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CD40-CD154 Blockade Facilitates Induction of Allogeneic Hematopoietic Chimerism and Transplantation Tolerance: A Dissertation

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CD40-CD154 BLOCKADE FACILITATES INDUCTION OF ALLOGENEIC HEMATOPOIETIC CHIMERISM AND TRANSPLANTATION TOLERANCE

A Dissertation Presented

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APPROVAL PAGE
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ABSTRACT

Allogeneic hematopoietic chimerism leading to central tolerance has significant therapeutic potential. Establishment of hematopoietic chimerism created by stem cell transplantation has been shown to prevent and cure a number of autoimmune diseases and induce the most robust and long-lasting form of transplantation tolerance known. However, the realization of the vast clinical potential of hematopoietic chimerism for induction of transplantation tolerance has been impeded by the toxicity of the host conditioning regimen and the development of graft-versus-host disease (GVHD). This thesis describes the development of stem cell transplantation protocols that 1) reduce the host conditioning regimen; and 2) abrogate the development of GVHD. When applied to the treatment of autoimmune diabetic NOD mice, a model of type 1 diabetes, stem cell transplantation was able to 3) prevent autoimmune recurrence; and 4) permit curative pancreatic islet transplantation.

I first describe a tolerance-based stem cell transplantation protocol that combines sub-lethal irradiation with transient blockade of the CD40-CD154 costimulatory pathway using an anti-CD154 antibody. With this protocol, I established hematopoietic chimerism in BALB/c mice transplanted with fully allogeneic C57BL/6 bone marrow. All chimeric mice treated with anti-CD154 antibody remained free of graft *vs.* host disease (GVHD) and accepted donor-origin but not third party skin allografts. It was similarly possible to create allogeneic hematopoietic chimerism in NOD/Lt mice with spontaneous autoimmune diabetes. Pancreatic islet allografts transplanted into chimeric NOD/Lt mice

were resistant not only to allorejection but also to recurrence of autoimmunity. I conclude that it is possible to establish robust allogeneic hematopoietic chimerism in sub-lethally irradiated mice without subsequent GVHD by blocking the CD40-CD154 costimulatory pathway using as few as two injections of anti-CD154 antibody. I also conclude that chimerism created in this way generates donor-specific allograft tolerance and reverses the predisposition to recurrent autoimmune diabetes in NOD/Lt mice, enabling them to accept curative islet allografts.

In order to further reduce the impediments associated with the implementation of allogeneic hematopoietic chimerism as a therapeutic modality, I adapted a costimulation blockade-based protocol developed for solid organ transplantation for use in stem cell transplantation. The protocol combines a donor-specific transfusion (DST) with anti-CD154 antibody to induce peripheral transplantation tolerance. When applied to stem cell transplantation, administration of DST, anti-CD154 antibody, and allogeneic bone marrow led to hematopoietic chimerism and central tolerance with no myeloablation (*i.e.* no radiation) and no GVHD in 3 different strains of mice. The development of donor-specific tolerance in this system was shown to involve deletion of both peripheral host alloreactive $CD8^+$ T cells and nascent intrathymic alloreactive $CD8^+$ T cells. In the absence of large numbers of host alloreactive $CD8^+$ T cells, the cell transfusion that precedes transplantation need not be of donor-origin, suggesting that both allo-specific and non-allo-specific mechanisms regulate engraftment. Agents that interfere with peripheral transplantation tolerance partially impair establishment of chimerism. I conclude that robust allogeneic hematopoietic chimerism and central tolerance can be

established in the absence of host myeloablative conditioning using a peripheral transplantation tolerance protocol.

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ABBREVIATIONS

APC, antigen-presenting cell

ATG, anti-thymocyte globulin

CMV, cytomegalovirus

CTL, cytotoxic T lymphocyte

DC, dendritic cell

DST, donor-specific transfusion

EBV, Epstein-Barr virus

GVHD, graft versus host disease

IDDM, insulin dependent diabetes mellitus

IL-2, interleukin-2

i.p., intraperitoneally

i.v., intravenous

mAb, monoclonal antibody

MHC, major histocompatibility complex

MST, median survival time

NK cell, natural killer cell

N.S., not significant

PBMC, peripheral blood mononuclear cells

sAg, superantigen

s.d., standard deviation

TCR, T cell receptor

CHAPTER I

GENERAL INTRODUCTION

Introduction

The modern era of transplantation has seen incredible advances due to the discovery of immunosuppressive drugs. Since the pioneering works of the 1950s, transplantation of a number of solid organs has become very successful and life-saving for almost all patients involved. But, even as the effectiveness and potency of the immunosuppressive drugs continually improve, the serious side effects that come with their usage are still ever present. These drugs induce a generalized suppression of the immune system in the patient that prevents rejection of the transplanted organ, but their ability to non-specifically affect the whole immune system is also their major toxicity. The field of transplantation is approaching a new era in which immunosuppressive drugs may no longer be needed. Researchers are now concentrating on targeting specific immune cells rather than inducing a generalized suppression of the patient's immune system. The most promising alternative to the use of immunosuppressive drugs for transplantation is the induction of transplantation tolerance.

The clinical definition of transplantation tolerance is the survival of foreign tissue in normal recipients in the absence of immunosuppression. For more then 50 years, the most robust and long-lasting form of transplantation tolerance has been the induction of allogeneic hematopoietic chimerism through stem cell/bone marrow transplantation. The

establishment of the chimeric state is thought to subsequently utilize the mechanism for deleting self-reactive lymphocytes (central tolerance) to also delete alloreactive T cells. In addition to the induction of allogeneic chimerism, stem cell transplantation has become an established clinical treatment for fatal disorders of the lymphohematopoietic system. Studies in both humans and animals have also demonstrated the ability of stem cell transplantation to cure several autoimmune diseases. In autoimmune disorders such as type I diabetes mellitus that require not only cure of the autoimmune process but also the replacement of damaged organ, allogeneic chimerism offers the possibility of achieving both goals simultaneously. The work presented in this thesis seeks to achieve these goals by creating clinically useful protocols and identifying mechanisms responsible for the generation of hematopoietic chimerism and the induction of tolerance.

History of Transplantation

From ancient times to the immunosuppression era

The earliest recorded attempts at tissue transplantation date back thousands of years. An ancient Chinese legend records the medical miracles of two famous surgeons, Pien Ch'iao and Hua T'o, during 2nd century B.C. They were reputed to have successfully transplanted tissues and organs, including the intricate heart, without inflicting pain to the patients. But these stories have been designated as more myth than history. However, the oldest record of successful transplantation surgeries can be found in the Sanskrit text of India, the *Sushruta Samhita*, written more than 2,000 years ago. The author, Sushruta, is described taking autogeneous pedicled grafts from the forehead, neck, and cheek to restore mutilations of the nose, ear, and lip. The work of Shushruta is one of the greatest of its kind in the Sanskrit literature and is of special importance in the history of surgery due to its emphasis on the need for extensive training to produce skillful surgeons (1). Not to be outdone by the East, the West also boasted feats of transplantation marvels. Among the classic legends, the Saints Cosmos and Damian were reputed to have transplanted the leg of a dead Moor to a patient whose leg had been amputated. This famous legend was a great favorite among medieval artists (1).

The modern revival of transplantation began with John Hunter (1728-1793), the father of experimental surgery, who moved the surgical field from a mechanical art to an experimental science. He believed that all living substances had the disposition to unite when brought into contact with one another and, thus, helped establish the scientific basis

of modern surgery (2). But it wasn't until the early 1900s that modern transplantation emerged as a promising field with the pioneering work of Nobel laureate Alex Carrel, who developed vascular suture techniques that are still used for transplantation today. With this technique, surgeons were finally able to transplant a variety of organs, such as the kidneys, the heart, the thyroid and parathyroids, and the ovaries (3). But the kidney became the pilot organ for research on transplantation because it could be removed easily and transplanted quickly. They were also convenient because excretory capacity allowed assessment of the transplant's renal function with simple serial observations. However, the continued failure to achieve allograft survival by the surgeons severely diminished the field of tissue transplantation by the 1920s. It would take 20 years and a world war for interest in the field to make a comeback.

The basic "laws of transplantation" were known as far back as 1912, as published by Georg Schone (4). His observations of the general rules of transplantation are still valid to this day:

1) autografts generally succeed
2) allografts usually fail
3) allograft rejection is not immediate, but delayed (takes time)
4) rejection of second graft is accelerated if recipient previously rejected the graft from same donor

Interestingly, most of his observations were based on results from tumor research that were expanded to encompass skin and other organs (4). It was not until World War II that the immunologic basis for allograft failures began to be understood. Peter Medawar,

a zoologist working in England, became interested in the problems of skin grafting after it was realized that seriously burned pilots required extensive medical procedures to survive. In his classic experiments, Medawar observed that the survival of autologous and allogeneic skin grafts on a human burn victim (5) and rabbits (6) experimentally supported the "laws of transplantation" observed decades earlier. Medawar's subsequent research helped establish the concept of immunological factors participating in graft rejection, thus bringing the field of immunology into transplantation.

In the next decade, the first successful kidney transplantation was performed between monozygotic twins in 1954 by Joseph Murray, who was later awarded the Nobel Prize for this achievement (1). Despite the success of this and other transplants between monozygotic twins, transplanting tissues between genetically disparate patients remained largely unsuccessful. A number of important observations in the mid-1950s helped elucidate the role of the immune system in the rejection of transplanted tissues. Rupert Billingham, Leslie Brent, and Peter Medawar reported the use of lymphoid cells from sensitized donors to "adoptively transfer" immunity against skin grafts and other tissues in the mouse (7). Other researchers also demonstrated that lymphocytes can directly attack transplanted tissue even in the absence of antibody (1). These studies led to the recognition that cellular, rather than humoral, factors were actively involved in transplantation rejection. These results also led to the recognition that regulation of these cellular factors through immunosuppression might overcome the "laws of transplantation".

Effective drugs to alter the immune system were not available at that time, so the first attempts at immunosuppression involved total body irradiation. Although irradiation proved to be effective in preventing the rejection of the grafts, it led to a very high mortality rate. In the early 1960s, Roy Calne introduced the first line of immunosuppressive drugs, 6-mercaptopurine, into clinical practice after successfully transplanting kidneys in dogs. This advance was followed soon thereafter by the development of a longer acting and less toxic derivative of the drug, azathioprine. During this decade, many other organs were transplanted for the first time: the liver by Thomas Starzl in 1963, the lung by James Hardy in 1963, the pancreas by Richard Lillehei in 1966, and the heart by Christiaan Barnard in 1967 (1).

Organ transplantation from unrelated donors was now possible, but graft survival remained poor, with the best center achieving 1-year survival rates of 50% for cadaver kidney transplants. Then, the chance discovery of a promising agent from a fungal strain changed dramatically the success rate for organ transplantation. Cyclosporine A, discovered by Jean-Francois Borel in 1972, was found to disrupt T lymphocyte activation by blocking the production of interleukin-2 (IL-2). The use of cyclosporine dramatically improved graft survival and quickly became the standard immunosuppression in transplant recipients. Over the last decade additional immunosuppressive agents have been discovered such as tacrolimus (FK-506), which inhibits both IL-2 production and IL-2 receptor expression, and sirolimus (rapamycin). Biological immunosuppressants have also come onto the field such as anti-thymocyte globulin (ATG) and OKT-3 anti-CD3 monoclonal antibody (mAb) that target the immune cells.

Problems with Immunosuppression

The achievements in organ transplantation after the introduction of immunosuppressive drugs have been amazing. Over the past two decades the development of new immunosuppressive drugs and a better understanding of how they work have led to marked improvement in patient survival and short-term graft survival. The 1-year survival of cadaveric renal allografts in the pre-cyclosporine era was 65%, whereas it now exceeds 80-85% (3;8). Similar success has been achieved in the transplantation of other major solid organs such as the liver, heart, lung and pancreas. But in spite of this success, immunosuppressive drugs have major residual problems that affect the life and livelihood of the patients.

There are now a number of different immunosuppressive drugs available for transplanted patients ranging from anti-metabolites such as azathioprine and mycophenolate mofetil, to calcineurin inhibitors such as cyclosporine and tacrolimus. But all these drugs are toxic to some degree to both the patient and the transplanted tissue. Anti-metabolites have been associated with bone marrow suppression and liver failure (9). The well known adverse effects of glucocorticoids include hypertension, hyperlipidemia, diabetes mellitus and osteopenia (10). Even though calcineurin inhibitors, specifically cyclosporine, revolutionized transplantation with their potent immunosuppressive activity, they have the most toxic effects among the different drugs. The most important are the neurologic, endocrinologic, hepatic, and renal toxicities. Patients can experience tremors and seizures due to affects on the nervous system and as many as 85% of renal transplant recipients will be treated for hypertension because of

cyclosporine A therapy (11). As transplant recipients survive longer, post-transplant diabetes mellitus (PTDM) has emerged as a major adverse effect of immunosuppressants. Corticosteroids have been shown to induce insulin resistance, while cyclosporine and tacrolimus are thought to inhibit insulin secretion and production, and also induce peripheral insulin resistance (12). Biological immunosuppressants, such as anti-lymphocyte serum (ALS) and ATG, do not have as much toxicity as their chemical counterparts, but they do significantly impair cell-mediated immunity resulting in opportunistic infections and an increased frequency of malignancies. The incidence of cytomegalovirus (CMV) infection was observed to be twice as high in liver allograft recipients treated with ALS compared to those treated with conventional immunosuppression (13). Another problem associated with these polyclonal antibodies is that there is inter-batch variation in their potency making effective treatment difficult.

Another major problem associated with immunosuppressive drugs is "chronic rejection." Even though the overall 1-year survival for cardiac transplantation is 79%, the patient half-life (time to 50% survival) is only 8.7 years. The decline in survival is almost a straight line from year one through year 14 with a constant mortality rate of 4% per year (14). The outlooks are very similar with the other major organs. A majority of transplanted grafts will function for a year, but a steady decline in survival can be seen as the years accumulate. The pathophysiology of chronic allograft rejection is poorly understood. It is widely acknowledged that both immunologic and nonimmunologic factors are likely to play a role in the pathogenesis (15).

The key mechanism of immunosuppressive drugs is their ability to block lymphocyte functions, but their caveat is that it is a generalized block that affects the whole immune system. People with an impaired immune system are generally more susceptible to virus infections than those who are fully immunocompetent. Patients with T cell deficiencies are more liable to experience infections with viruses, as well as intracellular bacteria, fungi and some protozoa. Infection with CMV is extremely common after transplantation, particularly in recipients who are seronegative for CMV and who receive seropositive organs. Because of the heightened immunosuppression and the risk of receiving seropositive grafts, endogenous viruses such as the herpesviruses (*i.e.*, CMV, EBV) and hepatitis viruses are commonly associated with transplant patients (16). Without a fully competent immune system, transplant patients depend on effective anti-viral or anti-bacterial drugs to combat any deadly microorganisms. But even though most infections can be treated, highly drug-resistant organisms have been detected with increasing frequency (17). The loss in the drugs' effectiveness would detrimentally affect the lives of these patients.

The increasing numbers of successful long-term transplant patients has heightened concern about the development of malignancies. The increased incidence of neoplastic diseases in transplant recipients is well recognized. The risk of lymphoproliferative diseases and skin cancer after transplantation ranges between 2 to 25 times the normal expectations (18), with the latter being the most common. The most important discoveries of the past two decades in cancer epidemiology relate to the carcinogenic effects of infectious pathogens. There is good evidence that Epstein-Barr

virus (EBV) is responsible for most lymphoproliferative diseases in immunosuppressed patients (19). Infection with the papillomavirus has also been thought to cause many of the cases of skin cancer in transplant recipients (16).

Another major issue is recurrence of disease in a successfully transplanted organ. Tissue-specific autoimmune diseases, like type I diabetes mellitus, can reoccur because of the persistent existence of destructive lymphocytes and antibodies in the host. A diabetic patient transplanted with both kidney and insulin-producing pancreas from an identical twin donor will indefinitely accept the kidney graft without immunosuppression due to lack of an alloresponse in the host because of the complete MHC match. But the insulin-producing beta cells in the pancreatic graft will be destroyed because of the continued existence of autoreactive lymphocytes (autoimmune recurrence) (20). Relatively high amounts of immunosuppressants are needed not only to prevent allorejection, but to also break the autoimmune memory. Unfortunately, standard regimens of immunosuppressive drugs were not effective for islet transplantation in diabetic patients for many years. Recently, however, a new protocol termed the "Edmonton protocol" has attained a high success rate for successful islet transplantation in diabetic patients (21). But further study is needed to determine if disease recurrence has truly been blocked in these cases. In diabetic animal models transplanted with syngeneic pancreatic islets and treated with a similar immunosuppressive therapy, initial graft survival is attained but eventually the islet grafts are rejected due to the persistent autoimmune response (22).

Immunosuppressive drugs have made an incredible impact in the transplantation field, but their deleterious side-effects have limited the full curative potential of organ transplantation. In an effort to find alternatives to generalized immunosuppression, intense research is now focused on the induction of transplantation tolerance.

Mechanisms of Tolerance

Definition of Tolerance

Tolerance is a process that comprises the whole repertoire of immune cells and regulatory pathways to prevent reactivity against a specific antigen (or antigens). The most basic concept of tolerance is the elimination or neutralization of lymphocytes specific for self-peptides and self-MHC to prevent autoimmunity (self-tolerance). This process involves both "central tolerance" and "peripheral tolerance" mechanisms. With the introduction of modern tissue transplantation, the term "transplantation tolerance" has emerged with two complementary definitions. It is defined clinically, or functionally, as the survival of foreign tissue in normal recipients in the absence of immunosuppression. It is also defined immunologically as the complete absence of a detectable immune response to a functional graft in the absence of immunosuppression (23). The rationale for this distinction is that experimental studies have shown that allogeneic grafts can survive and function *in vivo* even when donor-specific killing is observed using *in vitro* assays (split tolerance) (24). Additionally, prolonged survival of allografts has been demonstrated despite the presence of cellular infiltration (25). Since the success of tissue transplantation is determined by the survival of the graft rather than the cellular interactions involved, the functional definition is used more widely. In order to better understand how to induce transplantation tolerance, one has to first understand self tolerance. By manipulating Mother Nature's mechanism for tolerance against self, we can expand that tolerance to include foreign donors. The next few chapters will highlight

the mechanisms by which self tolerance is maintained in the body through central and peripheral tolerance mechanisms.

Central T-Cell Tolerance

Central tolerance refers to a tolerant state established primarily in the thymus through clonal deletion of developing antigen-reactive T cells. Central tolerance is the principal mechanism that eliminates autoreactive T cells and generates a state of self-tolerance. The human body can generate a diverse repertoire of T cells based on random gene rearrangement of the T cell receptor (TCR) DNA combined with junctional diversity. These "uneducated" T cells would not only recognize foreign antigen on self-MHC molecules, but would also recognize self antigens leading to autoimmunity. The potential antigenic diversity of T cells is tremendously reduced during maturation by a selection process in the thymus that allows only MHC-restricted (positive selection) and non-self-reactive (negative selection) T cells to survive.

