeneic bone marrow engraftment in LCMV-infected recipients

Although the removal of host IFN-αβ receptors prevented death in LCMV-infected allogeneic bone marrow recipients treated with sublethal irradiation and anti-CD154 mAb, the injected allogeneic cells still did not engraft. Therefore, the survival of these mice depended on the recovery of the host marrow. We next questioned whether failure of allogeneic bone marrow engraftment in the presence of LCMV infection was the result of cell-mediated immune rejection.

To identify the cell type responsible for graft failure we conducted a series of cell depletion studies focused on NK cells and T cell subsets. NK cells were of particular interest because they reportedly play a pivotal role in the rejection of murine allogeneic bone marrow transplants (232,255,256). Furthermore, we have shown that Poly I:C injection at the time of transplantation prevents the engraftment of allogeneic bone marrow into irradiated recipients, and Poly I:C is a potent activator of NK cells (257).

We therefore first tested whether NK cells play a role in the lack of allogeneic bone marrow engraftment in LCMV-infected mice. As shown in the upper half of Table 11, pre-transplantation administration of anti-NK1.1, anti-asialo-GMI or anti-CD122 mAbs had little or no effect on subsequent hematopoietic chimerism or survival in C57BL/6 recipients of BALB/c bone marrow and anti-CD154 mAb in the absence of LCMV.

The effects of these reagents on survival and chimerism in mice infected with LCMV on the day of bone marrow transplantation are shown in the lower half of Table
11. Once again, LCMV infection at the time of transplantation prevented the engraftment of allogeneic bone marrow and caused mice to die. Pre-transplantation administration of anti-NK1.1 mAb slightly improved the survival of these LCMV-infected mice (29% survival vs. 6% survival in untreated LCMV-infected controls), but, allogeneic bone marrow failed to engraft. In mice pre-treated with anti-CD122 mAb the majority of mice survived (7 of 9 mice, 78% survival), but again, allogeneic bone marrow engraftment failed to occur. Treatment of mice with anti-asialo-GM1 mAb, however, prevented LCMV-induced death and allowed allogeneic bone marrow engraftment. In their aggregate, these results suggest that NK cells are not responsible for preventing allogeneic bone marrow engraftment in LCMV-infected recipients of anti-CD154 mAb and irradiation.

Asialo-GM1 is expressed on the majority of NK cells in C57BL/6 mice, but is also expressed on activated T cells including those that express CD4, CD8, TCR-αβ and TCR-γδ (258). We therefore next focused on these 4 T cell subsets to determine what cell type was responsible for preventing the engraftment of allogeneic bone marrow in LCMV-infected mice.
Table 11. Chimerism and survival in recipients of bone marrow allografts treated to deplete NK cells

<table>
<thead>
<tr>
<th>LCMV Infection</th>
<th>Antibody</th>
<th>N</th>
<th>Chimerism at 2 weeks*</th>
<th>Survival at 6 Weeks</th>
<th>% of Donor-Origin PBMC in Chimeric Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>13</td>
<td>11/13</td>
<td>13/13</td>
<td>56 ± 25</td>
</tr>
<tr>
<td>Anti-NK1.1</td>
<td></td>
<td>9</td>
<td>11/14</td>
<td>14/14</td>
<td>57 ± 23</td>
</tr>
<tr>
<td>Anti-Asialo-GM1</td>
<td></td>
<td>9</td>
<td>9/9</td>
<td>9/9</td>
<td>73 ± 5</td>
</tr>
<tr>
<td>Anti-CD122</td>
<td></td>
<td>9</td>
<td>7/9</td>
<td>9/9</td>
<td>36 ± 20</td>
</tr>
<tr>
<td>LCMV Day 0</td>
<td>None</td>
<td>17</td>
<td>0/13</td>
<td>1/17</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Anti-NK1.1</td>
<td></td>
<td>14</td>
<td>0/4</td>
<td>4/14*</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Anti-Asialo-GM1</td>
<td></td>
<td>8</td>
<td>8/8</td>
<td>8/8</td>
<td>74 ± 23</td>
</tr>
<tr>
<td>Anti-CD122</td>
<td></td>
<td>9</td>
<td>0/8</td>
<td>7/9</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
Legend to Table 11: C57BL/6 mice were randomized into four groups. The first received no treatment. The remaining groups were treated with injections of the listed depleting reagents. Treatments were begun on days -8 to -1 before transplantation and completed by day +6 as described in Methods. On day 0 all mice received 6 Gy of radiation, 25 million BALB/c bone marrow cells and the first of 2 doses of anti-CD154. Mice in all four treatment groups were then randomized a second time, and half received LCMV immediately after bone marrow transplantation. The remaining half received no virus. The percentage of donor-origin PBMC was determined by flow cytometry 2 and 4-6 weeks after transplantation as described in Methods. Each data point represents the mean ± 1 s.d. *Some mice did not survive to the two week time point, the first time point at which the extent of chimerism was quantified in this experiment. a: p=N.S. vs. untreated LCMV-infected controls; b: p<0.001 vs. untreated LCMV-infected controls (Fisher exact statistic).
9. A population of CD8<sup>+</sup>TCR<sub>αβ</sub><sup>+</sup>asialo-GM<sub>1</sub><sup>+</sup> cells prevents allogeneic bone marrow engraftment in LCMV-infected recipients treated with 6 Gy and anti-CD154 mAb

Although the depletion of NK cells appears to have a positive effect on the survival of LCMV-infected mice, it did not enhance the engraftment of allogeneic bone marrow. Therefore, we next focused our experiments on T cell subsets. Recipient mice were either wild type C57BL/6 mice or C57BL/6 mice in which the CD4, CD8, TCR-<sub>αβ</sub> or TCR-γδ lymphocyte surface antigen gene was disrupted by homologous recombination. All mice were treated with our usual conditioning regimen of 6 Gy irradiation, 25 million BALB/c allogeneic bone marrow cells and anti-CD154 mAb.

As shown in the upper half of Table 12, the absence of CD4, CD8, TCR-<sub>αβ</sub>, or TCR-γδ cells had little or no effect on the generation of hematopoietic chimerism or survival of C57BL/6 knockout recipients of BALB/c bone marrow and anti-CD154 mAb in the absence of LCMV infection.