T cell precursors arising from bone marrow stem cells migrate to the cortex of the thymus where they proliferate into immature $CD4^{+}CD8^{+}$ thymocytes expressing T cell receptors (TCR) on the cell surface. Only thymocytes that express a functional TCR can survive and proceed to the next stage of development. These immature T cells are now able to interact with antigen-presenting cells (APC) that express self-MHC molecules bearing self-peptides (26). This MHC-peptide complex is recognized by the TCR, and signals transduced through the TCR constitute a key element in selection of the developing thymocytes. The current understanding is that the interaction of immature double-positive thymocytes with thymic epithelial cells, mediated by MHC-restricted T-

cell receptors, allow the cells to receive a protective signal permitting their survival, a process termed positive selection (27). In contrast, cells whose receptors are not MHC restricted would not interact with the epithelial cells and would consequently not receive the protective signal, thus leading to their death by apoptosis, commonly referred to as "death by neglect" (28;29).

The population of MHC-restricted thymocytes that survive positive selection comprises some cells with low-affinity receptors for self-antigens and other cells with high-affinity receptors. The latter group of cells undergoes negative selection by interacting with bone marrow-derived APCs comprised mostly of dendritic cells and macrophages (30;31). During negative selection, thymocytes bearing high-affinity receptors for self-antigen plus self-MHC molecules interact with APCs bearing class I and class II MHC molecules and undergo rapid apoptotic cell death (32-35). Tolerance to self-antigens is thereby achieved by eliminating autoreactive double-positive thymocytes and only allowing maturation of T cells that bear low or moderate affinity to a self-MHC-peptide complex. The process of clonal deletion in the thymus has been studied experimentally by following the intrathymic development of specific populations of T cells that express unique TCR Vβ sequences or transgenic TCR specific for a known peptide antigen.

The mechanism involved in clonal deletion to induce self tolerance postulates that all self-reactive T cells are eliminated before their migration into the periphery (34;36). Therefore, based on this mechanism, it can be hypothesized that introduction of transplantation antigen into the thymus should result in the selective deletion of all T cells

reactive to that antigen and should therefore induce tolerance (23). Several potent protocols for inducing transplantation tolerance have taken successful advantage of this process in murine models, such as intrathymic injections (37;38) and allogeneic chimerism (39;40). This subject will be discussed below with a major emphasis on induction of allogeneic hematopoietic chimerism.

Peripheral T-Cell Tolerance

Peripheral tolerance refers to a tolerant state that is primarily established outside of the thymus and its selection process. Even though intrathymic central tolerance is very efficient, it does not delete all T cells that recognize self-antigen/MHC complexes. It is evident that there are some autoreactive T cells circulating in the peripheral tissues, but in most individuals, they do not lead to the expression of autoimmune disease (41;42). Thus, it appears that there are peripheral mechanisms that control the destructive potential of T cells that have escaped negative selection in the thymus. It is believed that peripheral tolerance is induced and maintained by a series of distinct but interrelated mechanisms (23). The major mechanisms involved are clonal deletion, anergy, and immune suppression/regulation. Other processes such as immune deviation, T cell exhaustion, ignorance, and immune privileged sites are important, but are beyond the scope of this thesis and are not discussed further.

Clonal Deletion

Intrathymic clonal deletion has long been recognized as a primary mechanism of tolerance induction to self antigens, but clonal deletion of peripheral T cells has recently

also been recognized to be important in the induction of tolerance. Peripheral deletion of T cells was originally identified in systems in which strong responses against either superantigens (43-45) or conventional antigens (46;47) resulted in the disappearance of responding T cells. More recently, experimental studies using self antigens have demonstrated the deletion of self-reactive T cells in the periphery (41;42;48).

It has been suggested that apoptosis via the Fas/FasL pathway may play an important role in peripheral T cell deletion and tolerance (41;49). Surface expression of Fas (CD95) is upregulated on many activated T cells. The interaction between Fas and its counter-receptor FasL (CD95L) leads the cell expressing Fas to undergo programmed cell death by apoptosis (50). This process is thought to be important in the down-regulation of T cell responses (51) as evidenced by the lymphoproliferative disorders in Fas- or FasL-deficient mice and humans. So, not only are Fas and FasL important in peripheral deletion to regulate T cell homeostasis, they may also be crucial in the deletion of self-reactive T cells in the periphery (52).

Anergy

Anergy is a state in which T cells stimulated by TCR engagement are functionally unresponsive and refractory to activation (53). In addition to the primary signal generated by the TCR/MHC complex, T cells require a second costimulatory signal (*i.e.* CD28/B7 interaction) to become optimally activated. Further overview of this process will be discussed below in the section entitled "Co-stimulatory blockade and tolerance induction." Anergy is readily induced *in vitro* by T cells binding to their specific ligands in the absence of costimulation (54). Anergic cells are characterized by the inability to

produce IL-2 upon secondary challenge with antigen in conjunction with optimal costimulation. However, a common characteristic of anergic cells is their ability to reverse their unresponsiveness and proliferate in response to exogenous IL-2 (55). But anergy is not automatically maintained once induced; a continual presence of antigen is required to maintain their unresponsive state (56;57).

Induction of anergic T cells *in vivo* has been demonstrated in mice after administration of tolerizing doses of bacterial superantigens (sAgs), Mls-1a, and peptide antigens that do not cause deletion of antigen-reactive T cells (58). A number of differences have been shown between T cells anergized *in vivo* and T cell clones anergized *in vitro*. The former has been found to be more resistant to stimulation with exogenous IL-2 than the latter (59). This difference may reflect the constitutive expression of high affinity IL-2 receptors on the T cell clones, but not on normal T cells (58). But a common feature between the *in vivo* and *in vitro* anergized T cells is their profound defect in the expression of CD154 (60). CD154 is expressed mostly on activated $CD4^+$ T cells and plays a critical role in delivering costimulatory signals for T cell activation (61). More information on this costimulatory molecule will be discussed below.

T cell anergy has been suggested to play an important role in allograft tolerance in many systems. However, peripheral tolerance relying primarily on anergy may be unstable due to their readily reversible nature and the reliance on continued antigen stimulation to maintain the anergic state. Such tolerance may be easily broken through

exogenous stimuli. For example, it has been demonstrated that infections and consequent inflammation can break anergy-dependent tolerance (62;63).

Immune regulation / suppression

Lymphocyte subpopulations that were able to suppress immune responses were first described over thirty years ago (64). However, the inability to clone these suppressor T cells and to identify any specific cell markers led researchers to lose interest in this entire field of study in the 1980s (65;66). But recently there has been a resurgence of interest in this area after Sakaguchi and his colleagues demonstrated that the interleukin-2 receptor (IL-2) α-chain molecule, CD25, can be used as a marker for cells with suppressive activity (67;68). They showed that a minor population (~10%) of $CD4^+$ T cells expressing CD25 is important for the control of autoreactive T cells *in vivo*. Subsequently, other groups have identified additional cell populations that also have suppressive properties. The majority of regulatory T cells identified to date are within the $CD4^+$ population, but other T cell subsets, such as $CD8^+$, $CD8^+CD28^-$, and $TCR^+CD4^-CD8^-$ have also been shown to contain cells with regulatory capacities (69-72). Although the term 'regulatory T cell' has replaced the term 'suppressor T cell' in the immunology literature, the former can mean either the enhancement or suppression of the immune response. In the transplantation tolerance field where specific down-regulation of immune responses is desired, I will use the term regulatory T cells to describe their suppressive activity in this thesis.

Studies *in vitro* have shown that the $CD4^+CD25^+$ regulatory cells do not proliferate in response to stimulation unless exogenous IL-2 is added to the culture (73-75). Further studies have shown that suppression occurs only when the regulatory cells are stimulated through their T cell receptor (TCR) and are in direct contact with the population being regulated (73;76). The main mechanism of suppression seems to be the inhibition of IL-2 production in the responder population.

The role of cytokines in the function of the $CD4^+CD25^+$ regulatory cells remains to be defined. Regulatory cells from normal mice have been shown to transcribe IL-4, IL-10 and TGF-β genes more actively than the reciprocal $CD4^+CD25^-$ population (67). Additional studies, however, have shown that neutralization of these cytokines has little effect on suppression *in vitro* (76). *In vivo* experiments have been more convincing in demonstrating the role cytokines may have in regulatory functions. IL-10 production by $CD4^+CD25^+$ cells has been found to be important in regulation of cytokine production, as regulatory cells from IL-10 knockout animals do not show regulatory effects *in vivo* (77). Blockade of IL-10 and TGF-β using monoclonal antibodies also has been demonstrated to prevent regulation in many disease models such as ulcerative colitis (78;79), experimental autoimmune encephalomyelitis (80), autoimmune nephritis (81), and autoimmune thyroiditis (82).

Regulatory cells are also important in transplantation tolerance. The first suggestion for the existence of regulatory cells came from studies on neonatal tolerance. In neonates injected with allogeneic donor bone marrow, not only is the donor graft

tolerated, but the tolerance cannot be broken by an infusion of non-tolerized syngeneic lymphocytes (83). This result suggested the existence of an active suppressive mechanism. Further indications of the importance of regulatory cells in transplantation come from findings that alloreactive cells are still present in animals bearing intact grafts. This was demonstrated by the detection of *in vitro* proliferation and cytotoxic activity against donor graft antigens (83;84). The concept of active immune regulation in the periphery has been around for many years, but identifying the specific cells involved in the action proved to be difficult. It was not until the discovery of the $CD25^+$ molecule on $CD4^+$ cells as a marker for regulatory cells that researchers were able to target the cell population involved in transplantation. These cells have now been identified in mice with long-term surviving cardiac and pancreatic islet allografts (85;86) and have been shown to also suppress the rejection of allogeneic skin grafts (87). Additionally, cells within the $CD4^+CD45RB^{low}$ population in mice have recently been shown to possess regulatory properties in transplantation as adoptive transfer of these cells are able to prevent $CD4^+CD45RB^{high}$-mediated rejection of skin allografts (85). The importance of IL-10, TGF-β and CTLA-4 in transplantation tolerance by regulatory cells has also been demonstrated in mice and rats (85-88).

Type 1 Diabetes Mellitus: An Autoimmune Disease

Autoimmunity

As described above, the fundamental definition of tolerance is the elimination or neutralization of lymphocytes specific for self-peptides and self-MHC complexes by mechanisms accomplished in both the thymus and the peripheral tissues. The processes involving self-tolerance are ongoing recurring events that when interrupted by genetic and/or environmental factors can lead to expression of autoimmunity. Any defect in the tolerance process, from thymic selection to production of regulatory cells, can trigger autoimmunity.

Clinically, autoimmunity can be defined using three criteria: 1) direct evidence from transfer of disease by antibodies or immune cells; 2) indirect evidence based on reproduction of the autoimmune disease in experimental animals; and 3) circumstantial evidence from clinical clues such as pathology and familial inheritance (89). Based on these criteria, more than 40 human diseases can be classified as autoimmune disorders. This thesis work will concentrate on one of those disorders, type 1 diabetes mellitus, but it is expected that the work accomplished in this project will be applicable to the treatment of other autoimmune diseases.

Type 1, or juvenile, insulin-dependent diabetes is caused by an absolute deficiency in the production of insulin as a result of pancreatic beta cell destruction and consequent hyperglycemia. About one million people in the United States suffer from this disease comprising ~10-15% of the total diabetic population (90). Since the

discovery of insulin, deaths related directly to the absolute absence of insulin (diabetic ketoacidosis) have markedly decreased. But despite tight control of glucose levels, most patients with diabetes develop end-organ complications, including renal failure, proliferative retinopathy, peripheral neuropathy, and premature coronary disease (91).

The current consensus is that the islet beta cells are destroyed by an autoimmune response mediated by T lymphocytes that react specifically to one or more beta cell proteins. There are several lines of evidence to support the concept that type 1 diabetes is a T cell-mediated autoimmune disease. a) The presence of mononuclear cell infiltrate (insulitis) in the islets. b) A strong linkage between type 1 diabetes and certain alleles of the major histocompatibility complex (MHC). c) Autoantibodies that react with islet cell autoantigens. d) Immunosuppressive and immunomodulatory agents can slow the progressive destruction of the beta cells. e) Disease can be transferred through transplantation of bone marrow cells or immune cells from diabetic donors to non-diabetic recipients. f) Disease recurrence destroys insulin-producing transplant from non-diabetic identical twin (91;92).

Approaches to Treat Autoimmune Diseases

The primary treatment for most patients with type 1 diabetes, which has remained essentially the same for the last 80 years, is injection of exogenous insulin. But, as mentioned previously, insulin injections are not effective enough for the long-term well-being of the patient due to their inability to maintain the metabolic balance of glucose. Based on the aforementioned lines of evidence supporting the concept that type 1

diabetes is a disease in which the host's immune system inappropriately attacks healthy insulin-secreting beta cells, new strategies for therapeutic treatment are being developed and tested. Since the underlying cause of type 1 diabetes is the immune system, one such therapy takes the form of immunosuppression. Although directed at the abnormal immune function that underlies type 1 diabetes, this form of therapy is non-specific and consequently associated with toxic side effects and, until recently (21), with suboptimal clinical results. Additionally, in the case of type 1 diabetes, immunosuppression alone would only be effective if the patient still possessed sufficient beta-cell mass for adequate metabolic balance. Clinical trials to test this theory showed that ~30-50% of recently diagnosed patients who received the immunosuppressant cyclosporine A underwent remission from diabetes (93;94). But the patients displayed many of the toxic side-effects of the drug and remission was dependent on quick treatment after onset of the disease. Other forms of therapy for those patients fortunate enough to still retain some beta cell function include aggressive insulin treatment (95;96) and FcR non-binding humanized antibodies to CD3 (hOKT3) (97).

Autoimmune diseases, including type 1 diabetes, are all believed to involve lymphohematopoietic lineages descended from pluripotent hematopoietic stem cells that function abnormally. In both animal models and in humans, autoimmune diabetes has been shown to be transferred by bone marrow stem cells from diabetic donors (92;98). The replacement of these abnormal stem cells by cells derived from a normal population is the premise underlying stem cell transplantation therapy. The treatment is designed to replace host immune cells that have native or acquired autoimmunity and/or replace

missing or defective regulatory cell populations that are necessary to keep the autoreactivity in check. Laboratory studies have shown that development of autoimmune diabetes can be prevented if bone marrow cells from non-autoimmune-prone strains are transplanted into pre-diabetic NOD/Lt mice (99;100). More recently, Beihack *et al.* have shown that purified hematopoietic stem cells obtained from bone marrow can also stop the development of hyperglycemia in NOD mice even with significant levels of NOD T cells still circulating in the host (101).

In animal models, literally dozens of therapies prevent type 1 diabetes, but very few can cure established and advanced forms of the disease (102). Two major obstacles must be overcome to fully treat autoimmune diabetes. Firstly, in the advanced form of the disease the destroyed pancreatic islet beta cells must be replaced. Since the most likely donors will be genetically disparate, an allo-tolerant state must be achieved to prevent the rejection of the transplanted islets or pancreas. Secondly, the patient has to overcome recurrent autoimmunity. Even syngeneic transplantation of replacement tissue is unsuccessful because of the continued presence of the autoreactive T cells that initially destroyed the target tissue (20). Stem cell transplantation offers the possibility of overcoming both major obstacles simultaneously. Hematopoietic stem cell chimerism has been shown to reverse the autoimmune process and induce tolerance to donor allografts. Studies have shown that establishment of allogeneic hematopoietic chimerism by lethal myeloablative conditioning followed by stem cell transplantation allows restoration of normoglycemia in diabetic NOD/Lt by islet transplantation (101;103). In the present study, I document that a protocol consisting of a sub-lethal conditioning

regimen can also induce allogeneic hematopoietic chimerism in diabetic mice and permit them to permanently accept pancreatic islet allografts.

Stem Cell Transplantation for Tolerance Induction

History

In 1945, Owen observed that a natural hematopoietic chimeric state, in which two distinct types of erythrocytes coexisted, could be generated in fraternal freemartin cattle twins that shared a common placenta (104). Subsequently, Medawar demonstrated transplantation tolerance between the chimeric fraternal twins by transplanting skin grafts from each other (105;106). In their landmark study, Billingham, Brent, and Medawar were the first to actively induce allogeneic tolerance through the injection of tissue suspension containing bone marrow cells into embryos or neonatal mice (107) demonstrating for the first time neonatal tolerance. These studies represent the foundation upon which most of the subsequent research on tolerance induction by stem cell transplantation is based. The prevalent scientific explanation was that the neonatal immune system was not physiologically mature and was immunologically incompetent. Thus, the donor cells were not rejected and produced an environment conducive to the development of donor-specific tolerance.

Concept of Tolerance Induction through Hematopoietic Chimerism

Hematopoietic stem cells are single cells that are clonal precursors of more stem cells of the same type as well as being able to differentiate into progeny capable of regenerating adult tissues (108). The capacity of hematopoietic stem cells to induce transplantation tolerance results largely from their ability to induce intrathymic clonal

deletion of thymocytes with T cell receptors that recognize antigens expressed on the donor cells (30;109). The fundamental concept underlying this process is to replicate nature's major strategy for "self" tolerance as described above. A depiction of this is shown schematically in Figure 1.

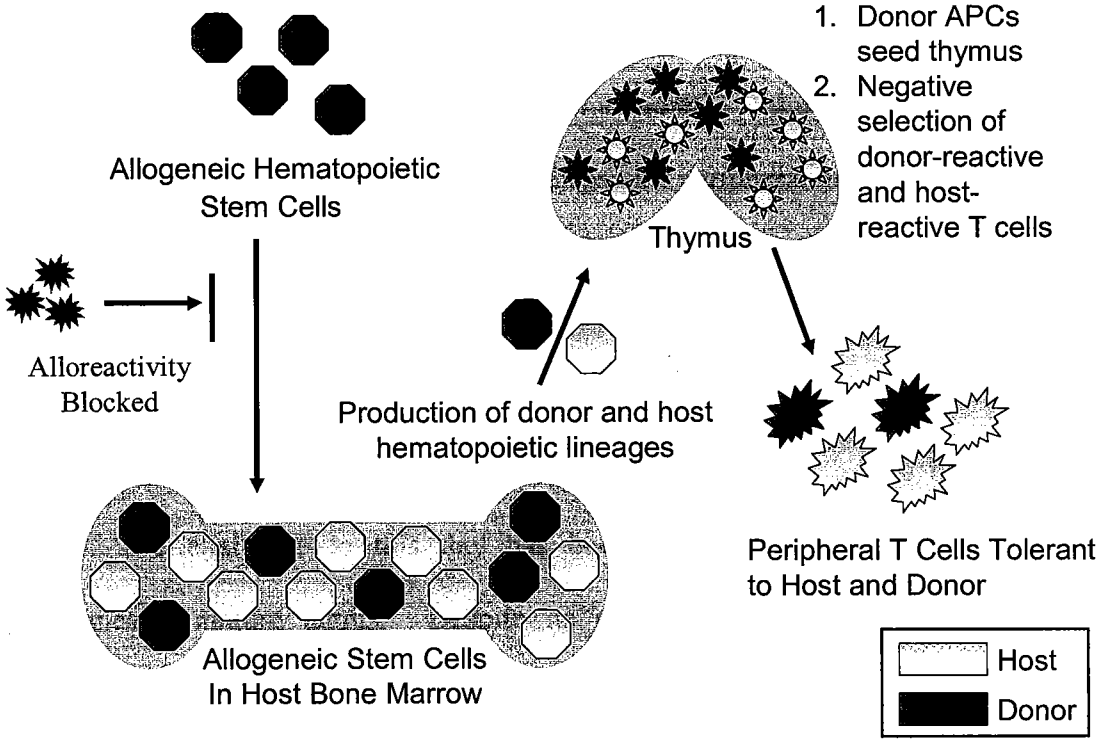

Figure 1. Hematopoietic Chimerism and Central Tolerance Induction.