In contrast to LCMV-infected wild-type control mice, which failed to develop allogeneic hematopoietic chimerism and died, allogeneic bone marrow transplantation into either LCMV-infected CD8 or TCR-αβ knockout mice resulted in greatly enhanced survival and robust hematopoietic chimerism. LCMV-infected CD8 knockout mice became chimeric in 100% of the mice tested (N=9), with a survival rate of 90% (N=10). Likewise, LCMV-infected TCR-αβ knockout mice had a high rate of both chimerism (89%, N=9) and survival (89%, N=9). Transplantation into LCMV-infected CD4 or TCR-γδ knockout mice resulted in slightly improved survival, but no engraftment of
donor bone marrow was detected. Together, these data strongly imply that a population of CD8$^{+}$TCR-αβ$^{+}$asialo-GM1$^{+}$ T cells prevents allogeneic bone marrow engraftment in LCMV-infected recipients treated with irradiation and anti-CD154 mAb.
Table 12. Chimerism and survival in CD4, CD8, TCR-αβ and TCR-γδ knockout mice

<table>
<thead>
<tr>
<th>LCMV Infection</th>
<th>Recipient</th>
<th>N</th>
<th>Chimerism at 2 weeks*</th>
<th>Survival at 6 Weeks</th>
<th>% of Donor-Origin PBMC in Chimeric Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>C57BL/6 wild type</td>
<td>12</td>
<td>6/12</td>
<td>12/12</td>
<td>54 ± 14</td>
</tr>
<tr>
<td></td>
<td>CD4 KO C57BL/6</td>
<td>9</td>
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<td>C57BL/6 TCR-γδ KO</td>
<td>8</td>
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<td>8/8</td>
<td>65 ± 24</td>
</tr>
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<td></td>
<td>C57BL/6 TCR-αβ KO</td>
<td>8</td>
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<td>8/8</td>
<td>88 ± 11</td>
</tr>
<tr>
<td></td>
<td>CD8 KO C57BL/6</td>
<td>10</td>
<td>10/10</td>
<td>10/10</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>LCMV Day 0</td>
<td>C57BL/6 wild type</td>
<td>12</td>
<td>0/8</td>
<td>0/12</td>
<td>&lt;1</td>
</tr>
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<td>9</td>
<td>0/4</td>
<td>2/9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 TCR-γδ KO</td>
<td>8</td>
<td>0/6</td>
<td>5/8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 TCR-αβ KO</td>
<td>9</td>
<td>8/9</td>
<td>8/9&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>9/9</td>
<td>9/10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81 ± 12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Indicates significantly different from controls.
<sup>b</sup> Indicates a trend.

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Legend to Table 12: Groups of C57BL/6 wild type and C57BL/6 CD4, CD8, TCR-αβ or TCR-γδ knockout mice were treated with 6 Gy of irradiation, 25 million BALB/c bone marrow cells and anti-CD154 mAb according to our standard transplantation protocol as described in Methods. Immediately after transplantation, mice in each of the five groups were randomized, and half of each group was injected with LCMV on the day of transplantation. The percentage of donor-origin PBMC was determined by flow cytometry 2-4 and 7 weeks after transplantation as described in Methods. Each data point represents the mean ± 1 s.d. *Some mice did not survive to the two week time point, the first time point at which the extent of chimerism was quantified in this experiment. a: p=N.S. vs. untreated control; b: p<0.001 vs. untreated control (Fisher exact statistic).
10. The prevention of allogeneic bone marrow engraftment does not require the Fas/FasL pathway or perforin

After discovering that CD8⁺TCR-αβ⁺asialo-GM1⁺ T cells are involved in the rejection of allogeneic bone marrow in mice treated with sublethal irradiation, anti-CD154 mAb, and an LCMV infection on the day of transplantation, we next sought to determine the mechanism responsible. T cells can destroy allogeneic bone marrow cells through several mechanisms, including the release of perforin, granzyme, or TNF-α, as well as by activating the Fas-FasL pathway (44,45).

To help determine which of these mechanisms may be involved, we performed allogeneic bone marrow transplants into Fas⁺br/ Fas⁻br, FasL⁺ld/ FasL⁻ld and pfp/pfp (perforin) C57BL/6 knockout mice. As shown in Table 13, the absence of Fas, FasL or perforin did not alter the engraftment of allogeneic bone marrow or the survival of uninfected mice. When these mice were infected with LCMV on the day of transplantation, all four groups contained undetectable levels of donor cell engraftment. Furthermore, the vast majority of these mice died 2-3 weeks after transplantation. These results suggest that neither the Fas/FasL pathway nor the release of perforin are required for the prevention of allogeneic bone marrow engraftment in recipient mice treated with sublethal irradiation, anti-CD154 mAb, and given an LCMV infection on the day of transplantation. Further experiments will be necessary in order to determine whether the release of granzymes or TNF-α are required for the rejection of allogeneic bone marrow in mice treated with sublethal irradiation, anti-CD154 mAb, and an LCMV infection on the day of transplantation.
Table 13. Chimerism and survival in Fas\textsuperscript{lpr}/Fas\textsuperscript{lpr}, FasL\textsuperscript{gld}/FasL\textsuperscript{gld} and pfp/pfp knockout mice

<table>
<thead>
<tr>
<th>LCMV Infection</th>
<th>Recipient</th>
<th>N</th>
<th>Chimerism at 8 weeks*</th>
<th>Survival at 8 Weeks</th>
<th>% of Donor-Origin PBMC in Chimeric Mice</th>
</tr>
</thead>
<tbody>
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<td>77 ± 16</td>
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<td>FasL\textsuperscript{gld}/FasL\textsuperscript{gld} C57BL/6</td>
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<td>5/5</td>
<td>5/5</td>
<td>90 ± 3</td>
</tr>
<tr>
<td></td>
<td>pfp/pfp KO C57BL/6</td>
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<td>4/4</td>
<td>4/4</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>LCMV Day 0</td>
<td>C57BL/6 wild type</td>
<td>5</td>
<td>0/5</td>
<td>0/5</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Fas\textsuperscript{lpr}/Fas\textsuperscript{lpr} C57BL/6</td>
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<td>0/5</td>
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<td>&lt;1</td>
</tr>
<tr>
<td></td>
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<td>4</td>
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</table>
Legend to Table 13: Groups of C57BL/6 wild type and Fas$^{lpr}$/ Fas$^{lpr}$, FasL$^{gld}$/ FasL$^{gld}$ and pfp/pfp C57BL/6 knockout mice were treated with 6 Gy of irradiation, 25 million BALB/c bone marrow cells and anti-CD154 mAb according to our standard transplantation protocol as described in Methods. Immediately after transplantation, mice in each of the four groups were randomized, and half of each group was injected with LCMV on the day of transplantation. The percentage of donor-origin PBMC was determined by flow cytometry 8 weeks after transplantation as described in Methods. Each data point represents the mean ± 1 s.d.
11. Chapter III Summary

Allogeneic bone marrow engraftment successfully occurs in C57BL/6 mice using a combination of sublethal irradiation and anti-CD154 mAb treatment. However, LCMV infection at the time of transplantation prevents the engraftment of bone marrow and causes death 2-3 weeks after transplantation. The death of these mice was associated with a severe hypoplasia in both spleen and femurs of LCMV-infected mice. Furthermore, death was dependent on the combined treatment of 6 Gy irradiation, LCMV infection at the time of transplantation, and an allogeneic bone marrow transplant.

The prevention of bone marrow engraftment and the induction of mouse mortality appear to be due to two distinct and separate mechanisms. Mortality requires the presence of host IFN-αβ receptor expression and rejection of allogeneic bone is due to a population of CD8+ T cells that express both TCR-αβ and asialo-GM1.
DISCUSSION

Currently, the transplantation of human hematopoietic stem cells relies on the combination of lethal conditioning and immunosuppression. The use of lethal conditioning leads to severe neutropenia, which results in a variety of bacterial, fungal and viral infections (163). Moreover, these infections have been implicated in allograft rejection, the enhancement of GVHD, and the loss of graft function (6,228-230).

In attempts to overcome these obstacles, a new wave of protocols for stem cell transplantation have focused on the induction of hematopoietic chimerism using sublethal irradiation. By reducing the toxicity involved, these protocols hope to substantially reduce the extent and duration of neutropenia and immune suppression associated with stem cell transplantation. Furthermore, the use of sublethal conditioning could make stem cell transplantation a realistic option for non-lethal malignancies, autoimmune diseases, and organ transplantation.

The use of costimulation blockade in combination with sublethal irradiation is one approach that has been used to induce hematopoietic chimerism. Costimulation blockade of T cell activation has been found to induce potent donor-specific non-responsiveness. Furthermore, costimulation blockade can facilitate the establishment of allogeneic hematopoietic chimerism, while also significantly reducing the toxicity of the conditioning regimen (4,181,184,185,259).

Despite the promise inherent in stem cell transplantation protocols based on costimulation blockade, significant issues of safety and durability remain to be assessed. In particular, patients treated with partial myeloablation combined with costimulation
blockade could be less resistant to viral infection and its associated pathophysiological effects. Furthermore, the ability of viruses to induce inflammatory cytokines, T cell growth and differentiation factors and virus-specific CTL that react to allogeneic targets could potentially compromise graft survival and overcome tolerance (5,260,261). Finally, many viruses can enhance GVHD after bone marrow transplantation (6,262).

To begin to investigate these issues, we have set up a model of allogeneic bone marrow transplantation based on sublethal conditioning and costimulation blockade that allows stable and long-term engraftment of allogeneic bone marrow in the absence of GVHD. We then used this model to test both the safety and efficacy of bone marrow transplantation based on costimulation blockade and sublethal conditioning by infecting mice with LCMV either at the time of tolerance induction or at selected time points after transplantation.

Using 6 Gy of irradiation, two doses of anti-CD154 mAb, and 25 million BALB/c bone marrow cells, hematopoietic chimerism was established in the majority of C57BL/6 recipient mice. Chimeric mice demonstrated long-term and stable mixed hematopoietic chimerism for up to one year after transplantation. Furthermore, the peripheral blood from chimeric mice contained high levels of donor-derived B and T cells, monocytes/macrophages and granulocytes, indicating that hematopoietic chimerism in these mice was multi-lineage.

To determine whether the conditioning regimen induced donor-specific tolerance, conditioned recipients were transplanted with both donor-origin and third party skin grafts. Skin grafts were used because they are considered one of the most stringent tests
for tolerance induction. All chimeric mice permanently accepted donor-specific BALB/c (H2d) skin grafts while readily rejecting third party CBA/JCr (H2k) skin grafts, demonstrating that T cell function was present and that mice were specifically tolerant to H2d-expressing cells.

A major complication in allogeneic hematopoietic chimeras is GVHD. In order to test for the presence of GVHD in our chimeric mice we performed histological analyses on small and large intestine, liver, lung and skin. None of these samples showed immune cell infiltration suggestive of GVHD.

Hematopoietic chimerism could be established in C57BL/6 mice using as little as 5 Gy of irradiation (Table I). However, only the combination of 6 Gy of irradiation or more in combination with anti-CD154 mAb consistently induced hematopoietic chimerism in the majority of recipient mice. Interestingly, anti-CD154 mAb was required for hematopoietic chimerism at 6 Gy, but not at 7 Gy. Although mice became chimeric at 7 Gy without anti-CD154 mAb, chimerism was transient and disappeared within 7 weeks of transplantation. Because our goal was to establish stable chimerism while reducing the toxicity of the host conditioning, the combination of 6 Gy irradiation and anti-CD154 mAb was used in all subsequent experiments.

The ability of anti-CD154 mAb to induce chimerism at 6 Gy suggests that anti-CD154 mAb lowers the required dose of irradiation necessary for successful bone marrow engraftment. Previous experiments transplanting C57BL/6 bone marrow into BALB/c recipients supports this concept. C57BL/6 bone marrow engrafted into BALB/c recipients treated with 5 Gy regardless of whether they received anti-CD154 mAb (190).
However, BALB/c recipients treated with only 4 Gy of irradiation required anti-CD154 mAb for engraftment. Therefore, in both C57BL/6 and BALB/c recipients, anti-CD154 mAb reduced the amount of irradiation necessary for hematopoietic chimerism by ~1 Gy.

Besides lowering the required dose of irradiation, the addition of anti-CD154 mAb has been implicated in the prevention of HVGD and GVHD. In a model of GVHD initiated by the injection of parental bone marrow into F1 recipient mice, the addition of anti-CD154 mAb prevented the induction of both acute and chronic forms of GVHD (263). Our laboratory has extended these observations by documenting that anti-CD154 mAb prevented both GVHD and HVGD in BALB/c hematopoietic chimeras created with a sublethal conditioning protocol (190). The experiments performed in this thesis confirm these results, as chimeric C57BL/6 mice that received 6 Gy of irradiation and anti-CD154 mAb showed no overt or histological evidence of GVHD.

It appears that there is an interplay between the amount of anti-CD154 mAb injected, the number of anti-CD154 mAb injections, the number of injected bone marrow cells, and the amount of irradiation that is necessary for successful engraftment of allogeneic bone marrow. Several laboratories have shown that increasing the amount of anti-CD154 mAb injections or increasing the amount of injected bone marrow cells lowers the minimum amount of irradiation necessary for allogeneic bone marrow engraftment (179, 182, 184). These results suggest that the amount and duration of anti-CD154 mAb, the level of irradiation and the number of injected allogeneic bone marrow cells can all interfere with the competition between donor and host hematopoietic stem cells in regard to their growth and expansion in the recipient.
Using our standard protocol of 25 million allogeneic bone marrow cells, 2 injections of anti-CD154 mAb and 6 Gy of irradiation, we observed three different outcomes. The majority of treated mice became chimeric and remained chimeric throughout the length of our studies. However, some mice failed to become chimeric, while other mice lost their chimerism shortly after transplantation. The latter tended to be mice with low initial engraftment that eventually disappeared. The loss of chimerism in these mice is most likely 1) the result of the few donor-derived cells that engraft being outcompeted by host marrow cells, or 2) the engraftment of progenitors and not true stem cells that accounted for the transient chimerism observed.

A low percentage of treated mice in our experiments never became chimeric. One explanation for the lack of chimerism in our experimental mice is the anti-CD154 mAb. Our laboratory is continuously generating new lots of purified antibody from ascites. Although quality control of each lot for concentration and endotoxin is performed, and the same lot of anti-CD154 mAb is always used within a single experiment, multiple lots of antibody have been used over the course of these experiments. Hence, any variability in the biological activity of anti-CD154 mAb may reflect subtle changes in the number of mice that become chimeric or in the level of donor cell engraftment that occurs. In support of this possibility, the majority of mice that failed to become chimeric were not dispersed evenly throughout the experiments, but rather were concentrated in just a few of the experiments performed. Unfortunately, the lots of anti-CD154 mAb used where mice failed to become chimeric were not available for retrospective analyses of purity or potency.
These data document a model system characterized by mixed hematopoietic chimerism and donor-specific transplantation tolerance in the absence of GVHD and minimal preparative risk to the recipient. Because the system accurately models an approach that could well be put into clinical practice, it was deemed appropriate for use in analyses of safety and durability in the presence of viral infection, which is a common complication of clinical bone marrow transplantation. We addressed the issue of safety by studying the effects of viral infection on mice undergoing treatment to induce hematopoietic chimerism. We also addressed issues of both safety and durability by examining the effect of delayed exposure to virus on mice in which mixed chimerism had been successfully established 15 or 50 days earlier.