Briefly, once the donor bone marrow is injected into the host the hematopoietic stem cells contained in the inoculum must survive and not be rejected by the host. Once the stem cells overcome this obstacle and engraft, they give rise to cells of all

hematopoietic lineages during the lifetime of the host. These progenitors include antigen presenting cells (APC) expressing donor antigens that migrate and seed the thymus. Antigens expressed on cells of hematopoietic origin within the thymus are the most effective mediators of negative selection (30;110). So, in addition to the deletion of self-reactive T cells in the thymus, donor-reactive T cells are also negatively selected due to the presence of the donor APCs. Additionally, the newly developing T cell repertoire in the chimeric host can also contain immunoregulatory cells that help to maintain allogeneic tolerance. Once a state of hematopoietic chimerism is successfully accomplished a robust form of central, and perhaps also peripheral, tolerance is achieved.

Problems to Overcome for Stem Cell Transplantation

The landmark experiments demonstrating that hematopoietic stem cells can induce tolerance in immunologically immature animals were performed more than 50 years ago. The challenge to this day has been to achieve a similar outcome in immunologically mature adults. Despite its potent and reliable ability to induce intrathymic tolerance, hematopoietic stem cell transplantation has not yet been applied to the induction of tolerance in man mainly due to the "conditioning" required of the host. Since the donor bone marrow cells would be seen as foreign by the recipient's immune system, any circulating mature donor-reactive T cells must be eliminated or inactivated to allow for the survival of the stem cells. The simplest and most widely-used approach to overcome the immunologic obstacle has been to pre-treat recipients with lethal whole body irradiation prior to bone marrow transplantation (39;111). This process would

eliminate almost all immune lymphocytes, including the donor-reactive T cells, and produce a "clean" environment for the engraftment of donor stem cells. The treatment would also be totally myeloablative in that almost all of the host hematopoietic stem cells would be destroyed or damaged. Implementation of this procedure in animal models to induce allogeneic chimerism and transplantation tolerance has been very successful (39;112). However, widespread utilization of this approach in humans is not feasible because the lethal radiation would be unduly toxic in individuals who do not have malignant diseases and who do not need stem cell transplantation for survival.

Another generally used procedure to prevent the rejection of donor hematopoietic stem cells has been the use of immunosuppressive drugs or nonspecific T cell elimination (113-116). The rationale underlying behind both methods is similar in that most tissues in the host would not be affected, but this procedure would eliminate the immune cells that mediate the rejection of the donor stem cells. However, the suppression of the immune system and use of toxic drugs creates major side effects detrimental to the recipient.

Once hematopoietic chimerism has been achieved, another major complication arises in the form of graft-*vs*.-host disease (GVHD), especially with the increased clinical use of grafts from mismatched and unrelated donors. GVHD occurs as a result of donor lymphocytes, present in the transplanted bone marrow, recognizing and attacking host antigens as foreign. Consequently, the effector phase of the disease involves tissue damage by cytotoxic T cells (CTL) and natural killer (NK) cells (117). Clinically relevant cases of GVHD occur in 20-50% of patients who receive stem cells from HLA-

identical sibling donors and in 50-80% of those receive who receive stem cells from HLA-mismatched siblings or from HLA-identical unrelated donors (118). Billingham in 1966 described the essential factors necessary for the development of GVHD: 1) the graft must contain immunologically competent lymphocytes; 2) the host must express transplantation antigens that are not present in the donor and that can lead to the stimulation of donor lymphocytes; and 3) the recipient has to be incapable of immunologically destroying the graft (119). GVHD is reduced significantly by non-specifically deleting donor T cells in the inoculum prior to transplantation, but this procedure leads to a higher incidence of graft failure. A explanation for this phenomenon may be due to the existence of "facilitating" $CD8^+$ T cells residing in the donor inoculum that aid in stem cell engraftment (120;121). Deletion of such cells may hinder the induction of allogeneic chimerism. *In vivo* blockade of CD40-CD154 interaction using anti-CD154 mAb has also been shown to prevent GVHD by blocking the induction of CD4 help and aborting CD8 alloresponsiveness (122-124). Further discussion on blockade of the CD40-CD154 pathway will be presented in the next section.

The major obstacles in inducing allogeneic tolerance through stem cell transplantation have been the conditioning regime necessary to prevent the rejection of the donor stem cells and the occurrence of GVHD once engraftment of the stem cells have taken place. Recent advances in the transplantation field have shown that these obstacles can be overcome by the use of reagents designed to stop T cell activation through costimulatory blockade.

Co-stimulatory Blockade and Tolerance Induction

T Cell Activation

The "two-signal" concept of T cell activation proposes that optimal activation of antigen-specific T cells requires interaction of the TCR with a peptide-MHC complex expressed on an APC (signal 1), along with an additional costimulatory signal (signal 2) (125). Antigen recognition alone, in the absence of signal 2, has been shown to induce a state of anergy by shutting down IL-2 production and down-regulating TCR expression (23;53). A number of molecules with costimulatory functions have been identified, but only a few have been shown to be essential for full T cell activation. Optimal T cell activation of naïve T cells appears to involve three receptor-ligand interactions (23). A depiction of this process is shown in Figure 2a. The first interaction is the binding of the TCR with the peptide-MHC complex (signal 1). The second is binding of CD154 on T cells to CD40 on APCs (co-activation). The third interaction is binding of CD80/CD86 (B7.1/B7.2) on the APC to CD28 on the T cell (signal 2 / costimulation). These three steps lead to the full activation of antigen-specific T cells. A number of strategies for inducing transplantation tolerance have been developed that involve blocking one or more of these interactions.

The B7.1/2-CD28/CTLA4 interaction is the best characterized of the T-cell co-stimulatory pathways and is crucial for T-cell activation. CD28 is constitutively expressed on T cells whereas the B7molecules on APCs must be induced by activation of the APC (126). The binding of CD28 on T cells to its ligands B7.1 and B7.2 has been

shown to enhance T cell proliferation and IL-2 production (127). The absence of the CD28-B7.1/2 interaction after TCR stimulation has been shown to prevent T cell activation and induce anergy (53;128). Cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed on T cells is a second receptor for B7.1/2. Unlike the constitutive expression of CD28, CTLA-4 is not expressed on the cell surface for 48-72 hours after T cell activation. CTLA-4 binds to B7.1/2 with 10- to 100-fold greater affinity than CD28 (129;130). CTLA-4 is thought to down-regulate activated T cells by inducing anergy, promoting the production of inhibitory cytokines, regulating the TCR signaling threshold, and by

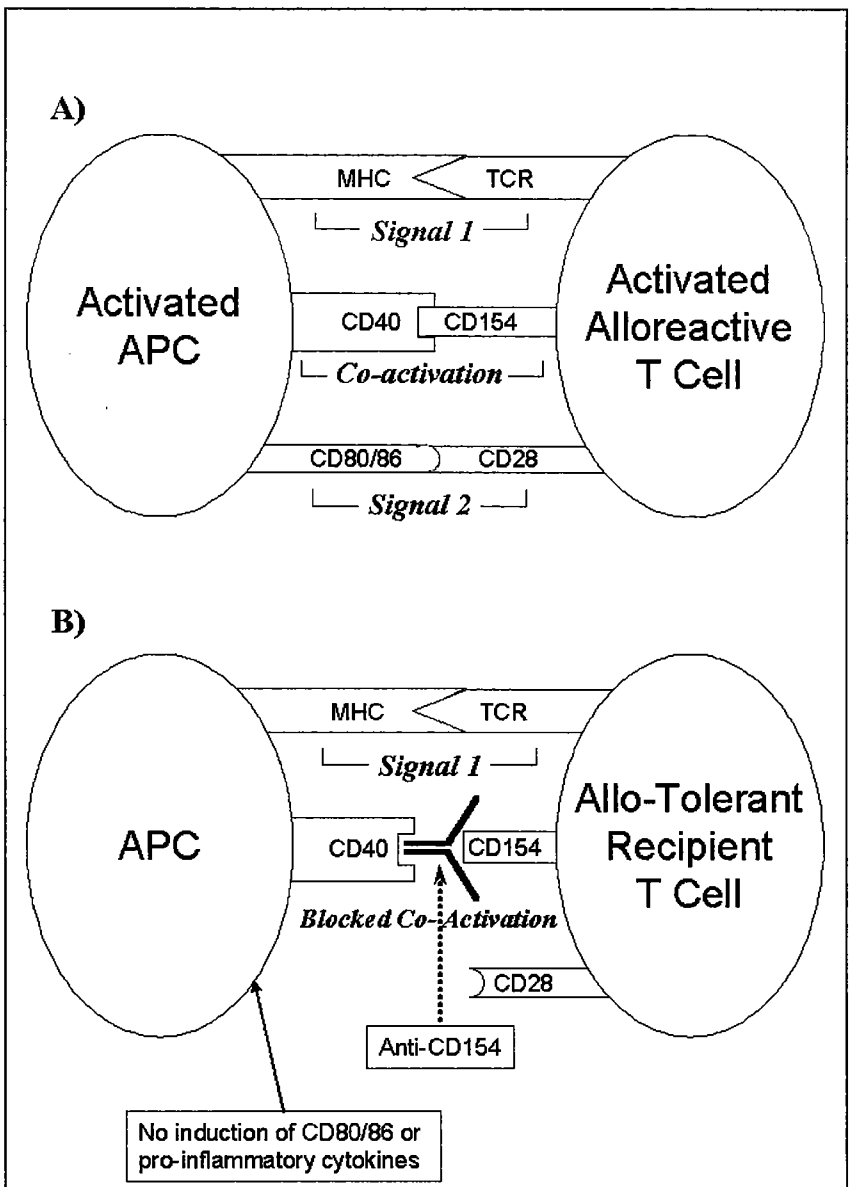

Figure 2. Allo-Tolerance Induction.

affecting cell-cycle progression (131). Its primary role in T cell activation is complex and remains to be fully understood.

A second important costimulatory signaling pathway is the interaction between CD40 and its ligand CD154 (CD40L). Antigen-specific stimulation of the TCR (signal 1) induces the rapid upregulation of CD154 on the surface of the T cell. The subsequent interaction of CD154 with its receptor CD40 activates the APC on which the receptor is expressed. The activation of the APC then induces the upregulation of B7.1/2 expression on its surface. The interaction of B7.1/2 costimulatory molecules with CD28 on T cells constitutes signal 2 required for T cell activation (23). CD40 is a member of the tumor necrosis factor (TNF) receptor family that is constitutively expressed on a wide variety of cells, including dendritic cells, B cells, macrophages, and endothelial cells (61). CD154 is a member of the TNF cytokine superfamily and is expressed mainly by activated $CD4^+$ T cells, but can be found on some $CD8^+$ T cells, mast cells, basophils, NK cells, and B cells (61). Preventing CD40-CD154 interaction has been shown to block primary and secondary immune responses to T cell dependent antigens, immunoglobulin class switching, germinal center formation, and maturation of B cell memory (132;133). Recent protocols have been developed that block the interaction of CD40 with its ligand CD154 to induce transplantation tolerance; this approach is termed "costimulation blockade."

Peripheral Transplantation Tolerance Induced by Costimulatory Blockade

The use of monoclonal antibodies against CD154 to block CD40-CD154 interaction has demonstrated marginal success for inducing allograft survival as a monotherapy (134). In contrast, the combination of anti-CD154 mAb plus a donor-specific transfusion (DST) in the form of donor spleen cells has led to very promising allograft survival outcomes. It was hypothesized that the "two-element" protocol would induce allograft survival by first initiating T-cell activation (signal 1) by the introduction of the DST and preventing costimulation (signal 2) by the injection of anti-CD154 mAb (135;136). A depiction of this process is shown in Figure 2b. The absence of costimulation would induce T-cell non-responsiveness to the donor alloantigen in the graft. This hypothesis has been tested in animal models and leads to indefinite survival of islet and cardiac allografts (135-137). Evaluation of the protocol using a more challenging assay of transplantation tolerance showed that murine skin allografts can survive for a prolonged period of time (~50 days) in euthymic mice, and indefinitely in thymectomized hosts (134;138). These results suggested that costimulatory blockade by anti-CD154 mAb in combination with DST effectively induces a state of peripheral transplantation tolerance, but does not induce central tolerance. Newly developing thymic emigrants were able to repopulate the periphery and over time, mediate the rejection of the donor grafts (139). Mechanistically, transplantation tolerance to skin allografts in mice using DST plus anti-CD154 mAb has been shown to be dependent on IFN-γ, CTLA4, regulatory $CD4^+$ T cells, and the disappearance of alloreactive $CD8^+$ T cells from the circulation (138-140).

Co-stimulatory Blockade and Allogeneic Chimerism

Non-myeloablative Chimerism Protocols

The need for exhaustive T cell depletion and severe myelosuppressive conditioning of the host with lethal irradiation or cytotoxic drug treatment for stem cell transplantation is not feasible for non-fatal, non-malignant diseases. As described next, the use of costimulatory blocking reagents such as anti-CD154 mAb as part of the stem cell transplantation protocol has allowed the induction of allogeneic chimerism to occur in the absence of these potentially toxic treatments.

Wekerle *et al.* showed that allogeneic chimerism and transplantation tolerance could be induced using costimulatory blockade (anti-CD154 mAb and CTLA4-Ig) plus sub-lethal doses of radiation (141). They demonstrated a thymus-independent deletion of donor-reactive host peripheral $CD4^+$ cells based on the disappearance of T cells expressing certain V regions of the β chain in their TCR that specifically recognize certain superantigens presented by donor MHC. The group further showed that newly developing thymocytes bearing these Vβ subunits that recognize superantigens are specifically deleted in the thymus after donor stem cell engraftment.

Although reduced, this protocol still required some myeloablative conditioning. The next goal was to develop protocols that would completely eliminate the requirement for irradiation for stem cell transplantation. Several reports have described approaches for the successful induction of allogeneic hematopoietic chimerism without any cytoreductive host treatment (142;143). However, they require supraphysiological doses

of donor bone marrow cells. One protocol required 2 x 10^8 unseparated bone marrow cells with anti-CD154 mAb and CTLA4-Ig (142), whereas a second protocol required almost the same dosage at 2 x 10^7 cells / injection for 8 injections spanning across 3 months (143). These protocols are by far the least toxic treatments described that successfully induce allogeneic stem cell engraftment, but the quantity of bone marrow cells needed is unlikely to permit extension of these protocols to the clinic.

This thesis describes the use of costimulatory blockade to induce allogeneic hematopoietic chimerism in autoimmune diabetic NOD/Lt mice and permanently restore normoglycemia by pancreatic islet transplantation. This thesis also introduces a chimerism protocol amenable for clinical use by incorporating peripheral transplantation tolerance induced by administration of DST plus anti-CD154 mAb to a stem cell transplantation protocol. Mechanisms important in the induction and maintenance of allogeneic hematopoietic chimerism are also investigated.

CHAPTER II

MATERIALS AND METHODS

Animals

C57BL/6 ($H2^b$), BALB/c ($H2^d$), CBA/JCR ($H2^k$) and B10.BR ($H2^k$) mice were obtained from the National Cancer Institute, Frederick, MD. NOD/Lt ($H2^{g7}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). To investigate the fate of specific alloreactive T cells, we established in our animal colony the KB5 TCR transgenic mouse, which has specificity to native $H2^b$ alloantigen (144;145). This TCR transgenic mouse was the generous gift of Dr. John Iacomini (Harvard Medical School, Boston, MA) who obtained it from the original developer, Dr. Andrew Mellor (Medical College of Georgia, Augusta, GA). The TCR transgene is expressed by $CD8^+$ cells in CBA ($H2^k$) mice and has specificity for H2-K^b. These transgenic T cells express a TCR that is recognized by the DES anti-clonotypic mAb.

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, GD7 virus, Reo-3 virus, mouse adenovirus, lymphocytic choriomeningitis virus, polyoma, *Mycoplasma pulmonis*, and *encephalitozoon cuniculi*. All animals were housed 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).

Antibodies and Flow Cytometry

FITC-conjugated anti-H2-K^b (clone AF6-88.5), PE-conjugated anti-H2-K^k (clone 36-7-5), and PE-conjugated anti-H2-K^d (clone SF1-1.1) mAbs were obtained from PharMingen (San Diego, CA). MR1 hamster anti-mouse CD154 mAb was produced as ascites in *scid* mice and purified using a Protein A Sepharose 4 Fast-flow purification column (Pharmacia Biotech) and quantified by optical density (139;146). Antibody concentration was determined by measurement of optical density and confirmed by ELISA (139). The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC) and was uniformly <10 units/mg of mAb (139).

Anti-CD4 (GK1.5), anti-CD8 (2.43) and anti-CD25 (PC61.5.3) antibodies were obtained from the American Type Culture Collection (Rockville, MD). Antibodies for *in vivo* depletion were produced as ascites in *scid* mice and purified using a Protein G PLUS purification column (Oncogene Research Products, Boston, MA). To *in vivo* deplete $CD4^+$ and $CD8^+$ cells, mice were injected intraperitoneally with 0.5 mg of mAb on three consecutive days. To *in vivo* deplete $CD25^+$ cells, mice were injected once intraperitoneally with 0.25 mg of mAb. A hybridoma cell line secreting hamster anti-mouse CTLA4 mAb (clone 9H10) was the gift of Dr. James Allison (University of California, Berkeley, CA). Anti-CTLA4 mAb was grown as ascites, purified using

Protein A purification column (Oncogene Research Products, Boston, MA), and injected intraperitoneally at a dose of 0.075 mg per mouse daily on 3 consecutive days.

Flow microfluorometry was performed as described (139;147). Briefly, 1 x 10^6 viable cells were reacted with the appropriate antibody for 20 min at 4°C. In experiments using the KB5 synchimeras, cells were reacted with the DES anti-clonotypic mAb for 20 min. at 4°C. Cells were then washed and reacted with FITC-conjugated anti-mouse IgG2a mAb (to visualize the bound DES antibody). Whole blood was processed using FACS™ lysing solution (Becton Dickinson, Sunnyvale, CA) in accordance with the protocol supplied by the manufacturer. Labeled cells were washed, fixed with 1% paraformaldehyde-PBS, and analyzed using a FACScan® instrument (Becton Dickinson). Lymphoid cells were gated according their light-scattering properties, and 30-50 x 10^3 events were acquired for each analysis.