The results of these studies were extremely surprising, as essentially all mice given an allogeneic bone marrow transplant and infected with LCMV on the day of transplantation died 2-3 weeks after transplantation. This is in stark contrast to uninfected controls, where only a few widely scattered deaths occurred.

Typically, an intraperitoneal injection of LCMV-Armstrong presents as a non-cytopathic infection that is cleared within two weeks (197). However, in mice treated with a sublethal dose of 6 Gy irradiation (10-11 Gy is lethal for C57BL/6 mice), injected with an allogeneic bone marrow transplant, and infected with LCMV at the time of transplantation, a lethal outcome was observed. This lethal outcome was not observed in mice that received syngeneic bone marrow and infected with LCMV on the day of transplantation.
In contrast, both syngeneic and allogeneic chimeras infected with LCMV 2 or 7 weeks after transplantation survived. The survival of mice that received a delayed LCMV infection suggests that LCMV causes the death of bone marrow transplant recipients only when infection occurs during the immediate period of allogeneic bone marrow transplantation. Further, it suggests that LCMV infection does not interfere with the maintenance of hematopoietic chimerism, indicating that once chimerism and tolerance are established they are unaffected by viral infection.

The differential survival of acutely infected syngeneic vs. allogeneic bone marrow recipients was not due to the differential ability of these mice to clear LCMV, as both syngeneic and allogeneic bone marrow recipients infected with LCMV on the day of transplantation were unable to clear the viral infection. The inability of these mice to clear a day 0 LCMV infection was not due to anti-CD154 mAb induced costimulation blockade, as LCMV-infected CD154 knockout mice generate a strong LCMV-specific CTL response that clears the LCMV infection (264). The persistence of virus in day 0 LCMV-infected mice is likely due to radiation-induced elimination of peripheral T cells, as treatment with 6 Gy induces a 50% decrease in splenic and femoral cell counts.

When LCMV infection was given 15 days after transplantation, allochimeric mice were still unable to clear the infection, whereas synchimeras successfully cleared the virus. Allochimeric mice were only able to clear the LCMV infection when it occurred 50 days after transplantation. As LCMV infection is cleared by LCMV-specific CD8+ CTL (215), it is likely that synchimeras recover the ability to produce this population of cells quicker than allochimeras. This reasoning is supported by our cellularity studies. Splenic
and femoral cell numbers in syngeneic chimeras returned to levels comparable to untreated control mice within 14 days of irradiation and transplantation (Figure 6). In contrast, the splenic cellularity of allogeneic chimeras took 7 weeks to recover 80% of the cell counts of untreated mice.

After documenting that LCMV infection abrogated the induction of hematopoietic chimerism but not the maintenance of chimerism, we next asked whether LCMV infection could interfere with either the induction or maintenance of donor-specific tolerance. We were unable to test the effect of LCMV infection on the induction of donor-specific tolerance in allogeneic chimeras as these recipients died soon after transplantation. In order to determine whether LCMV infection altered the maintenance of donor-specific tolerance, syngeneic and allogeneic chimeras that were stably chimeric were infected with LCMV immediately after being transplanted with donor-origin skin grafts. In both syngeneic and allogeneic recipients, LCMV infection at the time of skin grafting did not cause graft rejection suggesting that LCMV infection does not alter the maintenance of donor-specific tolerance.

In contrast to the findings presented here with LCMV-infected bone marrow transplant recipients, our laboratory has previously shown that LCMV-infection can abrogate the induction and maintenance of tolerance using a two-element protocol of DST and anti-CD154 mAb (238). In those experiments, LCMV infection enhanced the rejection of skin allografts, even when infection occurred 29 days after skin grafting. Only when LCMV infection occurred 51-57 days after skin grafting did LCMV-infected mice display similar graft survival to uninfected controls. These experiments suggest that
LCMV infection interfered with both the initiation and maintenance of donor-specific peripheral tolerance using a two-element protocol of DST and anti-CD154 mAb. In contrast, LCMV infection abrogated the induction of central donor-specific tolerance but did not alter the maintenance of central transplantation tolerance using allogeneic bone marrow, 6 Gy of irradiation, and anti-CD154 mAb.

The last goal of this thesis was to determine 1) the mechanism of LCMV-induced mortality in recipients of allogeneic bone marrow and a day 0 LCMV infection, and 2) the mechanism of LCMV-induced abrogation of allogeneic bone marrow engraftment.

Initially, we hypothesized that mortality was simply due to the lack of allogeneic bone marrow engraftment. This hypothesis would suggest that mice that receive irradiation, an acute LCMV infection, and anti-CD154 mAb treatment without receiving a bone marrow transplant would succumb to aplasia and death. Although 30% of the mice treated in this way died, the rate of death increased to >95% when an allogeneic bone marrow transplant was added to the conditioning regimen. When anti-CD154 mAb was removed from this conditioning regimen, approximately 20% of the mice still died suggesting that anti-CD154 mAb is not involved in the death of these recipients. Therefore, the combination of allogeneic bone marrow, 6 Gy of irradiation and an LCMV infection at the time of transplantation is sufficient to induce death.

To analyze why acutely infected allogeneic bone marrow recipients were dying, we performed histological and cellularity analyses at various time points after transplantation. Spleen and bone marrow cell counts were drastically reduced in acutely infected allogeneic bone marrow recipients compared to similarly treated LCMV-infected
syngeneic bone marrow recipients. The reduction in marrow cellularity averaged 86±12%, with a range of 60-97%, leading us to conclude that mice were dying from severe hematopoietic aplasia.

In the early 1970s a link between LCMV infection and hematopoietic aplasia was reported (265,266,266,267). In those experiments, LCMV-infected mice displayed severe decreases in both bone marrow and splenic cellularity, but this aplasia was only temporary and did not result in mortality. Ten days post-infection, splenic cell numbers returned to normal. In contrast, femoral cell counts remained depressed for up to three weeks after infection. The onset of hematopoietic aplasia was correlated with an increase in circulating IFN-γ and activated NK cells, indicating their possible role in LCMV-induced hematopoietic suppression.

Recently, it was discovered that LCMV infection of IFN-αβ receptor knockout mice (IFN-αβ R<sup>0/0</sup>) did not produce the typical hematopoietic stem cell depression seen in wild type control mice (251). This observation was unique to IFN-αβ R<sup>0/0</sup> mice, as LCMV-infection of mice deficient in NK cells, Fas, IFN-γ, perforin, CD4 or CD8 T cells all led to the characteristic decrease in femoral and splenic cell counts (251).

Based on these studies, we tested whether IFN-αβ was involved in LCMV-induced aplasia and death that occurred in acutely infected mice treated with 6 Gy of irradiation, anti-CD154 mAb, and given an allogeneic bone marrow transplant. Interestingly, all LCMV-infected IFN-αβ R<sup>0/0</sup> mice that received irradiation and anti-CD154 mAb survived. Histological examination of the femurs of LCMV-infected IFN-
αβ R0/0 mice showed normal cellularity 13 days after transplantation, suggesting that these mice had recovered from the effects of irradiation by this time and that host IFN-αβ receptor expression is involved in LCMV-induced aplasia.

Although LCMV-infected IFN-αβ R0/0 mice survived the conditioning regimen, allogeneic bone marrow was still unable to engraft into these mice. These results, for the first time, separated host death from allograft rejection. Therefore, host IFN-αβ receptor expression is necessary for LCMV-induced aplasia and mortality, and a second mechanism appears to be responsible for the rejection of allogeneic bone marrow.

After discovering that host IFN-αβ receptor expression is necessary for mortality, we tested whether circulating levels of IFN-αβ varied between LCMV-infected mice that received syngeneic or allogeneic bone marrow transplants. We hypothesized that allogeneic recipients that succumbed to LCMV-induced death would contain higher levels of circulating IFN-αβ than their syngeneic counterparts which survived. Surprisingly, this was not the case. Both syngeneic and allogeneic bone marrow recipients contained high levels of circulating IFN-αβ by day 2 post-infection, which dramatically decreased by day 14. At all time points tested, there was no significant difference in the circulating IFN-αβ levels between LCMV-infected recipients of syngeneic or allogeneic bone marrow.

To further elucidate the role of IFN-αβ in our studies, we injected mice conditioned with sublethal irradiation and anti-CD154 mAb with Poly I:C, a known inducer of type 1 interferon (268). All mice treated with irradiation, anti-CD154 mAb,
Poly I:C and then given an allogeneic bone marrow transplant survived. This was true for mice injected with Poly I:C only on the day of transplantation, as well as for mice that received two injections of Poly I:C (on day 0 and day +5).

Several differences may exist between Poly I:C injection and LCMV infection that would explain these results. First, the induction of type 1 interferon that results from Poly I:C injection is of less intensity and duration compared to the induction of these cytokines after LCMV infection (Dr. Craig Peacock, personal communications). A decrease in either the intensity or duration of IFN-αβ production could explain why Poly I:C injected mice survive, whereas LCMV-infected mice die.

A second possibility is that these two treatments result in IFN-αβ production at different sites within treated mice. Both LCMV infection and Poly I:C injection initiate the production of IFN-αβ. However, the ability of LCMV to infect bone marrow cells may also initiate a localized production of IFN-αβ, specifically in the bone marrow. In several studies, both in vivo and in vitro, IFN-αβ has been shown to directly suppress hematopoiesis (231,269-271). After irradiation-induced damage to the stem cells, this additional localized production of IFN-αβ may initiate the induction of aplasia and eventual death that is seen in LCMV-infected mice, but not in mice injected with Poly I:C. Furthermore, this localized production of type 1 interferon in the bone marrow would not have been detected in our experiments, where only circulating levels of IFN-αβ in the serum were examined.
A final possibility is that LCMV infection causes the induction of cytokines or a cell population that is not induced by Poly I:C infection. In our experiments, the removal of CD4+ T cells or NK cells did not alter LCMV-induced mortality, suggesting that these two cell populations are not involved. Two cytokines that may be involved are IFN-γ and TNF-α, as both cytokines inhibit hematopoietic progenitor cell growth (254), and therefore could combine with LCMV-induced IFN-αβ to produce recipient death. We have shown that IFN-γ is involved in LCMV-induced death, but have not looked to see whether production of this cytokine varies in LCMV-infection versus Poly I:C injection. Unfortunately, we have not yet determined whether TNF-α is involved in the ability of LCMV infection to cause allograft rejection or recipient death.

The final sets of experiments in this thesis were performed in order to determine the mechanism of allograft rejection in LCMV-infected mice treated with sublethal irradiation, anti-CD154 mAb and given an allogeneic bone marrow transplant. We first tested whether the rejection of bone marrow cells was specifically directed to allogeneic targets or whether the response was non-specific. When syngeneic and allogeneic bone marrow were simultaneously injected into uninfected recipients, 5 of 13 mice contained both allogeneic and syngeneic derived donor cells. In contrast, all 15 LCMV-infected mice that were injected with both syngeneic and allogeneic marrow contained syngeneic donor-derived cells, whereas none of these mice contained allogeneic donor-derived cells. This result suggests that our conditioning regimen induce a specific anti-allogeneic response in acutely infected mice treated with sublethal irradiation and anti-CD154 mAb. This allogeneic response was found to be dose-dependant, as the injection of 200 million
allogeneic bone marrow cells into LCMV-infected mice treated with irradiation and anti-CD154 mAb resulted in the engraftment of allogeneic bone marrow.

We next focused on the various cell populations that could be responsible for this LCMV-induced rejection of allogeneic bone marrow. NK cells were of particular interest because of their prominent role in the rejection of murine allogeneic bone marrow transplants (232,255,256). Furthermore, we have shown that Poly I:C infection at the time of transplantation prevents the engraftment of allogeneic bone marrow, and Poly I:C is a potent activator of NK cells (257). Using various antibodies to deplete NK cells, we have shown that the absence of NK cells does not prevent the rejection of allogeneic bone marrow associated with a day 0 LCMV infection. Mice depleted with either anti-NK1.1 mAb or anti-CD122 mAb had undetectable levels of donor cell engraftment.

One interesting observation from these NK cell depletion studies was the results obtained with anti-CD122 mAb depletion. In LCMV-infected recipients, CD122 depletion did not permit the engraftment of allogeneic bone marrow, but recipient survival was significantly enhanced. CD122, the interleukin-2 receptor beta chain, is found on monocytes/macrophages, NK cells, and activated CD8+ T cells (241-243). Monocytes and macrophages are known to produce IFN-αβ, and we have shown that LCMV-infected IFN-αβ R00 mice survive an LCMV infection. Therefore, we speculate that the beneficial effect of anti-CD122 antibody treatment on the survival of LCMV-infected allogeneic bone marrow recipients is due to the depletion of these IFN-αβ-producing myeloid lineage cells.
As opposed to the other NK cell depleting reagents, depletion of asialo-GM1\(^+\) cells prior to transplantation and a day 0 LCMV infection resulted in allogeneic bone marrow engraftment and recipient survival. Asialo-GM1 expression in C57BL/6 mice has been found on NK1.1\(^+\) cells, as well as on activated T cells that express TCR-\(\alpha\beta\), TCR-\(\gamma\delta\), CD4 or CD8 (258). As the experiments using anti-CD122 mAb and anti-NK1.1 mAb suggest that NK cells are not involved in LCMV-induced allogeneic bone marrow rejection, we next focused on the various populations of activated T cells that express asialo-GM1.

Using both depleting antibodies and knockout mice, the critical observation was that, after infection with LCMV, only three types of recipients survived and became chimeric: mice depleted of CD8\(^+\) T cells, CD8 knockout mice, and TCR-\(\alpha\beta\) knockout mice. These data indicate that the mediator of bone marrow allograft destruction in LCMV-infected mice treated with costimulatory blockade is a radioresistant CD8\(^+\) NK1.1\(^-\) asialo-GM1\(^+\) TCR\(\alpha\beta\)^+ T cell.