The relative percentages of host- and donor-origin cells in the various recipients of C57BL/6 (H2-K^{b+}) bone marrow were determined by flow microfluorometry. The percentage of peripheral blood mononuclear cells (PBMC) in chimeric mice expressing MHC class I was determined by dual labeling with anti-H2-K^b (donor) and anti-H2-K^d, or anti-H2-K^k, (recipient) antibodies. Because fewer than 100% of hematopoietic cells express MHC class I antigen, the relative percentage of donor-origin cells (H2-K^{b+}) in chimeric recipients was calculated as follows: [%H2-K^{b+} ÷ (%H2-K^{b+} + %H2-$K^{k+ \text{ or } d+}$)] x 100. In previous experiments, known mixtures of BALB/c and C57BL/6 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^{b+}) or host (H2-K^{d+}) cells was 0.5% (147).

Allogeneic Chimerism Induction Protocol #1: Radiation + Anti-CD154mAb + Bone Marrow

Marrow donors were female C57BL/6 mice. Donor femurs and tibias were flushed with RPMI medium using a syringe with a 24-gauge needle. Recovered cells were filtered through sterile nylon mesh (70 μm, Becton Dickinson, Franklin Lakes, NJ), counted, and re-suspended in RPMI. Recipients were BALB/c or NOD/Lt mice at least 6 weeks of age. In a preliminary experiment, the doses of radiation that produced 100% mortality in untreated control BALB/c and NOD/Lt mice were determined to be 7 Gy (N=5) and 10 Gy (N=5). Doses of 6 Gy (N=5) and 9 Gy (N=5) or less were uniformly non-fatal. All NOD/Lt recipients were spontaneously diabetic (plasma glucose concentration >250 mg/dl) at the time of bone marrow transplantation.

Before transplantation, recipients were randomized to receive anti-CD154 mAb plus radiation or radiation alone. All injections of mAb were given intraperitoneally. Whole body irradiation was administered using a ^{137}Cs source (Gammacell 40, Atomic Energy of Canada, Ottawa, ON, Canada). All recipients received a single intravenous injection of 25 x 10^6 donor bone marrow cells in a volume of 0.5-1.0 ml via the lateral tail vein within 1-5 hrs of irradiation. The doses and timing of the injections, the doses of radiation, and the timing of injections of mAb in relation to irradiation varied in different experiments and are described in detail in Chapter III. Blood samples were obtained from all bone marrow recipients 4-6 weeks after transplantation for determination of the percentages of donor and host cells by flow microfluorometry. Additional blood samples were obtained periodically as described in Chapter III. The presence of hematopoietic

chimerism was defined as the presence of ≥1% donor-origin peripheral blood mononuclear cells.

Allogeneic Chimerism Induction Protocol #2: Radiation + DST + Anti-CD154mAb + Bone Marrow

Recipients were BALB/c, CBA/J, B10.BR, and NOD/LT mice at least 6 weeks of age. Before bone marrow transplantation, recipients were randomized to receive low-dose radiation, anti-CD154 mAb and/or donor-specific transfusion (DST). All injections of mAb were given intraperitoneally at a dose of 0.5 mg on days –7, -4, 0 and +3 relative to bone marrow transplantation for a total of 4 doses. In some groups, C57BL/6 splenocytes (10^7) from female donors were injected intravenously in a volume of 0.5 ml into the various recipients. The DST was given 7 days prior to bone marrow transplantation.

Whole body irradiation was administered using a ^{137}Cs source (Gammacell 40, Atomic Energy of Canada, Ottawa, ON, Canada). All recipients received a single intravenous injection of 25 x 10^6 donor bone marrow cells in a volume of 0.5-1.0 ml via the lateral tail vein within 1-5 hrs of irradiation. Marrow donors were female C57BL/6 mice. Donor femurs and tibias were flushed with RPMI medium using a syringe with a 24-gauge needle. Recovered cells were filtered through sterile nylon mesh (70 μm, Becton Dickinson, Franklin Lakes, NJ), counted, and re-suspended in RPMI.

Blood samples were obtained from all bone marrow recipients 4-6 weeks after transplantation for determination of the percentages of donor and host cells by flow microfluorometry. Additional blood samples were obtained periodically as described in

Chapter IV. The presence of hematopoietic chimerism was defined as the presence of ≥1% donor-origin peripheral blood mononuclear cells.

Allogeneic Chimerism Induction Protocol #3: DST + Anti-CD154mAb + Bone Marrow

Bone marrow recipients were BALB/c, CBA/J, and B10.BR mice at least 6 weeks of age. Additional bone marrow recipients were KB5 synchimeric mice (described below). Except as noted in specific experiments, bone marrow recipients were treated with our standard protocol for peripheral transplantation tolerance induction (138-140). Relative to the transplantation of allogeneic bone marrow on day 0, mice received a single intravenous donor-specific transfusion (DST, 1 x 10^7 spleen cells) on day -7 and four injections of MR1 anti-CD154 mAb (0.5 mg/dose) on days -7, -4, 0, and +3 (138-140). The allograft consisted of 50 x 10^6 or 100 x 10^6 donor bone marrow cells in a volume of 0.5-1.0 ml, injected via the lateral tail vein.

Femurs and tibias from bone marrow donor mice were flushed with RPMI medium using a syringe equipped with a 24-gauge needle. Recovered cells were filtered through sterile nylon mesh (70 μm, Becton Dickinson, Franklin Lakes, NJ), counted, and suspended in RPMI for injection. Recipient and donor marrow combinations are indicated in each Table.

Blood samples were obtained from all bone marrow recipients at the times indicated after bone marrow transplantation for determination of the percentages of donor and host cells by flow microfluorometry. Additional blood samples were obtained

periodically as described in Chapters V and VI. The establishment of hematopoietic chimerism was defined as the presence of ≥1% donor-origin peripheral blood mononuclear cells.

Generation of KB5 TCR Transgenic Hematopoietic CBA Synchimeras

To examine the fate of both mature and developing alloreactive $CD8^+$ T cells in a normal microenvironment, we used TCR transgenic hematopoietic chimeras (139) generated by injecting small numbers of bone marrow cells from KB5 transgenic donors into sub-lethally irradiated syngeneic CBA non-transgenic hosts. We refer to these as "synchimeric" mice (139). In this model system there is continual production of low levels of tracer transgenic cells in a host's cellular microenvironment that is essentially unaltered by transgene expression.

Donor marrow was collected from male and female KB5 x CBA/JCr/F1 mice ($H2^k$). Donor femurs and tibias were flushed with RPMI medium using a syringe with a 24-gauge needle. Recovered cells were filtered through sterile nylon mesh (70 μm, Becton Dickinson, Franklin Lakes, NJ), counted, and re-suspended in RPMI. Recipients were male CBA/JCr mice 4-7 weeks of age. Recipient mice were treated with 2 Gy whole body gamma irradiation using a ^{137}Cs source (Gammacell 40, Atomic Energy of Canada, Ottawa, ON, Canada). They were given a single intravenous injection of 0.5 x 10^6 transgenic bone marrow cells in a volume of 0.5 ml via the lateral tail vein. The transgenic T cells that develop from KB5 bone marrow express an anti-H2-K^b specific TCR that is recognized by the clonotypic mAb DES (144). Previously published data

(139) and results shown in Chapter VI show the generation of stable synchimeras with 5-8% circulating $DES^{+}CD8^{+}$ cells in the lymphocyte population of PBMC 8 weeks after bone marrow injection.

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 as described (138). Skin grafts were dressed with Vaseline™-impregnated gauze and an adhesive bandage for the first week 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 (138).

Diagnosis of Diabetes and Pancreatic Islet Cell Transplantation

NOD/Lt mice were monitored twice weekly for the presence of glycosuria. Diabetes in glycosuric mice was diagnosed on the basis of two plasma glucose concentrations ≥250 mg/dl on different days (Glucose Analyzer2™, Beckman Instruments, Fullerton, CA). Prior to islet transplantation, diabetic NOD/Lt mice were treated with timed release insulin pellets (1 pellet per animal; Linplant™, Linshin Canada Inc.) implanted subcutaneously. Pellets were removed on the day of islet transplantation.

Pancreatic islets were harvested from C57BL/6 donor mice by collagenase digestion as described (135;148). Islets (20 per g body weight) were transplanted into the renal sub-capsular space of diabetic NOD/Lt recipients, which were monitored twice weekly thereafter for the presence of glycosuria. Graft failure was defined as the presence

of a plasma glucose concentration ≥250 mg/dl on two successive days. To confirm graft function in the case of recipients that were normoglycemic at the conclusion of an experimental protocol, nephrectomy of the kidney containing the islet graft was performed and the animal was allowed to recover. The graft was scored as having been functional if hyperglycemia recurred (≥250 mg/dl).

Histology

Samples of transplanted skin, host skin, small intestine, large intestine, and liver were recovered from selected experimental mice, fixed and stored in 10% buffered formalin solution, then embedded in paraffin and processed for light microscopy. Kidneys into which islet grafts had been transplanted were processed in the same way. Sections for routine light microscopy were stained with hematoxylin and eosin. Sections of islet graft-bearing kidneys were stained immunohistochemically for the presence of insulin and glucagon as described (149). Portions of islet graft-bearing kidneys were also snap frozen and processed for immunohistochemistry as described (150). Sections were reacted with antibodies directed against CD3, CD4, CD8, and CD19. Histological analyses were performed by a qualified pathologist (BAW) who was unaware of the treatment status of specimen donors.

Statistical Analysis

Comparisons of three or more means used oneway analyses of variance and the least significant difference procedure for *a posteriori* contrasts (151). Comparisons of two means used unpaired t-tests without assuming equal variance (152). In experiments

in which large variances were observed, groups were compared non-parametrically with the Mann-Whitney U or Kruskall-Wallis tests (153). Analysis of contingency tables used the χ^2 statistic or, in the case of 2 x 2 tables, the Fisher exact statistic (153). Average duration of graft survival is presented as the median survival time (MST). Skin allograft survival among groups was compared using the method of Kaplan and Meier (154); the equality of allograft survival distributions for animals in different treatment groups was tested using the log rank statistic (155). P values <0.05 were considered statistically significant.

CHAPTER III

ALLOGENEIC CHIMERISM INDUCED BY SUB-LETHAL RADIATION AND ANTI-CD154 ANTIBODY

Introduction

Stem cell transplantation leading to allogeneic hematopoietic chimerism has been proposed as a method for both the generation of transplantation tolerance and the treatment of autoimmune diseases. As stated above, implementation of clinical trials has in part been impeded by the toxicity of conditioning procedures and by the development of graft-versus-host disease (GVHD). The theory is that procedures that induce peripheral tolerance to allogeneic grafts may facilitate in the induction of allogeneic chimerism. One class of tolerance induction protocols is based on blockade of the CD40-CD154 T cell costimulatory pathways using anti-CD154 mAb. So, the hypothesis is that blockade of the CD40-CD154 interaction will eliminate the need for lethal myeloablative preparation to induce chimerism and simultaneously prevent GVHD. I have tried to develop a clinically relevant hematopoietic chimerism protocol by using anti-CD154 mAb in conjunction with sub-lethal radiation and fully allogeneic bone marrow cells. Additionally, I have explored the use of this chimerism protocol as a treatment for type 1 diabetes using the NOD/Lt mouse model for autoimmune diabetes.

Results

Blockade of CD40-CD154 Interaction Permits Establishment of C57BL/6 Hematopoietic Chimerism in BALB/c Mice Without Lethal Conditioning

We first established that it is possible to generate hematopoietic chimerism in BALB/c ($H2^d$) mice transplanted with fully allogeneic C57BL/6 ($H2^b$) bone marrow using the combination of sub-lethal irradiation and a brief course of anti-CD154 mAb. As shown in Table 1, chimerism could be established using as little as 4 Gy of conditioning radiation and as few as two doses of anti-CD154 mAb. The percentage of successful transplantation procedures yielding detectable chimerism using 4 Gy varied as a function of the dose and frequency of treatment with anti-CD154 mAb. Treatment with only two doses of 0.5 mg of mAb was optimal, yielding chimerism in 100% of nine mice in two independent trials. Chimerism could also be established without blockade of CD154 in recipients given 5 Gy but not 4 Gy of conditioning radiation.

The data shown in Table 1 were obtained 4-6 weeks after transplantation. All mice documented to be chimeric at this time point were subsequently reanalyzed at varying intervals 49 to 338 days after transplantation. Analysis of PBMC demonstrated that, without exception, they remained chimeric to the same degree as indicated in Table 1.

Mice were ultimately killed at varying intervals 27-48 weeks after transplantation for histological studies and flow microfluorometric analyses of spleen, thymus, and bone marrow. In mice that were given 4 Gy or 5 Gy of conditioning radiation and 4 or 14

Table 1: Hematopoietic Chimerism in BALB/c Mice Given Varying Sub-lethal Doses of Radiation and C57BL/6 Bone Marrow in the Presence or Absence of Anti-CD154

Radiation (Gy)	*Anti-CD154 mAb*		*Number of Chimeric Mice (%)*		*Percentage of Donor Origin PBMC in Chimeric Mice*
	Number of Injections	*Dose*			
3	None	—	0/10	(0%)	—
	4	0.25 mg	0/5	(0%)	—
	14	0.25 mg	0/5	(0%)	—
	2	0.5 mg	0/5	(0%)	—
4	None	—	0/10	(0%)	—
	4	0.25 mg	2/4	(50%)	Both >99%
	14	0.25 mg	4/9	(44%)	All >99%
	2	0.5mg	9/9	(100%)	All >99%
5	None	—	10/10	(100%)	All >99%
	4	0.25 mg	4/4	(100%)	All >99%
	14	0.25 mg	8/9	(89%)	All >99%
	2	0.5 mg	9/9	(100%)	All >99%

Legend to Table 1: BALB/c ($H2^d$) mice were irradiated, transfused intravenously with 25 x 10^6 C57BL/6 ($H2^b$) bone marrow cells, and injected intraperitoneally with anti-CD154 mAb as indicated. The schedule of anti-CD154 mAb (relative to irradiation and bone marrow transplantation on day 0) was as follows. Recipients given 2 injections were treated on days 0 and +3, recipients of 4 injections on days –3, 0, +3, +7, and recipients of 14 injections on days –3, 0, and twice weekly thereafter. The percentage of $H2^b$ donor origin peripheral blood mononuclear cells (PBMC) was determined by flow cytometry 4-6 weeks after irradiation and bone marrow transplantation. The presence of chimerism was defined as ≥1% donor-origin cells.

doses of anti-CD154 mAb, >99% of splenic B cells, $CD4^+$ T cells, and $CD8^+$ T cells detected were of donor-origin (N=9). In contrast, the percentage of donor-origin thymocytes in chimeric recipients was generally much smaller, 57 ± 27% (range 17 to >99%, N=9). Bone marrow cells were >99% donor-origin. We also analyzed two chimeric mice that had received only 5 Gy of conditioning radiation and no anti-CD154 mAb. In these mice >99% of B cells, $CD4^+$ T cells, and $CD8^+$ T cells were of donor-

origin. The percentages of donor-origin thymocytes in these two mice were >99% and 60%. Bone marrow cells were >99% donor-origin in both.

The diagnosis of GVHD was made either clinically or histologically. Throughout the period of observation (up to 338 days) there was no clinical evidence of GVHD in any chimeric bone marrow recipient that had received any dose of radiation plus any of the anti-CD154 mAb regimens (N=36, Table 1). This was true in the case of mice that were not further manipulated (N=22) and mice that were given donor-specific skin allografts (N=14, described below). The absence of GVHD was confirmed by histological study of skin, liver, and small and large intestine in a subset of the animals that were skin graft recipients (N=9). Two of the nine mice showed rare apoptotic bodies in intestinal crypts, but cryptitis was absent. Similarly, the livers of two of nine mice showed scattered peripheral lymphocytes, but bile duct inflammation was not evident. Skin biopsies were unremarkable.

Among the ten chimeric BALB/c recipients that had been given 5 Gy of conditioning radiation but no anti-CD154 mAb, seven survived >10 weeks. The other three exhibited clinical signs of GVHD before death; these included weight loss, loss of hair, hunched posture, and erythematous encrusted ears. The remaining seven animals were confirmed still to be chimeric 7-21 weeks after bone marrow transplantation and given skin allografts as described in the next section. Among these, five subsequently died with clinical evidence of GVHD. Two surviving mice in this group were killed on day 112 after skin grafting for flow cytometric and histological analyses. Both were

documented to have remained chimeric, and neither showed histological evidence of GVHD.

Donor-specific Skin Allografts Uniformly Survive in Chimeric Mice that Receive Anti-CD154 Antibody

We next measured the survival of donor-origin skin allografts on a subset of the bone-marrow transplanted mice presented in Table 1. Selected mice from among those tested for chimerism 4-6 weeks after bone marrow transplantation were given C57BL/6 skin grafts 3 to 15 weeks later.

Donor-specific (C57BL/6) skin allograft survival on chimeric mice given any of the anti-CD154 mAb treatment regimens plus either 4 Gy (N=6) or 5 Gy (N=8) was significantly prolonged (MST >183 d and >112 d, respectively). Graft survival in these two groups was statistically similar, and the groups were combined in subsequent analyses (Figure 3, Group 1). Median skin allograft survival was statistically significantly longer on mice that had received both radiation and anti-CD154 mAb (MST=166 d, N=14, Figure 3, Group 1) than on chimeric mice that had received only 5 Gy of radiation (MST=9 d, N=7, Figure 3, Group 2). As can be seen in the figure, skin graft survival on three of the seven chimeric animals that had received radiation alone was prolonged. One of these three mice had clinical signs of GVHD and died with its graft intact. The other two were killed for histological study 112 days after skin grafting when one of them became ill. As noted at the end of the previous section, both of these mice were chimeric, and neither showed histological evidence of GVHD. The skin graft from one animal showed scattered mononuclear cells around hair follicles and sweat glands, and rare

apoptotic keratinocytes and intradermal mononuclear cells. The other graft was unremarkable, with no evidence of mononuclear infiltration.

Skin allograft survival on irradiated mice that had failed to become chimeric (<1% donor cells) was uniformly brief regardless of whether or not they had received anti-CD154 mAb (Figure 3, Groups 3, 4; MST=9 d for both groups).

We next determined if skin allograft survival on chimeric BALB/c recipients of C57BL/6 bone marrow was donor-specific. A subset of mice that were documented to be chimeric after treatment with both conditioning irradiation (4 or 5 Gy) and anti-CD154 mAb (2 doses) was selected at random 14-15 weeks after bone marrow transplantation. They were given either C57BL/6 ($H2^b$, donor-specific) or CBA/JCR ($H2^k$, 3rd party) skin allografts. As expected, donor-specific (C57BL/6) skin allograft survival on chimeric mice was significantly prolonged (MST >65 d, N=3). In contrast, CBA/JCR skin allografts survived only briefly (MST=15 d, N=4).