This conclusion is supported by the finding that anti-CD8 mAb treatment facilitates the induction of mixed hematopoietic chimerism in sub-lethally irradiated mice given anti-CD154 mAb plus allogeneic bone marrow (180). Furthermore, asialo-GM1\(^+\)CD8\(^+\) T cells have been implicated as the cause of allograft rejection in mice treated with the combination of anti-CD154 and CTLA4-Ig (258). These two studies indicate an important role for alloreactive CD8\(^+\) T cells in allogeneic bone marrow rejection. Here, we show that this host alloreactive activity can be greatly amplified by
virus infection to overcome costimulation blockade and prevent allogeneic marrow engraftment.

Similar to the results seen with anti-CD122 mAb, LCMV-infected TCR-γδ knockout mice showed a marked increase in survival compared to wild-type controls. We propose that this increased survival is due to the ability of TCR-γδ cells to produce IFN-γ (272, 273). IFN-γ can act either alone or synergistically with IFN-αβ to suppress the growth of hematopoietic stem cells (250, 269). Therefore, the loss of IFN-γ producing TCR-γδ cells may decrease the severity of hematopoietic suppression resulting in the increased survival of LCMV-infected TCR-γδ knockout mice.

In our final experiments, we sought to determine the mechanism by which CD8⁺TCR-αβ⁺asialo-GM1⁺ T cells prevent allogeneic bone marrow engraftment. T cells can destroy allogeneic bone marrow cells through several mechanisms, including the release of perforin, granzyme, or TNF-α, as well as by activating the Fas-FasL pathway (44, 45). Utilizing knockout mice that lacked functional Fas, FasL or perforin, we observed that LCMV infection at the time of transplantation resulted in the lack of allogeneic bone marrow engraftment in all three groups of knockout mice. Furthermore, the vast majority of infected mice died soon after transplantation, suggesting that neither the Fas/FasL pathway nor the release of perforin is the sole mechanism by which CD8⁺TCR-αβ⁺asialo-GM1⁺ T cells prevent allogeneic bone marrow engraftment in LCMV-infected mice. Further studies will be necessary in order to determine whether
granzyme or TNF-α is involved in LCMV-induced abrogation of allogeneic bone marrow engraftment.

Recently, it has been documented that mice conditioned with busulfan and treated with anti-CD154 mAb, CTLA4-Ig, and a bone marrow allograft lose that graft (but do not die) if infected with LCMV (274). In that report, the mechanism of allogeneic stem cell graft failure was not identified, but evidence was presented to suggest that both CD4+ and CD8+ T cells were important and the authors hypothesized that the mechanism was dependent on dendritic cells. In contrast, we clearly demonstrate that CD8+TCR-αβ-asialo-GM1+ T cells are involved in the rejection of allogeneic bone marrow grafts. The survival of mice treated with busulfan, in contrast to the death of mice treated with sublethal irradiation, may be due to the differential effects of busulfan (a hematopoietic stem cell selective toxin) versus irradiation (generalized toxin). Alternatively, the different mechanisms involved in graft rejection between these two systems may induce distinct responses with regards to cytokine release and/or cellular activation.

The discovery that CD8+TCR-αβ-asialo-GM1+ T cells prevent the engraftment of allogeneic bone marrow raises two interesting questions. The first is how our conditioning regimen of irradiation and anti-CD154 mAb tolerizes these alloreactive T cells, thus allowing allogeneic bone marrow engraftment in uninfected mice. The second question is how does LCMV infection override tolerance induction and activate the alloreactive T cells.

In previous experiments, our laboratory has documented the complete deletion of peripheral alloreactive CD8+ T cells after treatment with DST and anti-CD154 mAb
These experiments were performed using splenocytes as DST. In a similar manner, one would expect that in these experiments injected donor bone marrow cells would act as DST and induce the complete deletion of alloreactive CD8⁺ T cells.

Recently however, we have discovered that bone marrow, when used as DST, does not induce the complete deletion of peripheral alloreactive T cells (E. Seung-unpublished observations). In a preliminary experiment, bone marrow and anti-CD154 mAb deleted ~50% of the peripheral alloreactive T cells. Therefore, allogeneic bone marrow and anti-CD154 mAb does not cause the complete deletion of alloreactive CD8⁺ TCR-αβ⁺ asialo-GM1⁺ T cells. The question remains, what happens to the ~50% of peripheral alloreactive T cells that persist in mice given anti-CD154 mAb and an allogeneic bone marrow transplant.

One possibility is that the remaining T cells are eliminated when recipient mice receive 6 Gy of irradiation. In our experiments, treatment of C57BL/6 mice with 6 Gy reduced femoral and splenic cellularity by 50-60%. Therefore, it is possible that half of the alloreactive T cells are eliminated after irradiation and the other half are deleted following exposure to anti-CD154 mAb and allogeneic bone marrow.

Alternatively, the alloreactive T cells that remain following irradiation may be tolerized following exposure to allogeneic bone marrow and anti-CD154 mAb. Exposure of alloantigen to host T cells (Signal 1) under conditions where CD154 can not interact with its receptor, CD40 (Signal 2) can render allo-specific T cells anergic, while all other T cells would remain unaffected (115).
A second question that needs to be addressed is how do alloreactive T cells become activated following LCMV infection. As mentioned above, half of the alloreactive T cells in uninfected mice will be destroyed following irradiation, and the other half are most likely deleted following exposure to donor bone marrow and anti-CD154 mAb. It is unlikely that LCMV infection interferes with the irradiation-induced elimination of alloreactive T cells. Therefore, the real question is how does LCMV prevent the deletion of alloreactive T cells caused by exposure to anti-CD154 mAb and allogeneic bone marrow.

Previously, we have demonstrated the complete deletion of peripheral alloreactive CD8+ T cells after treatment with DST and anti-CD154 mAb (120). Additionally, we have shown that LCMV infection at the time of tolerization prevented this deletion (275). In fact, LCMV infection caused an increase in the levels of alloreactive CD8+ T cells. Preventing the induction of peripheral clonal deletion and tolerance has also been observed in mice treated with LPS (276-278), Poly I:C, or LCMV at the time of tolerance induction (278). In the experiments presented here, both Poly I:C and LCMV were able to prevent the engraftment of allogeneic bone marrow, confirming that Poly I:C and LCMV are able to overcome the induction of tolerance. However, the mechanism by which viruses, Poly I:C and LPS are able to overcome tolerance induction is still unknown.

One likely candidate for the ability of LPS, Poly I:C or viruses to overcome tolerance induction are cytokines. Some of the redundant cytokine pathways that may be involved are IL-1, IL-2, IL-12, TNF and IFN-γ (277,279). In one study, the prevention of
superantigen-induced deletion by LPS was associated with the production of TNF-α (277). The role of TNF-α or IFN-γ is particularly intriguing as TNF-α and IFN-γ can inhibit hematopoietic progenitor cell growth (254). IFN-γ can also act synergistically with IFN-αβ to suppress hematopoietic stem cell growth (250). Therefore, these two cytokines could potentially play a role in both overcoming tolerance induction and in LCMV-induced death. However, IFN-γ is not involved in overcoming tolerance induction in our system, as pretreatment with anti-IFN-γ mAb did not prevent allograft rejection induced by LCMV. In contrast, IFN-γ likely plays a role, along with IFN-αβ, in LCMV-induced aplasia as pretreatment of LCMV-infected mice with anti-IFN-γ mAb prevented LCMV-induced death. Additional experiments will be necessary in order to determine which cytokines, if any, are involved in preventing tolerance induction in LCMV-infected mice treated with sublethal irradiation and anti-CD154 mAb.

Recently, the role of dendritic cells in the ability of viruses to overcome tolerance induction has been investigated. Dendritic cells can be stimulated by various cytokines (IL-1, TNF-α and IFN-αβ), LPS, Poly I:C or soluble CD154 (280,281). Dendritic cell stimulation leads to the upregulation of MHC class II molecules and costimulatory molecules (CD80, CD86 and CD54) (282-284) as well as the release of several cytokines (TNF-α, IL-8, IL-10, IL-12) (283-285). Following activation and maturation, dendritic cells migrate to the draining lymph nodes, and acquire the capacity to produce IL-12 (286). CD40-CD154 interactions are critical for dendritic cell activation, as CD40 ligation is sufficient for dendritic cell maturation (284), and to enable dendritic cells to
prime CD8+ T cells (287). Furthermore, when CD40-CD154 interactions are blocked, dendritic cells fail to mature, (288) are unable to localize to draining lymph nodes (289) and show decreased survival (290).

In the last few years, it has also been documented that LCMV infection can activate dendritic cells in the absence of CD40-CD154 interaction (291). Dendritic cells isolated from CD40 knockout mice 6 days after LCMV infection expressed upregulated CD80 and CD86 surface expression. Therefore, CD80 and CD86 surface expression on LCMV-activated dendritic cells would be capable of activating the alloreactive T cells in our LCMV-infected mice treated with costimulation blockade.

The mechanism by which LCMV infection activates dendritic cells is currently unknown. One possibility is IFN-αβ, as LCMV infection greatly increases the production of IFN-αβ, and this cytokine is capable of activating dendritic cells (280). Recent experiments by Ruedl et al. however, have shown that either CD4+ or CD8+ T cells can activate dendritic cells in LCMV-infected mice (291). Although unsuccessful in discovering the mediator by which CD8+ T cells activate dendritic cells, they have eliminated several molecules and cytokines including: CD28, CD154, TRANCE, TNF, IL-1, IL-4, IL-6, IL-7, IFN-αβ and IFN-γ (291). The cytokine or factor involved is, therefore, not a commonly known activator of dendritic cells, and may even be a molecule that has not yet been discovered.

Although LCMV-induced activation of dendritic cells is one method of activating the alloreactive T cells in our LCMV-infected mice given sublethal irradiation and costimulation blockade, CD4+ T cell help may also be involved. Normally, anti-CD154
mAb would prevent the activation of CD4+ T cells by blocking the CD40-CD154 interaction. However, in LCMV-infected CD154 knockout mice, CD4+ T cells can still produce IFN-γ, suggesting that these cells are being activated in a CD40-independent manner. This CD4-CD154 independent mechanism of dendritic cell activation was soon solved with the discovery of TRANCE (TNF-related activation-induced cytokine) (292,293). Blocking the TRANCE-TRANCE-R interaction completely prevented the production of IFN-γ from CD4+ T cells isolated from LCMV-infected CD40 deficient mice (294), suggesting that the TRANCE pathway is capable of activating CD4+ T cells in the absence of CD40-CD154 interactions.

Therefore, there are two distinct pathways that could lead to LCMV-induced activation of dendritic cells, which in turn activate alloreactive T cells. One pathway involves the activation of CD4+ T cells through the TRANCE pathway, and the second pathway involves the activation of CD8+ T cells by an unknown mechanism. In our model of LCMV-induced abrogation of tolerance, CD4+ T cells are not necessary. Allograft rejection occurred in LCMV-infected CD4 knockout mice, suggesting therefore, at least with our conditioning regimen, that CD8+ T cells are sufficient to induce dendritic cell activation.

Our working model of how sublethal irradiation and anti-CD154 mAb induces hematopoietic chimerism and donor-specific tolerance is presented in Figure 10. Treatment of C57BL/6 mice with 6 Gy of irradiation has two effects. First, it destroys some of the host bone marrow cells, thus creating space for injected donor-derived bone marrow cells to engraft and expand. Second, irradiation deletes a portion of the peripheral
alloreactive T cells capable of destroying the injected donor bone marrow. Following irradiation, donor bone marrow cells and anti-CD154 mAb are injected into recipients. These injections expose the remaining host alloreactive T cells to donor alloantigen (Signal 1) under conditions where CD154 is unable to interact with its receptor, CD40 (Signal 2). As a result, alloreactive T cells receive Signal 1 without receiving Signal 2 and are deleted. With the complete deletion of host alloreactive T cells, donor bone marrow is not destroyed by alloreactive CD8^+TCR-αβ^+asialo-GM1^+ T cells. Stem cells in the injected donor bone marrow are therefore free to localize to the host marrow where space for their engraftment and expansion has been provided by irradiation. Donor dendritic cell precursors then migrate from the marrow to engraft in the thymus, where they develop into thymic dendritic cells and mediate negative selection of developing donor-reactive host T cells. The continued presence of both donor and host origin dendritic cells in the thymus ensures that both donor-reactive and host-reactive T cells are continually deleted in the thymus by negative selection, and as a result, permanent donor-specific state of central tolerance is obtained.

Figure 11 depicts our working model of how LCMV infection at the time of transplantation prevents the engraftment of allogeneic bone marrow and causes host death in recipients treated with sublethal irradiation and costimulation blockade. Initially, as in uninfected recipients (Figure 10), mice are treated with 6 Gy irradiation, creating space in the donor bone marrow and removing some of the peripheral alloreactive T cells. Next, the remaining peripheral alloreactive T cells are exposed to a combination of donor bone marrow, anti-CD154 mAb, and an LCMV infection. As was the case with
uninfected mice, alloreactive T cells receive Signal 1 (alloantigen) while unable to receive Signal 2 (anti-CD154 mAb). In this case however, LCMV infection causes the activation and upregulation of CD80 and CD86 on dendritic cells, possibly through signals mediated by CD8\(^+\) T cells despite blockade of CD40-CD154 interactions. Activated dendritic cells can therefore display alloantigen, as well as costimulatory molecules such as CD80 and CD86, to induce the activation of alloreactive CD8\(^+\)TCR-\(\alpha\)\(\beta\)+asialo-GM1\(^+\) T cells. This stimulation of alloreactive T cells by activated dendritic cells prevents the alloreactive T cells from being deleted. As a result, alloreactive CD8\(^+\)TCR-\(\alpha\)\(\beta\)+asialo-GM1\(^+\) T cells are available to recognize and destroy the injected donor bone marrow cells, thus preventing the establishment of hematopoietic chimerism. Furthermore, the activation of alloreactive T cells induces the release of cytokines, such as IFN-\(\gamma\) and possibly TNF-\(\alpha\). These cytokines then act in combination with LCMV-induced IFN-\(\alpha\)\(\beta\) to initiate a massive aplasia of hematopoietic stem cells, which results in the death of LCMV-infected mice that receive costimulation blockade and a normally sublethal dose of irradiation.
Specific Tolerance