Histological analysis of transplanted skin was performed on a subset of 11 mice with intact grafts that had survived for 64-259 days. These included 9 chimeric recipients that had received radiation plus anti-CD154 mAb and 2 that had received radiation alone. In all instances, there was no evidence of inflammation suggestive of graft rejection.

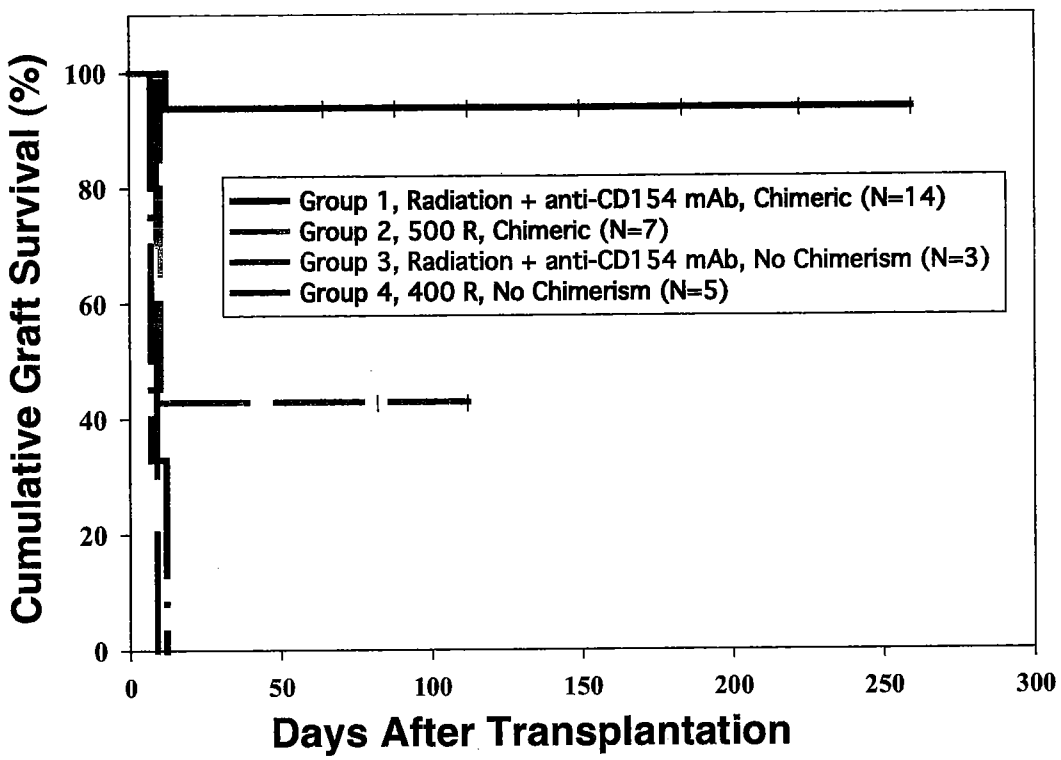

Figure 3. ***C57BL/6 Skin Allograft Survival in BALB/c Recipients of C57BL/6 Bone Marrow.*** Both chimeric and non-chimeric BALB/c mice from among those described in Table 1 were selected at random and transplanted with a C57BL/6 skin graft 7 to 19 weeks after bone marrow transplantation as described in Methods. For purposes of analysis, mice were divided into four groups. Mice in Group 1 had received either 4 or 5 Gy of conditioning radiation plus anti-CD154 mAb and were chimeric. MST of grafts in this group was >166 days. Mice in Group 2 had received 5 Gy of conditioning radiation but no anti-CD154 mAb and were chimeric. MST of grafts in this group was 9 days. Mice in Group 3 had received either 4 or 5 Gy of radiation plus anti-CD154 mAb, but were not chimeric. Mice in Group 4 were given 4 Gy of radiation but no anti-CD154 mAb and were not chimeric. MST of grafts in both Group 3 and Group 4 was 9 days. Graft survival in Group 1 was significantly longer than in any other group ($p<0.0001$). Censored data, *i.e.* mice that died or were killed for histological study or were still alive at the end of the experiment are indicated by vertical lines.

Blockade of CD40-CD154 Interaction Permits Establishment of C57BL/6 Hematopoietic Chimerism in Sublethally Conditioned NOD/Lt Mice

We next established that it is possible to generate hematopoietic chimerism in NOD/Lt ($H2^{g7}$) mice transplanted with allogeneic C57BL/6 ($H2^{b}$) bone marrow using the combination of sub-lethal irradiation and a brief course of anti-CD154 mAb. In this

experiment, all NOD/Lt recipients had developed spontaneous autoimmune diabetes prior to conditioning and bone marrow transplantation. Consistent with a previous report (103), a preliminary experiment demonstrated that NOD/Lt mice are relatively radioresistant; the lethal radiation dose was 10 Gy. We tested for the generation of chimerism in NOD/Lt mice using 6 to 9 Gy of conditioning radiation.

As shown in Table 2, hematopoietic chimerism in NOD/Lt mice could be generated using as little as 6 Gy of conditioning radiation provided that the mice were also treated with anti-CD154 mAb. As few as two doses of anti-CD154 mAb were sufficient. Chimerism could also be established without blockade of CD154, but required a minimum of 7 Gy of conditioning radiation.

The percentage of transplantation procedures yielding detectable chimerism in mice given both radiation and anti-CD154 mAb varied with the dose and frequency of treatment with the mAb. As was true in the case of BALB/c recipients of C57BL/6 bone marrow, treatment with two doses of 0.5 mg of mAb was optimal at each dose of radiation.

The data shown in Table 2 were obtained 4-6 weeks after transplantation. Mice initially observed to be chimeric at this time point were subsequently reanalyzed at varying intervals 49 to 331 days after transplantation. With the exception of one mouse, they were all documented to have remained chimeric to the same extent as indicated in Table 2. In the one exceptional mouse, the percentage of donor-origin cells at 4 weeks was atypically low (24%) and no donor-origin cells could be detected at 7 weeks.

Table 2: Hematopoietic Chimerism in NOD/Lt Mice Given Varying Sub-Lethal Doses of Radiation and C57BL/6 Bone Marrow in the Presence or Absence of Anti-CD154

Radiation (Gy)	*Anti-CD154 mAb*		*Number of chimeric mice (%)*		*Percentage of Donor Origin PBMC in Chimeric Mice*
	Number of Injections	*Dose*			
6	None	—	0/6	(0%)	—
	14	0.25 mg	3/6	(50%)	24%, 93%, >99%
	2	0.5 mg	8/12	(67%)	95%, 97%, Six >99%
7	None	—	4/4	(100%)	All >99%
	14	0.25 mg	1/3	(33%)	All >99%
	2	0.5 mg	5/6	(83%)	All >99%
8	None	—	2/2	(100%)	Both >99%
	14	0.25 mg	0/2	(0%)	—
	2	0.5 mg	3/3	(100%)	All >99%
9	14	0.25 mg	6/6	(100%)	All >99%
	2	0.5 mg	3/3	(100%)	All >99%

Legend to Table 2: Spontaneously diabetic NOD/Lt mice ($H2^{g7}$) were irradiated, transfused intravenously with 25 x 10^6 C57BL/6 ($H2^b$) bone marrow cells, and injected intraperitoneally with anti-CD154 mAb as indicated. The schedule of anti-CD154 mAb (relative to irradiation and bone marrow transplantation on day 0) was as follows. Recipients given 2 injections were treated on days 0 and +3, and recipients of 14 injections on days –3, 0, and twice weekly thereafter. The percentage of $H2^b$ donor origin peripheral blood mononuclear cells (PBMC) was determined by flow cytometry 4-6 weeks after irradiation and bone marrow transplantation. The presence of chimerism was defined as ≥1% donor-origin cells.

Mice were ultimately killed at varying intervals 16-42 weeks after transplantation for histological studies and flow microfluorometric analyses of spleen, thymus, and bone marrow. Except for the one animal described above, >99% of splenic B cells, $CD4^+$ T cells, and $CD8^+$ T cells detected were of donor-origin. In the one exceptional case, no donor-origin cells could be detected in blood, spleen, bone marrow, or thymus 20 weeks after transplantation. As was also true of the BALB/c recipients of C57BL/6 bone

marrow (Table 1), the average percentage of donor-origin thymocytes in chimeric NOD/Lt recipients (Table 2) was lower, 61 ± 37% (range 25-99%, N=5).

The diagnosis of GVHD in these mice was made either clinically or histologically. Throughout the period of observation (up to 331 days) there was no clinical evidence of GVHD in any chimeric NOD/Lt bone marrow recipient that had received any dose of radiation plus any of the anti-CD154 mAb regimens (N=29, Table 2). This was true in the case of mice that were not further manipulated (N=10) and mice that were given donor-specific allografts (N=19, described below). The absence of GVHD was confirmed by histological study of skin, liver, and small and large intestine in a subset of the animals that were allograft recipients (N=11). Five of eleven mice showed scattered peri-portal lymphocytes. The skin and intestine were unremarkable.

Among the six chimeric NOD/Lt recipients that had been given 7 or 8 Gy of conditioning radiation but no anti-CD154 mAb, three were found dead within 6 weeks of bone marrow transplantation. These mice died before they could receive islet grafts, and the causes of death are not known. The three remaining mice were given islet grafts as described in the next section. Two mice survived >23 weeks after bone marrow transplantation without clinical evidence of GVHD. The third died with clinical evidence of GVHD shortly after islet grafting.

Donor-specific Islet Allografts Uniformly Survive in Chimeric NOD/Lt Mice and Reverse Autoimmune Disease

We next measured the survival of donor-origin islet allografts on a subset of the bone-marrow transplanted NOD/Lt mice presented in Table 2. Selected mice from among

those tested for chimerism 4-6 weeks after bone marrow transplantation were given C57BL/6 islet allografts 1 week later. These mice had spontaneously developed autoimmune diabetes before bone marrow transplantation. They were treated with slow-release insulin pellets until the day of islet transplantation, at which time the pellets were removed.

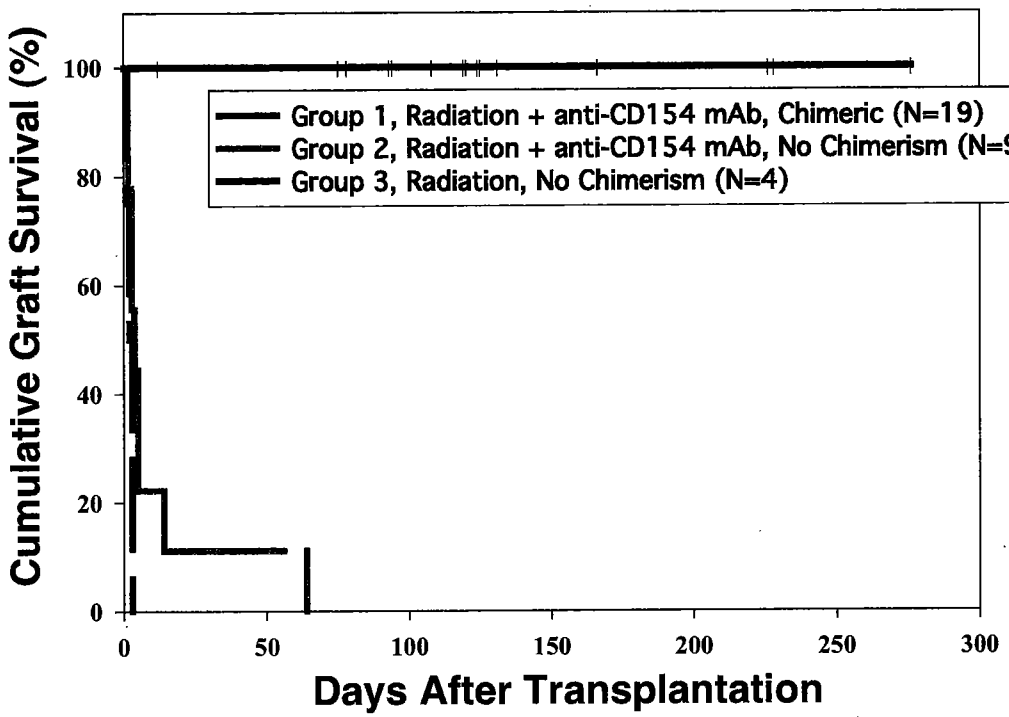

Figure 4. ***C57BL/6 Islet Allograft Survival in Diabetic NOD/Lt Recipients of C57BL/6 Bone Marrow.*** Both chimeric and non-chimeric NOD/Lt mice from among those described in Table 2 were selected at random and transplanted with a C57BL/6 islet graft 5-7 weeks after bone marrow transplantation as described in Methods. All mice were diabetic at the time of both bone marrow and islet transplantation; their diabetes was treated with sustained-release insulin pellets until islet transplantation, at which time the pellets were removed. For purposes of analysis, mice were divided into three groups. Mice in Group 1 had received 6 to 9 Gy of conditioning radiation plus anti-CD154 mAb and were chimeric. Median survival of islet grafts in this group was >120 days. Mice in Group 2 had received 6 to 8 Gy of conditioning radiation and anti-CD154 mAb and were non-chimeric. Mice in Group 3 had received 6 Gy of radiation but no anti-CD154 mAb and were non-chimeric. MST of grafts in Groups 2 and 3 was 3 days. Graft survival in Group 1 was significantly longer than in the other groups ($p<0.0001$). Graft survival in Groups 2 and 3 was statistically similar. Censored data, *i.e.* mice that died or were nephrectomized at the end of the experiment, are indicted by vertical lines.

Islet allograft survival in chimeric diabetic NOD/Lt mice given any of the anti-CD154 mAb treatment regimens plus any dose of conditioning radiation was significantly prolonged. Through the end of the experiment, none of these 19 islet recipients rejected its graft (MST >120 d, Figure 4, Group 1). One mouse died unexpectedly 12 days after islet transplantation, but was normoglycemic 2 days before death. The mouse did not exhibit signs of GVHD, and the cause of death is not known. To confirm graft function, 11 of the 18 long-term islet allograft recipients underwent unilateral nephrectomy of the islet graft-bearing kidney 75-276 d after islet transplantation. Hyperglycemia recurred in all nephrectomized mice, confirming the prolonged survival and function of the transplanted islets.

As noted at the end of the previous section, only three NOD/Lt mice that had become chimeric after irradiation alone survived long enough to receive islet grafts. Two received NOD-*scid* islet grafts; one received a C57BL/6 islet graft. The NOD-*scid* graft recipients died 40 and 185 days after transplantation. Both were normoglycemic immediately before death, but the mouse that died on day 40 showed evidence of GVHD. The single recipient of C57BL/6 islets remained normoglycemic and clinically well until the conclusion of the experiment 260 days after islet grafting.

Islet allograft survival in irradiated mice that had failed to become chimeric (<1% donor cells) was uniformly brief. This was true both for recipients that had received anti-CD154 mAb (N=9, MST=3 d, Figure 4, Group 2) and for those that had not (N=4, MST= 3 d, Figure 4, Group 3).

Histological analysis of transplanted islets was performed on the 11 nephrectomy specimens described above (Figure 5). In some cases, light microscopy revealed a very sparse mononuclear cell infiltrate in the renal capsule or adjacent to the islet graft, but no mononuclear cell infiltrates were observed in the grafts themselves. Immunohistochemical analyses using antibodies specific for CD3, CD4, CD8, and CD19 confirmed that few or no lymphoid cells were present in the transplanted tissue. Consistent with the observation that hyperglycemia recurred after nephrectomy, immunohistochemical staining demonstrated that insulin was present in each specimen. Immunoreactive glucagon was also detectable in each specimen.

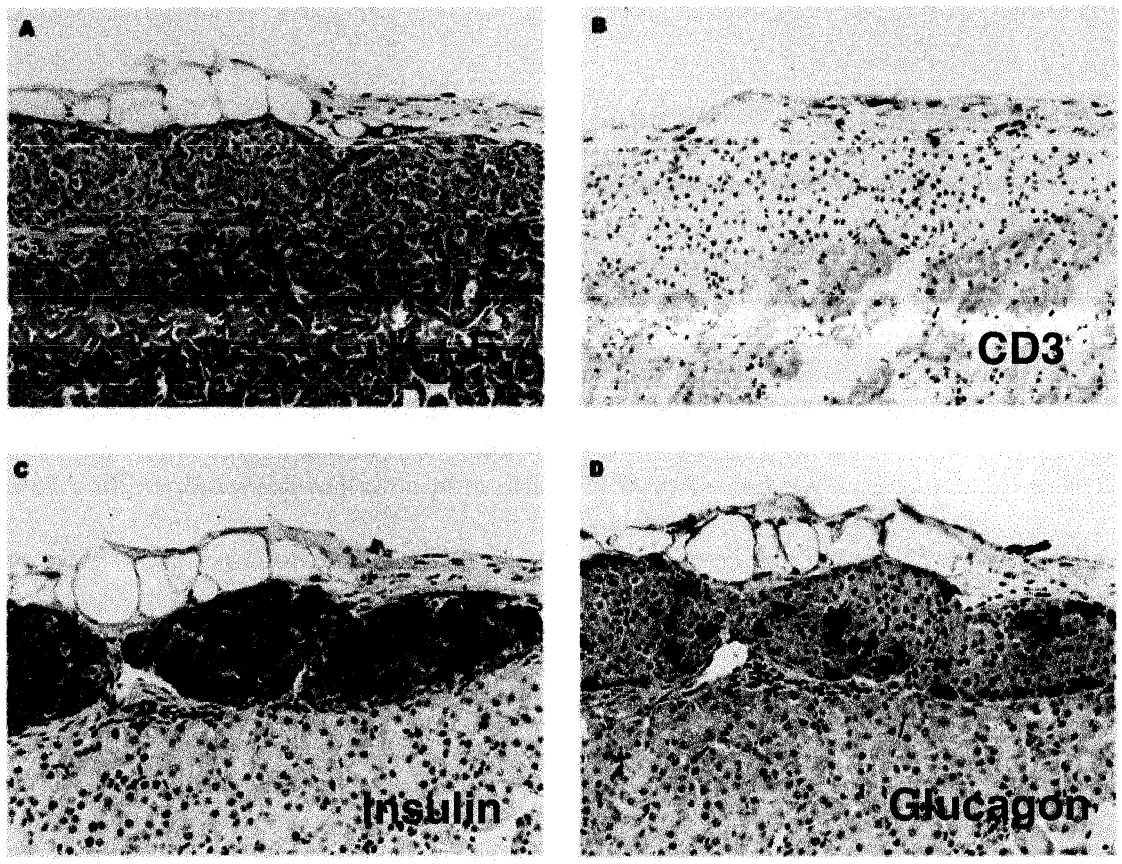

Figure 5. ***Histology of a Representative C57BL/6 Islet Allograft from a Chimeric NOD/Lt Recipient.*** Shown are histological sections of C57BL/6 islet tissue that had been transplanted into the renal sub-capsular space of a chimeric NOD/Lt mouse. The NOD/Lt recipient had developed spontaneous autoimmune diabetes and had been treated with radiation and anti-CD154 mAb and C57BL/6 bone marrow ~4 weeks before islet transplantation (see Methods). This recipient was normoglycemic for 124 days after transplantation and became hyperglycemic immediately after recovery of the kidney bearing the islet tissue shown here. A: Hematoxylin and eosin stained section showing normal appearing islet tissue. An inflammatory infiltrate (x 150) was not evident. B: A frozen section stained immunohistochemically for the presence of the $CD3^+$ lymphocytes. There is no appreciable $CD3^+$ T cell infiltrate. (x 150) C and D: Sections stained immunohistochemically for the presence of insulin and glucagon, respectively. Abundant hormone-containing cells are visible (x 150).