Figure 10: Induction of Donor Specific Tolerance

STEP 1:
6 Gy irradiation

STEP 2:
-BALB/c BM
-Anti-CD154 mAb

Periphery:
-Allo-reactive CD8+ T cells

BALB/c Skin Graft

Allo-chimeric C57BL/6 mouse

C57BL/6 Mouse

BM Compartment
**Legend Figure 10:** This figure schematically depicts our working model of how sublethal irradiation and costimulation blockade induces hematopoietic chimerism and donor-specific tolerance in C57BL/6 mice.
Figure 11: LCMV Induces Graft Rejection and Host Death

Mechanism 2: Aplasia/Death

C57BL/6 BM → IFN-αβ → Aplasia/Death

STEP 1: 6 Gy

STEP 2A: LCMV → Cytokines → Allo-reactive CD8+ T cells

STEP 2B: BALB/c BM + anti-CD154 → IFN-γ, (TNF-α) → BALB/c BM rejection
Legend Figure 11: This figure schematically depicts our working model of how an LCMV infection at the time of bone marrow transplantation prevents the engraftment of allogeneic bone marrow and causes host death in C57BL/6 recipients treated with sublethal irradiation and costimulation blockade.
In summary, we have documented that a conditioning regimen of 6 Gy irradiation, anti-CD154 mAb and BALB/c allogeneic bone marrow can create stable hematopoietic chimerism and donor-specific tolerance in C57BL/6 recipients. The induction of tolerance and chimerism in our hematopoietic chimeras was extremely stable two weeks after transplantation, as LCMV infection at this time point did not prevent the maintenance of chimerism or tolerance. The recovery of anti-viral immunity in chimeric mice was slightly delayed, as 7 weeks of recovery after transplantation was necessary to recover the ability to clear an LCMV infection.

As opposed to their maintenance, the induction of tolerance and chimerism was extremely vulnerable in our recipient mice treated with costimulation blockade and sublethal irradiation. LCMV infection on the day of transplantation prevented the engraftment of allogeneic bone marrow. Furthermore, these mice succumbed to aplasia-induced death 2-3 weeks after transplantation. We have documented that the mediator of graft rejection is a CD8⁺TCR-αβ⁺asialo-GM1⁺ T cell and the mediator of death is the host response to IFN-αβ. It is important to note that both graft loss and a fatal outcome occurred after challenge with a virus that is usually non-cytopathic. It is of concern that more virulent agents might have similar adverse consequences in the context of less stringent conditioning or at later time points after bone marrow transplantation. Clinical application of stem cell transplantation protocols based on costimulation blockade and tolerance induction may therefore require initial patient isolation to facilitate the procedure and to protect recipients.
FUTURE DIRECTIONS

In this thesis work we have discovered that two different mechanisms are involved in LCMV infection preventing the engraftment of allogeneic bone marrow and causing death in sublethally irradiated recipients treated with costimulation blockade. We provide evidence that host response to IFN-αβ and IFN-γ are involved in LCMV-induced aplasia, while alloreactive CD8⁺TCR-αβ⁺asialo-GM1⁺ T cells are the mediator of allograft rejection. However, the experiments performed in this thesis leave many unanswered questions that require additional research. First, what is the mechanism by which IFN-αβ in LCMV-infected mice induces aplasia. Second, how do alloreactive CD8⁺TCR-αβ⁺asialo-GM1⁺ T cells become activated and reject the allogeneic marrow graft. Third, what is the role of cytokines in this process. Fourth, what is the potential role of virus-specific CD8⁺ T cells that cross-react with alloantigen that, following virus infection, become memory alloreactive CD8⁺ T cells.

Although we have clearly shown that host IFN-αβ receptor expression is necessary for LCMV-induced aplasia and death, we have not discovered what other cytokines may be involved in this process. Cytokines such as IFN-γ and TNF-α have been shown to suppress hematopoietic stem cell growth (254). Although we have evidence that IFN-γ is involved, we do not know its exact role in death, and the involvement of TNF-α has yet to be determined. Many viral infections can impair hematopoiesis and induce bone marrow failure in stem cell transplant recipients treated with conventional procedures; these include Epstein-Barr virus (295), cytomegalovirus...
(296,297), human herpesvirus 6 and 7 (298,299) and human immunodeficiency virus (300,301) among others. The ability of each of these viruses to induce IFN-αβ, IFN-γ and TNF-α may be a common factor in the ability of these viruses to impair hematopoiesis and cause graft failure. Our finding, that LCMV can induce hematopoietic aplasia and death even in recipients of sublethal irradiation is of concern and suggests that these viruses may still be problematic even in newer sublethal conditioning regimens. Therefore, it would be of interest to know the exact relationship between the viral infections associated with stem cell therapy and their corresponding cytokine production profiles. With this knowledge, clinicians could take appropriate measures to prevent hematopoietic suppression and aplasia from becoming life-threatening complications after stem cell transplantation using sublethal conditioning and costimulation blockade.

Another area of future research could be directed at the alloreactive CD8^+ TCR-αβ^+ asialo-GM1^+ T cell population that induces allograft rejection in our model. Interestingly, this population is capable of causing allograft rejection immediately after receiving 6 Gy of irradiation, suggesting that this population of T cells is radio-resistant. If these CD8^+ T cells are radioresistant, it would be of interest to learn whether they become activated and expand in order to destroy the donor allogeneic bone marrow, or whether their existing numbers in the periphery are sufficient for allograft rejection. Our studies suggest that this alloreactive response is dose-dependent, as 200 million donor allogeneic bone marrow cells induced engraftment in LCMV-infected mice. Therefore, it seems likely that this population of T cells does not expand, but rather is capable of
causing allograft rejection without expansion. Future research would help to further our understanding of these alloreactive radioresistant T cells. This research could ultimately help clinicians to recover T cell immunity as quickly as possible in patients who receive myeloablative therapies, and thus help to reduce the period of risk of infection that typically follows stem cell transplantation.

Another area of future research involves the role of virus-activated CD8+ T cells that are cross-reactive to alloantigen. Based on previous experiments in our laboratory and the laboratory of our collaborator, Dr. Raymond Welsh, it appears that virus-activated CD8+ T cells may express cross-reactivity to alloantigens, and memory alloreactive T cells could be generated by virus infection. Memory T cells are extremely hard to tolerize using a DST and anti-CD154 mAb protocol (Dr. Markees, personal communications). It would be interesting to see whether LCMV-immune mice could be tolerized using our method of sublethal irradiation, anti-CD154 mAb and allogeneic bone marrow; particularly, the ability of a second cross-reactive virus-infection at the time of transplantation to cause allograft rejection and death in LCMV-immune mice. Although we have discovered that a primary LCMV immune response can cause allograft rejection and death in our sublethally irradiated mice, we do not know the results of a memory LCMV immune response at the time of transplantation. Understanding the role of memory T cells in tolerance induction, allograft rejection and death is imperative if stem cell transplantation therapies are to be safe for patients with latent infections such as CMV.
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