Skin Allograft Tolerance is Donor-specific in Allogeneic NOD/Lt Hematopoietic Chimeras bearing Functional Donor-specific Islet Allografts

We next determined whether the state of transplantation tolerance in chimeric NOD/Lt recipients of C57BL/6 bone marrow was donor-specific, as was the case for the chimeric BALB/c recipients described above. A subset of NOD/Lt mice that had been treated with conditioning radiation plus anti-CD154 mAb and were normoglycemic >100 days after transplantation of C57BL/6 islets were selected at random. They were given either a C57BL/6 ($H2^b$, donor-specific) or a CBA/JCR ($H2^k$, 3rd party) skin allograft. Survival of C57BL/6 skin allografts was significantly prolonged (MST >128 d, N=3). In contrast, CBA/JCR skin allografts survived only briefly (MST=15 d, N=3, Table 3). None of the islet allografts failed in either group during the period of observation, and recurrence of hyperglycemia after unilateral nephrectomy was observed in all mice. Histological analysis of the donor-specific C57BL/6 skin grafts obtained at the conclusion of the experiment showed no evidence of inflammation suggestive of graft rejection.

Table 3: Skin Allograft Survival on Chimeric NOD/Lt Mice Bearing Donor-Specific Islet Allografts

Bone Marrow Donor	Islet Allograft Donor	Skin Allograft Donor	Skin Allograft Survival (days)
C57BL/6	C57BL/6	C57BL/6	>26, >128, >128
C57BL/6	C57BL/6	CBA/JCr	13, 15, 23

Legend to Table 3: Chimeric NOD/Lt mice bearing C57BL/6 islet allografts were selected at random from among those presented in Figure 4 (Group 1). They were given either a C57BL/6 ($H2^b$, donor-specific) or a CBA/JCr ($H2^k$, 3rd party) skin allograft as described in Methods. None of the islet allografts failed in either group during the period of observation.

Summary

I describe a tolerance-based stem cell transplantation protocol that combines sub-lethal irradiation with transient blockade of the CD40-CD154 costimulatory pathway using an anti-CD154 antibody. With this protocol, I established hematopoietic chimerism in BALB/c mice transplanted with fully allogeneic C57BL/6 bone marrow. The percentage of donor-origin mononuclear cells in recipients was >99%. In addition, all chimeric mice treated with anti-CD154 antibody remained free of graft *vs.* host disease (GVHD) and accepted donor-origin but not third party skin allografts. It was similarly possible to create allogeneic hematopoietic chimerism in NOD/Lt mice with spontaneous autoimmune diabetes. Pancreatic islet allografts transplanted into chimeric NOD/Lt mice were resistant not only to allorejection but also to recurrence of autoimmunity. I conclude that it is possible to establish robust allogeneic hematopoietic chimerism in sub-lethally irradiated mice without subsequent GVHD by blocking the CD40-CD154 costimulatory pathway using as few as two injections of anti-CD154 antibody. I also conclude that chimerism created in this way generates donor-specific allograft tolerance and reverses the predisposition to recurrent autoimmune diabetes in NOD/Lt mice, enabling them to accept curative islet allografts.

CHAPTER IV

DST + ANTI-CD154 ANTIBODY LOWERS RADIATION REQUIREMENT FOR ALLOGENEIC CHIMERISM INDUCTION IN NORMAL MICE

Introduction

The major objective in stem cell transplantation is the survival of the injected allogeneic hematopoietic donor cells so that they can engraft and establish a chimeric state in the host. Earlier attempts to prolong the survival of the donor stem cells involved total myeloablative regimes and immunosuppressive drugs. Recently, I have developed an allogeneic chimerism induction protocol that requires only sub-lethal radiation by using anti-CD154 mAb. Even though the use of lethal radiation was eliminated, a high dose was still required to establish hematopoietic chimerism. In an effort to lower the radiation requirement, I adapted the protocol involved in peripheral tolerance induction that has been used in our lab to the induction of hematopoietic stem cell engraftment. Our lab has published that DST plus anti-CD154 mAb greatly prolongs the survival of allogeneic grafts and deletes alloreactive $CD8^+$ T cells. I hypothesized that DST plus anti-CD154 mAb would also prolong the survival of allogeneic stem cells facilitating their ability to engraft in the host. I theorized that the deletion of the alloreactive $CD8^+$ T cells before bone marrow introduction would lower the radiation requirements.

Results

Large Doses of Bone Marrow Cells with Anti-CD154 mAb Does Not Lower Radiation Dose Requirements

In an effort to determine if increasing the amount of bone marrow cells injected into the host would decrease the dose of radiation required for chimerism induction, I varied the radiation dose and bone marrow cell numbers. As shown in Table 4, one group of BALB/c mice was given 4 Gy of radiation, 25 x 10^6 bone marrow cells from C57BL/6 donors, and a short course of anti-CD154 mAb. As expected, all of the BALB/c mice in this group became chimeric with 90.8% of their PBMC being of donor-origin. Increasing their bone marrow dose to almost 10 times the standard dose and decreasing their irradiation to 2, 1 or 0 Gy did not induce chimerism induction in any of the BALB/c mice (Table 4). In an effort to see if extending the injection of bone marrow might facilitate chimerism induction, BALB/c mice were given 40 x 10^6 C57BL/6 bone marrow cells each day for five consecutive days for a total of 200 x 10^6 donor bone marrow cells in combination with anti-CD154 mAb treatment. None of the mice turned chimeric using this protocol.

Table 4: Mega-dose Bone Marrow Transplantation with Anti-CD154 mAb in BALB/c Mice using C57BL/6 Donors

Radiation (Gy)	Bone Marrow Dose	Anti-CD154 mAb	Number of Chimeric Mice (%)	Percentage of Donor-Origin PBMCs in Chimeric Mice
4	$25x10^6$	+	5 / 5	90.8%
2	$200x10^6$	+	0 / 3	---
1	$200x10^6$	+	0 / 3	---
0	$200x10^6$	+	0 / 8	---
0	5 X $40x10^6$	+	0 / 5	---

Legend to Table 4: BALB/c ($H2^d$) mice were randomly grouped to receive 0, 1, 2 or 4 Gy of irradiation. The mice were then transfused intravenously with C57BL/6 ($H2^b$) bone marrow cells at 3 different dose regimens: $25x10^6$ cells on Day 0; $200x10^6$ cells on Day 0; and $40x10^6$ cells/day on Days 0, 1, 2, 3 and 4. The hosts were also injected intraperitoneally with 2 injections of anti-CD154 mAb at 0.5 mg per injection on days 0 and +3 (relative to bone marrow transplantation on day 0). PBMCs from the hosts were analyzed 4-6 weeks after bone marrow transplantation.

DST in Conjunction with Anti-CD154 mAb Lowers the Radiation Requirement for Allogeneic Chimerism in Normal Inbred Mice

I hypothesized that the procedure to induce peripheral transplantation tolerance could help induce allogeneic hematopoietic chimerism with less radiation then previously required using anti-CD154 mAb plus bone marrow cells. BALB/c mice were given donor-specific transfusion (DST) consisting of 10 x 10^6 spleen cells plus anti-CD154 mAb, irradiated at varying doses, and injected with 25 x 10^6 C57BL/6 bone marrow cells. As shown in Table 5, 4 Gy of radiation, which is sufficient for BALB/c mice to become chimeric by injection of bone marrow and anti-CD154 mAb (Table 4), was also sufficient to induce allogeneic chimerism in all of the mice treated with DST, anti-CD154 mAb,

and bone marrow. Most of the PBMC analyzed (99.7%) was of donor-origin. Lowering the radiation down to 2 and 1 Gy resulted in 89% (8/9) of the BALB/c mice becoming chimeric with 28.4% and 21.0% donor-origin cells, respectively. Unexpectedly, 3 Gy of radiation did not lead to any chimeric mice in the 3 BALB/c mice treated. More animals need to be tested to determine if the result is due to statistical insufficiency in light of the fact that almost all of the mice treated with 2 or 4 Gy of radiation became chimeric. An additional experiment to determine the effect of increasing the bone marrow cell dose to 50 x 10^6 with 1 Gy, DST and anti-CD154 mAb produced chimeric mice in 100% (5/5) of the BALB/c and increased the percentage of donor-origin cells in the PBMC to ~37%.

I next determined if the protocol could induce allogeneic chimerism in other

Table 5: DST plus Anti-CD154 mAb and Bone Marrow Regime Lowers Radiation Requirement for Hematopoietic Chimerism Induction in BALB/c Mice Using C57BL/6 Donors

Radiation (Gy)	DST donor	Anti-CD154 mAb	Bone Marrow Donor	Bone Marrow Dose	Number of Chimeric Mice (%)	Percentage of Donor-Origin PBMCs in Chimeric Mice
4	C57BL/6	+	C57BL/6	$25x10^6$	3 / 3	99.7%
3	C57BL/6	+	C57BL/6	$25x10^6$	0 / 3	---
2	C57BL/6	+	C57BL/6	$25x10^6$	8 / 9	28.4%
1	C57BL/6	+	C57BL/6	$25x10^6$	8 / 9	21.0%
1	C57BL/6	+	C57BL/6	$50x10^6$	5 / 5	37.2%

Legend to Table 5: BALB/c ($H2^d$) mice were given DST by receiving 10 x 10^6 splenocytes from C57BL/6 ($H2^b$) donors on day –7 intravenously. They were injected intraperitoneally with 4 injections of anti-CD154 mAb at 0.5 mg per injection on days –7, -4, 0, +3 (relative to bone marrow transplantation on day 0). The hosts were irradiated with 1, 2, 3 or 4 Gy on Day 0. They were then transfused intravenously with 25 x 10^6 or 50 x 10^6 C57BL/6 ($H2^b$) bone marrow cells. PBMCs from the hosts were analyzed 6-7 weeks after bone marrow transplantation.

strains of mice. B10.BR mice were treated with C57BL/6 DST plus anti-CD154 mAb. The mice were separated into groups based on the dose of irradiation and the number of C57BL/6 bone marrow cells injected. As shown in Table 6, 100% of B10.BR mice given 25 x 10^6 bone marrow cells and 4 or 3 Gy of radiation became chimeric with 78.6% and 61.5% donor-origin cells in their PBMC, respectively. In an effort to increase the likelihood of obtaining chimeric mice with even lower doses of radiation, the bone marrow dosage for the next two groups was increased to 50 x 10^6 cells. As seen in Table 6, 2 and 1 Gy of radiation also led to chimerism in all of the B10.BR hosts.

Table 6: DST plus Anti-CD154 mAb and Bone Marrow Regime Lowers Radiation Requirement for Hematopoietic Chimerism Induction in B10.BR Mice Using C57BL/6 Donors

Radiation (Gy)	DST donor	Anti-CD154 mAb	Bone Marrow Donor	Bone Marrow Dose	Number of Chimeric Mice (%)	Percentage of Donor-Origin PBMCs in Chimeric Mice
4	C57BL/6	+	C57BL/6	$25x10^6$	5 / 5	78.6%
3	C57BL/6	+	C57BL/6	$25x10^6$	5 / 5	61.5%
2	C57BL/6	+	C57BL/6	$50x10^6$	5 / 5	74.3%
1	C57BL/6	+	C57BL/6	$50x10^6$	5 / 5	57.1%

Legend to Table 6: B10.BR ($H2^k$) mice were given DST by receiving 10 x 10^6 splenocytes from C57BL/6 ($H2^b$) donors on day –7 intravenously. They were injected intraperitoneally with 4 injections of anti-CD154 mAb at 0.5 mg per injection on days –7, -4, 0, +3 (relative to bone marrow transplantation on day 0). The hosts were irradiated with 1, 2, 3 or 4 Gy on Day 0. They were then transfused intravenously with 25 x 10^6 or 50 x 10^6 C57BL/6 ($H2^b$) bone marrow cells. PBMCs from the hosts were analyzed 5-6 weeks after bone marrow transplantation.

A third strain of mice was tested using the protocol with low dose of irradiation to confirm that the results were not strain biased. CBA/J mice were given DST plus anti-

CD154 mAb and 50 x 10^6 C57BL/6 bone marrow cells along with 2 or 1 Gy of radiation. 100% of the mice in both groups became chimeric with both expressing ~30% donor-origin cells in their PBMC (Table 7).

Table 7: DST plus Anti-CD154 mAb and Bone Marrow Regime Facilitates Induction of Hematopoietic Chimerism in CBA/J Mice Using C57BL/6 Donors with Low-dose Irradiation

Radiation (Gy)	DST donor	Anti-CD154 mAb	Bone Marrow Donor	Bone Marrow Dose	Number of Chimeric Mice (%)	Percentage of Donor-Origin PBMCs in Chimeric Mice
2	C57BL/6	+	C57BL/6	$50x10^6$	5 / 5	33.0%
1	C57BL/6	+	C57BL/6	$50x10^6$	9 / 9	35.6%

Legend to Table 7: CBA/J ($H2^k$) mice were given DST by receiving 10 x 10^6 splenocytes from C57BL/6 ($H2^b$) donors on day –7 intravenously. They were injected intraperitoneally with 4 injections of anti-CD154 mAb at 0.5mg per injection on days –7, -4, 0, +3 (relative to bone marrow transplantation on day 0). The hosts were irradiated with 1 or 2 Gy on Day 0. They were then transfused intravenously with 50 x 10^6 C57BL/6 ($H2^b$) bone marrow cells. PBMCs from the hosts were analyzed 5-6 weeks after bone marrow transplantation.

DST plus Anti-CD154 mAb Does Not Permit Establishment of Allogeneic Chimerism in NOD/Lt with less than 6Gy of Radiation Pretreatment

The promising results obtained in normal inbred strain mice using DST and anti-CD154 mAb led to our testing the protocol in NOD mice. As shown in Table 8, the protocol consisting of allogeneic bone marrow cells and anti-CD154 mAb yielded chimeric mice only when at least 6 Gy of irradiation was used; 5 Gy did not produce any chimeras. The addition of DST into the protocol also yielded chimeras in 60% of the

mice irradiated with 6 Gy, but none of the mice treated with 5 Gy showed donor engraftment. The results indicate that the peripheral tolerance protocol does not lower the radiation requirement in autoimmune-prone NOD mice.

Table 8: DST plus Anti-CD154 mAb and Bone Marrow Regime Does Not Facilitate Induction of Hematopoietic Chimerism in NOD Mice Using C57BL/6 Donors with Low-dose Irradiation

Radiation (Gy)	DST donor	Anti-CD154 mAb	Bone Marrow Donor	Bone Marrow Dose	Number of Chimeric Mice (%)	Percentage of Donor-Origin PBMCs in Chimeric Mice
6	---	+	C57BL/6	$25x10^6$	5 / 5	99.0%
6	C57BL/6	+	C57BL/6	$25x10^6$	3 / 5	99.0%
5	---	+	C57BL/6	$25x10^6$	0 / 5	---
5	C57BL/6	+	C57BL/6	$25x10^6$	0 / 5	---

Legend to Table 8: NOD ($H2^{g7}$) mice were transfused with 25 x 10^6 C57BL/6 ($H2^b$) bone marrow cells on Day 0. They were also irradiated with 6 or 5 Gy on Day 0. Half the mice were given C57BL/6 DST and injected intraperitoneally with 4 injections of anti-CD154 mAb at 0.5 mg per injection on days –7, -4, 0, +3 (relative to bone marrow transplantation on day 0). The other half were injected with 2 injections of anti-CD154 mAb at 0.5 mg per injection on days 0 and +3. PBMCs from the hosts were analyzed 6 weeks after bone marrow transplantation.

Summary

A protocol used to induce peripheral tolerance to allogeneic skin and islet grafts is shown to be effective in enhancing the engraftment of allogeneic bone marrow cells to induce hematopoietic chimerism with only a low dose of radiation conditioning. As shown in Table 4, the previous protocol consisting of bone marrow cells and anti-CD154 mAb did not induce allogeneic chimerism at lower radiation doses even when the bone marrow cell dose was increased to almost 10 times the standard dosage. By incorporating DST into the protocol, we were able to induce mixed allogeneic chimerism in 3 different strains of mice (BALB/c, B10.BR and CBA/J) using C57BL/6 donors with as little as 1 Gy of radiation. Even at this low radiation dose, the percentage of donor-origin cells in the hosts was moderately high, ranging from 21-57%. However, the extension of this protocol to NOD mice did not reduce the radiation required for the generation of chimerism.

CHAPTER V

ALLOGENEIC CHIMERISM WITHOUT MYELOABLATIVE PRE-TREATMENT

Introduction

The previous experiments documented that DST plus anti-CD154 mAb can greatly reduce the radiation required to induce hematopoietic chimerism using fully allogeneic bone marrow cells. A major objective in stem cell transplantation is to find a protocol using the least amount of myeloablation to the host, but still lead to engraftment of the donor stem cells. So, the hypothesis is that by first inducing peripheral tolerance using DST plus anti-CD154 mAb, allogeneic chimerism could be established using no myeloablative treatment (*i.e.* no radiation). I also explored the requirements and cellular mechanisms involved in the induction of allogeneic chimerism using this protocol.

Results

Establishment of Allogeneic Hematopoietic Chimerism in the Absence of Host Myeloablative Conditioning in BALB/c Mice

We first tested our peripheral transplantation tolerance-induction protocol for its ability to facilitate the generation of hematopoietic chimerism in BALB/c ($H2^d$) mice.

BALB/c mice were treated with a C57BL/6 ($H2^b$) DST plus anti-CD154 mAb and injected 7 days later with 50 x 10^6 allogeneic C57BL/6 bone marrow cells in the absence of any myeloablative conditioning. As shown in Table 9 (Group 1), 89% of treated BALB/c mice became chimeric. The percentage of donor-origin PBMC in these mice 8 to 9 weeks after bone marrow transplantation averaged ~9%. PBMC were re-analyzed at intervals 12 to 30 weeks after transplantation, and this level of chimerism remained stable throughout the period of observation (data not shown). There was no evidence of chimerism in any BALB/c mice treated with bone marrow and anti-CD154 mAb monotherapy (Table 9, Group 2).

Table 9: Hematopoietic Chimerism in Recipients of C57BL/6 Bone Marrow

Group	*Host*	*Myeloablative Conditioning*	*DST*	*Anti-CD154 mAb*	*Bone Marrow Dose (x 10^6)*	*Frequency of Chimerism (%)*	*Donor Origin PBMC in Chimeric Mice (%)*
1	BALB/c	No	Yes	Yes	50	17/19 (89%)	9.2 ± 3.2
2	BALB/c	No	No	Yes	50	0/10 (0%)*	—
3	BALB/c	Yes	Yes	Yes	50	5/5 (100%)	37.2 ± 4.3%
4	CBA/J	No	Yes	Yes	50	17/25 (68%)	8.3 ± 4.5
5	CBA/J	No	No	Yes	50	0/9 (0%)*	—
6	CBA/J	No	Yes	Yes	100	5/5 (100%)	16.5 ± 5.7%
7	CBA/J	Yes	Yes	Yes	50	9/9 (100%)	35.6 ± 5.2%
8	B10.BR	No	Yes	Yes	50	14/14 (100%)	16.9 ± 12.4
9	B10.BR	No	No	Yes	50	15/15 (100%)	19.7 ± 10.9
10	B10.BR	No	No	No	50	0/8 (0%)*	—

Legend to Table 9: BALB/c ($H2^d$), CBA/J ($H2^k$) or B10.BR ($H2^k$) mice were randomized to the indicated treatment groups and injected with C57BL/6 ($H2^b$) bone marrow cells at the dose indicated on day 0. Mice treated with a donor-specific transfusion (DST) received 10^7 C57BL/6 spleen cells on day –7 relative to bone marrow transplantation. Mice treated with anti-CD154 mAb received 4 doses of 0.5 mg intraperitoneally on days –7, -4, 0, +3. Hematopoietic chimerism was defined as the presence of >0.5% donor-origin (H2-K^{b+}) PBMC as described in Methods. *The percentage of donor-origin PBMC in all non-chimeric mice was in all cases below the limit of detection (<0.5%).

The Levels of Hematopoietic Chimerism Achieved Generate Donor-Specific Transplantation Tolerance in the Absence of GVHD

Although we achieved allogeneic hematopoietic chimerism, the levels of chimerism were relatively low. To document that these levels were sufficient to generate transplantation tolerance, subsets of both chimeric and non-chimeric BALB/c mice from Table 9 (Groups 1 and 2) were transplanted with C57BL/6 skin allografts 9 weeks after injection of C57BL/6 bone marrow. Median survival time (MST) of skin allografts in the chimeric mice was >89 days (38, >56, 80, 80, >98, >98, >112, >112 days). In contrast, most of non-chimeric mice rejected skin allografts rapidly (11, 11, 11, 11, 49 days, $p<0.001$). To document that this state of transplantation tolerance was donor-specific, additional chimeric BALB/c mice were transplanted 9 weeks after bone marrow transplantation with third-party skin allografts from CBA/J donors. Survival of these 4 allografts was very brief, all of them rejecting on day 14 (p=N.S. vs. non-chimeric mice).

Animals were observed for signs of GVHD throughout the period of observation (up to 30 weeks). There was no sign of illness in any chimeric bone marrow recipients given the anti-CD154 mAb regimen. Thirty weeks after transplantation, 4 chimeric BALB/c mice were selected at random and studied histologically. There was no evidence of GVHD in samples of skin, liver, or small or large intestine in any of the mice.

Allogeneic Stem Cell Engraftment Using anti-CD154 mAb and DST but no Myeloablation can be Achieved in CBA/J and B10.BR Recipient Mice

To determine if the engraftment of allogeneic bone marrow cells in mice treated with DST plus anti-CD154 mAb is strain-dependent, we performed the same experiment

using two different strains of mice as recipients. When tested 8 to 9 weeks after administration of a C57BL/6 DST, anti-CD154 mAb, and C57BL/6 bone marrow cells, 68% of CBA/J mice (Table 9, Group 4) and 100% of B10.BR mice (Group 8) became chimeric. In both cases, the frequency of chimerism was statistically similar to that achieved using BALB/c recipients (Group 1, p=N.S.). The percentages of donor-origin PBMCs in chimeric CBA/J mice (~8%) were similar to those in chimeric BALB/c mice (p=N.S.) but levels in both BALB/c and CBA recipients were significantly less ($p<0.01$) than levels achieved in B10.BR mice (~17%).

Like BALB/c mice, CBA/J recipients of bone marrow and anti-CD154 mAb but no DST did not become chimeric (Table 9, Group 5). In contrast, B10.BR mice treated in the same way uniformly became chimeric (Group 9). As was true for B10.BR recipients given both anti-CD154 mAb and a DST (Group 8), ~20% of their PBMC were of donor-origin. In both groups of B10.BR chimeras, the percentage of donor-origin cells varied widely from 2.6 to 46%. B10.BR mice treated with a bone marrow graft but neither anti-CD154 mAb nor DST failed to become chimeric (Group 10).

To determine if the result obtained from B10.BR (Table 9, Group 9) was due to the similarities in the minor MHCs between the donor and host, a strain of mice that did not share a common ancestor was used as a donor. DBA/2 ($H2^d$) cells were injected into B10.BR hosts. As shown in Table 10, all of the B10.BR recipients of DBA/2 DST plus anti-CD154 mAb and DBA/2 bone marrow cells readily exhibited engraftment with ~40% of their PBMC being of donor-origin (Group 2). Interestingly, even though all of the mice that received just anti-CD154 mAb and DBA/2 bone marrow also turned

Table 10: Hematopoietic Chimerism in B10.BR Recipients of DBA/2 Bone Marrow

Group	*Host*	*DST Donor*	*Bone Marrow Donor*	*Frequency of Chimerism (%)*	*Percentage of Donor Origin PBMC in Chimeric Mice (%)*
1	B10.BR	C57BL/6	C57BL/6	5 / 5 (100%)	20.2 ± 8.0
2	B10.BR	DBA/2	DBA/2	5 / 5 (100%)	39.1 ± 20.4
3	B10.BR	None	DBA/2	4 / 4 (100%)	6.1 ± 0.8

Legend to Table 10: B10.BR ($H2^k$) mice were randomized into 3 groups. All groups were injected intraperitoneally with 4 doses of 0.5 mg anti-CD154 mAb on days –7, -4, 0, +3. The control group received the standard protocol of DST on day -7 and 50 x 10^6 bone marrow cells on day 0 from C57BL/6 donors. The 2nd group received the same treatment, but from DBA/2 ($H2^d$) donors. The 3rd group just received bone marrow cells from DBA/2 donors. Chimerism was defined as >0.5% donor-origin PBMC as determined by flow cytometry analysis.

chimeric, the percentage of donor-origin cells was drastically smaller (~6%) than Group 2 ($p<0.02$) and also than those that received anti-CD154 mAb and C57BL/6 bone marrow without DST (Table 9, Group 9, $p<0.03$).

Increasing Bone Marrow Cell Dose or Adding Minimal Myeloablative Conditioning Increases Levels of Chimerism

Because hematopoietic chimerism can be established in the absence of myeloablative conditioning if very high numbers of bone marrow cells are transplanted (142;143), we studied the effect of increasing the donor inoculum in mice treated with both DST and anti-CD154 mAb. Transplantation of 100 x 10^6 C57BL/6 bone marrow cells into CBA/J recipients was associated with uniform generation of chimerism (Table 9, Group 6), and the percentage of donor-origin PBMC in these mice was on average double that observed in CBA/J recipients of 50 x 10^6 C57BL/6 cells (Table 9, Group 4, $p<0.03$).

The addition of minimal myeloablation also improved outcome. Both BALB/c (Table 9, Group 3) and CBA/J (Group 7) recipients uniformly became chimeric if treated with 1 Gy of whole body irradiation prior to DST, anti-CD154 mAb, and infusion of 50 x 10^6 C57BL/6 bone marrow cells. In both cases, donor-origin cells comprised more than a third of the PBMC population 6-7 weeks after bone marrow injection, and these percentages were statistically significantly greater than the percentages achieved without conditioning (Table 9, Groups 1 and 4, $p<0.001$ for both comparisons).

Timing of DST and anti-CD154 mAb Treatment is Important for Generation of Allogeneic Chimerism

In studies of solid organ transplantation tolerance induction, we have shown that administration of DST plus anti-CD154 mAb leads to the deletion of peripheral host alloreactive $CD8^+$ T cells, an effect that is maximal ~3 days after the initiation of treatment (140). We hypothesized that deletion of host alloreactive $CD8^+$ T cells would define the optimal time point at which allogeneic bone marrow chimerism could be achieved in the absence of myeloablative conditioning. We tested this hypothesis by varying the timing of DST plus anti-CD154 mAb treatment in relation to C57BL/6 bone marrow transplantation into CBA/J recipients. In these experiments, the first of the four injections of anti-CD154 mAb was always given immediately before the DST.

When DST was given 10 or 14 days before bone marrow transplantation, 60% and 80% of recipients, respectively, became chimeric (Table 11, Groups 1 and 2); this rate of success was comparable to that achieved when DST was injected 7 days before transplantation (68%, Table 9, Group 4, p=N.S.). The percentage of donor-origin PBMC

Table 11: Hematopoietic Chimerism in CBA/J Recipients of C57BL/6 Bone Marrow

Group	*Day of DST Injection*	*Frequency of Chimerism (%)*	*Percentage of Donor Origin PBMC in Chimeric Mice (%)*
1	-14	4/5 (80%)	10.2 ± 1.9
2	-10	3/5 (60%)	10.4 ± 4.2
3	-5	0/5 (0%)*	—
4	-3	0/5 (0%)*	—

Legend to Table 11: Groups of CBA/J ($H2^k$) mice were randomized and transplanted with 50 x 10^6 C57BL/6 ($H2^b$) bone marrow cells on day 0. All mice also received a single C57BL/6 DST consisting of 10^7 spleen cells on days -3, -5, -10, or -14 relative to bone marrow transplantation. In addition, all mice were injected intraperitoneally with 4 doses of 0.5 mg anti-CD154 mAb on days 0, +3, +7, and +10 relative to the DST. The temporal relationship of the DST and anti-CD154 mAb injections was the same as in Table 9; only the timing of the bone marrow graft was varied. Chimerism was defined as the presence of >0.5% donor-origin (H2-K^{b+}) PBMC 6 weeks after transplantation. *$p<0.01$ *vs.* Table 9, Group 2.

detected 6 or more weeks after transplantation in the mice that became chimeric was ~10%, irrespective of the timing of the DST.

In contrast, when DST was injected 5 or 3 days before bone marrow transplantation, no recipients became chimeric (Table 11, Groups 3 and 4). Given that host alloreactive $CD8^+$ T cells are deleted in mice treated with DST plus anti-CD154 mAb at these time points (140), the result was unexpected.

Summary

The induction of peripheral tolerance before donor bone marrow transplantation is shown to facilitate the establishment of allogeneic chimerism without the need for radiation pre-treatment. The protocol is shown not to be strain-specific as two other strains of mice are also able to engraft donor stem cells without myeloablation. The establishment of chimerism also induced transplantation tolerance, as expected. Interestingly, a minimum of 7 days was required between the start of the peripheral tolerance protocol and donor bone marrow transplantation to achieve allogeneic chimerism.

CHAPTER VI

MECHANISMS REGULATING ALLOGENEIC STEM CELL ENGRAFTMENT IN NON-MYELOABLATED HOSTS

Introduction

The data presented in Chapter V clearly demonstrate that allogeneic hematopoietic chimerism can be generated without cytoreductive conditioning using a peripheral transplantation tolerance protocol consisting of DST and anti-CD154 mAb. In an effort to determine the mechanism involved in the induction and maintenance of this chimeric state, I employed TCR transgenic mice to determine the fate of the alloreactive $CD8^+$ T cells in the host. I also used depleting antibodies to determine the cell subsets required for engraftment and rejection of donor bone marrow cells. The hypothesis is that the induction and maintenance of the chimeric state not only involves the permanent deletion of alloreactive $CD8^+$ T cells in the host, but may also involve the regulation of other cell populations.

Results

The Combination of DST, Anti-CD154 mAb, and Bone Marrow Engraftment Leads to Permanent Deletion of Host Alloreactive $CD8^+$ Peripheral T Cells

In studies of peripheral tolerance induction using DST plus anti-CD154 mAb, we have documented that host alloreactive $CD8^+$ T cells are deleted rapidly, that these cells then reappear over time, and that their reappearance is associated with rejection of healed-in skin allografts (139). Given the apparent permanence of hematopoietic chimerism in mice treated with DST, anti-CD154 mAb, and bone marrow, we hypothesized that establishment of chimerism would lead to permanent deletion of peripheral alloreactive $CD8^+$ T cells. To test this hypothesis, we used KB5 synchimeric mice. These mice circulate small numbers of TCR transgenic alloreactive $CD8^+$ T cells that are continuously replenished over time as newly generated KB5 T cells are released from the thymus (139).

KB5 synchimeric mice were randomized into 4 groups. Mice in group 1 (Figure 6) were untreated. Mice in Groups 2 and 3 received anti-CD154 mAb plus either a C57BL/6 bone marrow (Group 2) or C57BL/6 spleen cell DST (Group 3). Mice in Group 4 received anti-CD154 mAb plus a C57BL/6 spleen cell DST and C57BL/6 bone marrow.

The level of alloreactive DES^+CD8^+ T cells in the peripheral blood of these 4 groups of mice is shown in Figure 6. The level of DES^+CD8^+ T cells in control mice

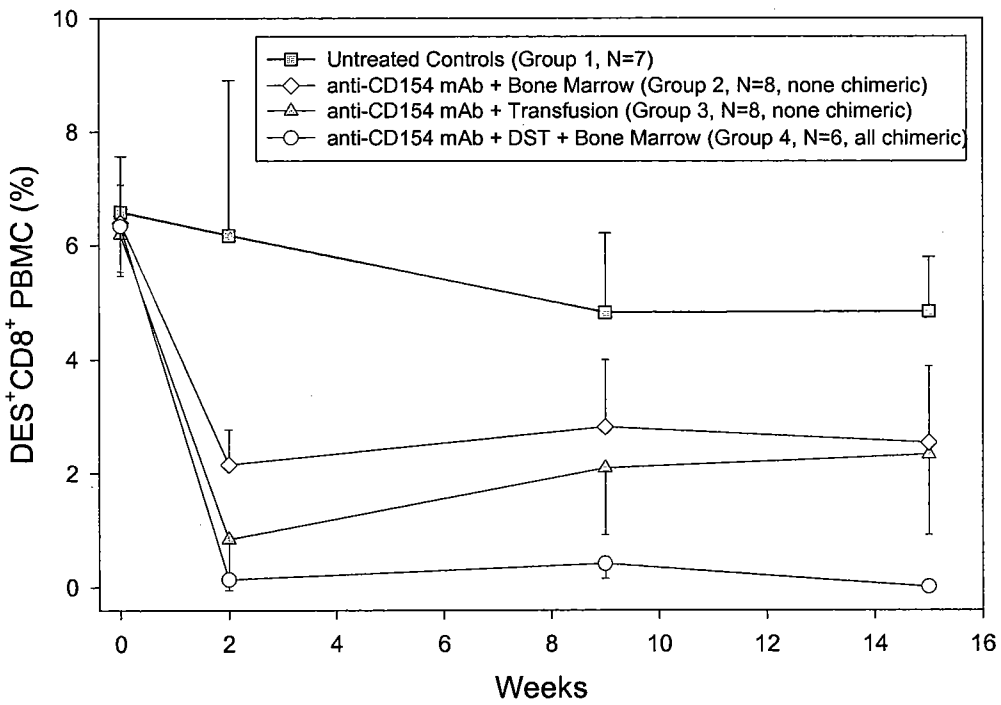

Figure 6. ***Permanent Deletion Of Allo-Reactive T Cells In Chimeric Mice.*** Deletion of peripheral host alloreactive $CD8^+$ T cells. KB5 CBA synchimeras were randomized into 4 cohorts. Group 1 was untreated. Group 2 was injected with 4 doses of anti-CD154 mAb on days 0, +3, +7, and +10 relative to injection of 50 x 10^6 C57BL/6 bone marrow cells on day 7. Group 3 received 4 doses of 0.5 mg of anti-CD154 mAb at the same intervals plus a transfusion of C57BL/6 spleen cells on day 0. Group 4 received a donor-specific transfusion of C57BL/6 spleen cells on day 0 and anti-CD154 mAb on days 0, +3, +7, and +10 relative to injection of 50 x 10^6 C57BL/6 bone marrow cells on day 7. The percentage of DES^+CD8^+ cells in the blood was determined on day 0 before any treatment and then at the indicated times. Within two weeks of treatment, the percentage of DES^+CD8^+ cells was significantly lower in all treatment groups compared with controls ($p<0.001$). Thereafter, the percentage of DES^+CD8^+ cells in Groups 2 and 3 tended to rise towards that observed in controls, but even at week 15 the percentage remained less than in controls ($p<0.001$). In contrast, the percentage of DES^+CD8^+ cells in Group 4 remained extremely low throughout the course of the experiment, and at week 15 was significantly lower than in all other groups ($p<0.001$ for each comparison). In 5 of 6 mice, in Group 4, no alloreactive $CD8^+$ T cells were detected (<0.2%) for the duration of experiment. In one mouse, 2.6% DES^+CD8^+ T cells were detected at week 9, but were undetectable (<0.2%) in this mouse when analyzed at week 15. With respect to chimerism, defined as ≥0.5% donor-origin ($H2\text{-}K^{b+}$) PBMC 9 weeks after transplantation, all mice in group 4 were chimeric and none in Groups 2 or 3 were chimeric.

during the period of observation was ~5 to ~6.5%; these levels are comparable to those we have reported previously in this model system (139).

None of the mice in Groups 2 and 3 became chimeric; no donor-origin cells being detectable at any time point throughout the 15 week period of observation. As expected,

and consistent with previous reports (139), the level of alloreactive DES^+CD8^+ T cells in mice treated with anti-CD154 mAb and a splenocyte DST (Group 3) was much lower within two weeks of transfusion (~0.8%). Thereafter the levels rose slowly and had recovered to ~2.4% by week 15. The behavior of mice treated with anti-CD154 mAb and bone marrow (Group 2) was similar, although the initial decline was less dramatic than that associated with the use of a splenocyte DST.

The results for the mice treated with anti-CD154 mAb, a splenocyte DST, and then with a bone marrow allograft (Group 4) were much different. As expected, 6 of 8 (75%) became chimeric. The percentage of donor-origin PBMC 9 weeks after transplantation was high ($22.3 \pm 13.2\%$) and remained at about this level throughout the 15 week period of observation. In striking contrast to the outcome in the other Groups, the level of DES^+CD8^+ T cells in the six chimeric mice fell within 2 weeks to levels that were below the limit of detection and remained at that low level throughout the period of observation ($p \leq 0.001$ vs. all other groups at week 15).

Normal Distribution of CD4 and CD8 Cells in the Thymus of KB5 Synchimeric Mice

The long-term absence of peripheral alloreactive DES^+CD8^+ cells in mice with hematopoietic chimerism suggested that they might be undergoing intrathymic deletion. Before proceeding to test this possibility, we first analyzed overall thymic maturation in the untreated KB5 synchimeric mouse. It was important to do so because the KB5 transgenic mice used to generate synchimeras have an abnormally large population of single-positive $CD8^+$ cells in the thymus (156). We observed, however, that KB5

synchimeric mice exhibit a normal distribution of total $CD4^+$ and $CD8^+$ thymocytes (157-159). The thymocytes of untreated KB5 synchimeric mice consisted of a large population of double-positive cells (81.5 ± 4.3%, N=14) and smaller populations of $CD4^+CD8^-$ single positive cells (9.4 ± 1.4%, N=14) and $CD4^-CD8^+$ (4.4 ± 1.6%, N=14) single positive cells. Representative histograms are shown in Figure 7. These percentages of single and double positive thymocytes are typical of those observed in normal untreated adult mice (158;160;161).

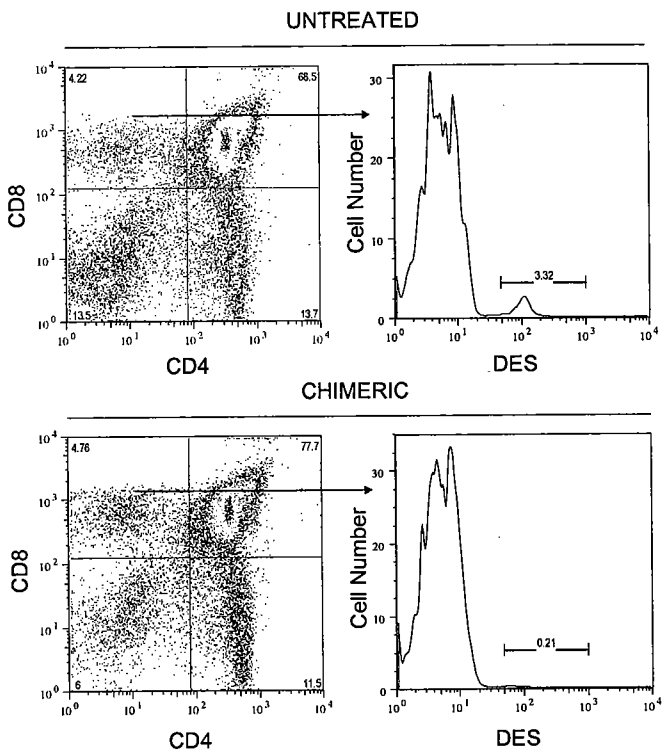

Figure 7. ***Intrathymic Deletion Of Alloreactive T Cells In Chimeric Mice.*** Intrathymic deletion of host alloreactive $DES^+CD8^+CD4^-$ thymocytes. KB5 CBA synchimeras were randomized into 2 groups. Group 1 (upper panels) was left untreated. Group 2 (lower panels) was injected with a C57BL/6 DST on day -7 and anti-CD154 mAb on days -7, -4, 0, and +3 relative to injection of 50 x 10^6 C57BL/6 bone marrow cells on day 0. Thymi were recovered 35 weeks after bone marrow transplantation and analyzed by flow microfluorometry for the percentage of $DES^+CD8^+CD4^-$ thymocytes as described in Methods. Shown in the left column are representative dot plots; the percentage of cells expressing CD4 and CD8 is indicated in each quadrant. Shown in the right column are histograms indicating the number of DES^+ cells in the $CD8^+CD4^-$ quadrant. Shown are representative data; the complete dataset is given in Table 12.

$DES^+CD8^+CD4^-$ Thymocytes are not deleted by Treatment with DST plus Anti-CD154 mAb

Having determined that the overall distribution of thymocyte $CD4^+$ and $CD8^+$ phenotypes in synchimeric mice is normal, we next measured the percentages of DES^+CD8^+ thymocytes following costimulation blockade and splenocyte transfusion. Before any treatment, the percentage of $CD8^+$ $CD4^-$ thymocytes that were also DES^+ was 26.4 ± 20.8% (Table 12, Group 1). $DES^+CD8^+CD4^-$ thymocytes were also readily detectable at statistically similar levels 4 and 7 days after treatment with DST and anti-CD154 mAb (Table 12, Groups 2 and 3, p=N.S.). In contrast, it is known that that peripheral DES^+CD8^+ cells are deleted within 3 days of treatment with DST plus anti-CD154 mAb, well before graft placement (140).

Bone Marrow Cell Engraftment in Mice Treated with DST plus Anti-CD154 mAb Leads to Intrathymic Deletion of $DES^+CD8^+CD4^-$ Cells

We next tested the hypothesis that successful generation of hematopoietic chimerism subsequent to treatment with DST and anti-CD154 mAb would lead to the deletion of $DES^+CD8^+CD4^-$ alloreactive thymocytes. As shown in Table 12, 15 days after bone marrow transplantation the percentage of $DES^+CD8^+CD4^-$ thymocytes in mice treated with DST and anti-CD154 mAb (Table 12, Group 5) was ~50% less than in age-matched mice that had been given DST and anti-CD154 mAb but no graft (Group 4), but at this time point the difference was not statistically significant (p=0.08). By 21-30 days after bone marrow injection, it was possible to distinguish chimeric and non-chimeric

Table 12: Hematopoietic Chimerism and Host Alloreactive $CD8^+$ T Cells in KB5 Synchimeric Recipients of C57BL/6 Bone Marrow

Group	*Transfusion Donor*	*Bone Marrow Donor*	*N*	*Chimeric*	*Time of Analysis Relative to completion of treatment (Days)*	*Host $CD8^+CD4^-$ Thymocytes that were DES^+ (%)*
1	None	None	11	—	Same approximate age as group 1 No treatment given	26.4 ± 20.8[a]
2	C57BL/6	None	3	—	-3 d	18.6 ± 4.8
3	C57BL/6	None	7	—	0 d	25.7 ± 11.2
4	C57BL/6	None	4	—	+15 d	38.2 ± 21.7
5	C57BL/6	C57BL/6	4	*See legend	+15 d	16.2 ± 13.8[b]
6	C57BL/6	None	6	No	+21-30 d	30.4 ± 13.9
7	C57BL/6	C57BL/6	3	No	+21-30 d	30.4 ± 8.9
8	C57BL/6	C57BL/6	3	Yes	+21-30 d	All <0.2[c]
9	None	None	3	—	Age matched, never treated	2.8 ± 0.6
10	C57BL/6	C57BL/6	2	No	35 weeks	4.4, 18.3
11	C57BL/6	C57BL/6	3	Yes	35 weeks	All <0.2[d]

Legend to Table 12: CBA/J ($H2^k$) mice ~4 weeks of age were irradiated (2 Gy) and injected with bone marrow from KB5 CBA/J TCR transgenic donors as described in Methods. Eight to 10 weeks later, with no additional irradiation, these KB5 CBA/J synchimeras received a donor-specific transfusion consisting of 10^7 C57BL/6 ($H2^b$) spleen cells on day -7 plus 4 intraperitoneal doses anti-CD154 mAb (0.5 mg/dose) on days –7, -4, 0, +3 relative to intravenous injection of 50 x 10^6 C57BL/6 bone marrow cells on day 0. Thymi were recovered at the indicated time points relative to marrow transplantation on day 0 and the percentage of host anti-donor alloreactive $DES^+CD8^+CD4^-$ thymocytes was measured by flow microfluorometry. a: p=N.S. vs. groups 2 and 3. b: p=0.08 vs. group 4. c: $p<0.01$ vs. groups 6 and 7. d: $p<0.05$ vs. combined groups 9 and 10. *At the 15 day time point, it cannot reliably be determined if mice are chimeric.

recipients. At this time point levels of $DES^+CD8^+CD4^-$ thymocytes remained at high baseline levels in both mice that had received DST plus anti-CD154 mAb but no graft (Table 12, Group 6) and in mice that had received DST plus anti-CD154 mAb plus a graft but had not become chimeric (Group 7). In contrast, $DES^+CD8^+CD4^-$ thymocytes

were undetectable (<0.3%) in all chimeric mice (Group 8, $p<0.01$ vs. both Groups 6 and 7).

Additional mice were studied 35 weeks after treatment to assess the durability of alloreactive thymocyte deletion. We first noted that the percentage of $DES^+CD8^+CD4^-$ thymocytes in age-matched but untreated synchimeras had spontaneously fallen over time, but were nonetheless readily detectable. The decline was from ~26% at baseline (Table 12, Group 1) to ~3% 8 to 9 months later (Table 12, Group 9, $p=0.01$). $DES^+CD8^+CD4^-$ thymocytes were also readily detectable in two recipients of DST, anti-CD154 mAb, and bone marrow that had not become chimeric (Group 10). In contrast, no $DES^+CD8^+CD4^-$ thymocytes could be detected in any of three recipients of DST, anti-CD154 mAb, and bone marrow that had become and remained chimeric (Table 12, Group 11, $p<0.05$). A representative histogram documenting the disappearance of $DES^+CD8^+CD4^-$ thymocytes in one of the chimeric mice from Group 11 is shown in Figure 7. Although lacking in $DES^+CD8^+CD4^-$ thymocytes, the thymi of these mice 35 weeks after bone marrow transplantation were clearly allochimeric. The presence of both host ($H2\text{-}K^k$) and donor ($H2\text{-}K^b$) thymocytes was confirmed by flow microfluorometry (data not shown).

MHC-Matching of the DST and Bone Marrow Donors is not Required for Allogeneic Bone Marrow Engraftment in Normal Mice

Induction of peripheral transplantation tolerance using DST and anti-CD154 mAb requires that the MHC of the transfusion donor be the same (*i.e.*, "donor specific") as that

of the graft donor (138). We hypothesized that successful generation of hematopoietic chimerism using our protocol would also require MHC-matching of the transfusion and bone marrow cells, *i.e.* that the transfusion had to be "donor-specific". To test this hypothesis, CBA/J mice ($H2^k$) were treated with a C57BL/6 ($H2^b$) non-donor specific spleen cell transfusion plus anti-CD154 mAb and then injected with BALB/c ($H2^d$) bone marrow cells. Unexpectedly, all became chimeric (Table 13, Group 1). To verify this unexpected outcome, we reversed the DST and bone marrow donors. CBA mice ($H2^k$) were treated with BALB/c ($H2^d$) spleen cell transfusion plus anti-CD154 mAb and then injected with C57BL/6 ($H2^b$) bone marrow cells. Again, the majority (90%) of these mice became chimeric (Table 13, Group 2).

Reduction of High Numbers of Host Alloreactive $CD8^+$ T Cells is Required for Bone Marrow Engraftment in KB5 Synchimeras

To begin to determine the role of the spleen cell transfusion in facilitating subsequent engraftment of bone marrow cells, we used KB5 synchimeras. In addition to circulating their normal complement of alloreactive T cells, these mice also circulate large numbers (6-8%) of DES^+CD8^+ alloreactive (anti-H2-K^b) T cells (140). Using our standard protocol, which is known to delete DES^+CD8^+ peripheral T cells (140), we observed that 2 of 3 KB5 synchimeras given a C57BL/6 DST, anti-CD154 mAb, and C57BL/6 bone marrow cells became chimeric (Table 13, Group 3). Confirming the results obtained in normal CBA/J mice (Group 1), all KB5 synchimeras treated with C57BL/6 spleen cell transfusion plus anti-CD154 mAb and then given BALB/c ($H2^d$) bone marrow became chimeric (Group 4). We then reversed the MHCs of the transfusion

Table 13: DST Facilitates Hematopoietic Cell Engraftment by Both Allo-specific and Allo-non-specific Mechanisms

Group	*Host*	*DST Donor*	*Bone Marrow Donor*	*Frequency of Chimerism (%)*	*Percentage of Donor Origin PBMC in Chimeric Mice (%)*
1	CBA/J	C57BL/6	BALB/c	9/9 (100%)	2.8 ± 0.8
2	CBA/J	BALB/c	C57BL/6	9/10 (90%)	7.4 ± 2.1
3	KB5 CBA/J Synchimera	C57BL/6	C57BL/6	2/3 (67%)	22.1 ± 10.4
4	KB5 CBA/J Synchimera	C57BL/6	BALB/c	5/5 (100%)	27.1 ± 5.9
5	KB5 CBA/J Synchimera	BALB/c	C57BL/6	0/4 (0%)*	<0.5

Legend to Table 13: CBA/J ($H2^k$) and KB5 CBA/J TCR transgenic synchimeric mice ($H2^k$) were injected intravenously with 10^7 spleen cells as a DST on day -7 and intraperitoneally with 4 doses of 0.5 mg anti-CD154 mAb on days –7, -4, 0, +3 relative to intravenous injection of 50 x 10^6 C57BL/6 ($H2^b$) or BALB/c ($H2^d$) bone marrow cells on day 0. Chimerism was defined as >0.5% donor-origin PBMC as determined by flow cytometry analysis. *$p<0.01$ *vs.* Group 4.

and bone marrow donors and found, in contrast, that no KB5 synchimeric mice became allochimeric when treated with BALB/c spleen cell transfusion plus anti-CD154 mAb, and then given C57BL/6 ($H2^b$) bone marrow cells (Table 13, Group 5, $p<0.01$ *vs.* Group 4). The result suggests that, in the presence of large numbers of allospecific T cells in the recipient, as in the synchimera with large numbers of anti-H2-K^b T cells, the transfusion may need to be matched to that allospecificity.

Host $CD8^+$ Cell Deletion is not Sufficient for Optimal Engraftment of Allogeneic Bone Marrow Cells

We have previously shown that, in part, the role of DST in our costimulation blockade protocol for peripheral tolerance induction is to enhance the deletion of host alloreactive $CD8^+$ T cells (139;140). The phenotyping analyses of KB5 synchimeras in which hematopoietic chimerism was generated successfully suggest that deletion of both

peripheral host alloreactive $CD8^+$ T cells and host alloreactive thymocytes is required. We next tested the hypothesis that host $CD8^+$ T cell deletion is required but not sufficient for establishing hematopoietic chimerism; we did so by replacing the DST in our protocol with a depleting anti-CD8 mAb. We observed that the frequency of chimerism in BALB/c recipients treated with anti-CD8 mAb and anti-CD154 mAb before transplantation of C57BL/6 bone marrow was much lower (22% Table 14, Group 1) than in recipients treated with anti-CD154 mAb and DST (Table 9, Group 1, $p<0.001$). The result suggests that the role of DST in facilitating engraftment of allogeneic bone marrow cells involves mechanisms in addition to the deletion of host alloreactive $CD8^+$ T cells.

Table 14: Frequency of Chimerism in BALB/c Recipients of C57BL/6 Bone Marrow Treated with Monoclonal Antibodies

Group	*DST Donor*	*Recipient Treatment*	*Frequency of Chimerism (%)*	*Percentage of Donor Origin PBMC in Chimeric Mice (%)*
1	None	Anti-CD8 mAb	2/9 (22%)*	5.6 ± 1.0
2	None	Anti-CD122 mAb	9/9 (100%)**	6.8 ± 2.4
3	C57BL/6	Anti-CTLA4 mAb	2/9 (22%)*	3.8 ± 2.5
4	C57BL/6	Anti-CD4 mAb	6/9 (67%)	2.7 ± 1.2*
5	C57BL/6	Anti-CD25 mAb	8/9 (89%)	6.0 ± 1.6

Legend to Table 14: BALB/c mice were randomized and injected with 50 x 10^6 C57BL/6 ($H2^b$) bone marrow cells on day 0. All mice were injected intraperitoneally with 4 doses of 0.5 mg anti-CD154 mAb on days –7, -4, 0, +3 relative to bone marrow transplantation. In the groups indicated, anti-CD8 (0.5 mg/dose), anti-CD4 (0.5 mg/dose), or anti-CTLA4 (0.075 mg/dose) mAb was injected intraperitoneally on days -7, -6, and -5 relative to bone marrow transplantation. Anti-CD122 mAb (1 mg/dose) was injected intraperitoneally on days -8 and -1 relative to bone marrow transplantation. Mice in Groups 3, 4, and 5 received a single donor specific transfusion consisting of 10^7 C57BL/6 spleen cells on day -7 relative to bone marrow cell transplantation. *$p<0.005$ *vs.* Table 9, Group 1; **$p<0.005$ *vs.* Group 1.

Hematopoietic Chimerism is Established in Mice Treated with Anti-CD154 mAb, Anti-CD122, and Bone Marrow Cells

NK cells, which are $CD122^+$, are known to be important in the rejection of allogeneic bone marrow (162-165). CD122 is expressed on most NK cells, activated macrophages, and a subset of activated $CD8^+$ T cells, and anti-CD122 mAb has been shown to delete NK cell activity *in vivo* (166-168). To begin to investigate the role of NK cell depletion in allogeneic bone marrow transplantation in mice treated with costimulation blockade, BALB/c mice were given anti-CD154 mAb, anti-CD122 mAb, and 50 x 10^6 C57BL/6 bone marrow cells. Surprisingly, hematopoietic chimerism was established in 100% of these recipients (9/9, Table 14, Group 2). This rate of successful engraftment is comparable to that achieved using DST in place of anti-CD122 mAb (89%, Table 9, Group 1, p=N.S.) and significantly greater than that achieved using anti-CD8 mAb in place of DST (Table 14, Group 1, $p<0.01$).

Interventions that Abrogate Peripheral Tolerance Induction Reduce Engraftment of Bone Marrow

We have previously shown that injection of anti-CTLA4 mAb at the time of peripheral tolerance induction with DST and anti-CD154 mAb prevents deletion of alloreactive $CD8^+$ T cells and shortens skin allograft survival (138;140). Treatment with anti-CD4 mAb at the time of tolerance induction also shortens skin allograft survival (138). We therefore tested the hypothesis that these interventions would also interfere with the generation of hematopoietic chimerism in bone marrow recipients treated with DST and anti-CD154 mAb. As shown in Table 14 (Group 4), treatment with anti-CD4

mAb had little effect, and two thirds of recipients became chimeric, albeit with a level of chimerism that was quite low (~2.7%, $p<0.001$ vs. Table 9, Group 1). Similarly, in a cohort of recipients treated with an anti-CD25 mAb known to delete $CD4^+CD25^+$ regulatory T cells, nearly all (89%, N=9) became chimeric (Table 14, Group 5). In these mice the level of chimerism (6.0%) was greater than in the anti-CD4 treated mice ($p<0.005$), but not as high as in recipients treated with only DST and anti-CD154 mAb (~9%, Table 9, Group 1, $p<0.025$). Only in the case of treatment with anti-CTLA4 mAb was there a significant reduction in the percentage of mice that became chimeric (22%, Table 14, Group 3). The results suggest that the mechanism by which the combination of DST plus anti-CD154 mAb generates peripheral transplantation tolerance is distinct but overlaps with the mechanism by which it generates hematopoietic chimerism.

Summary

Establishment of allogeneic hematopoietic chimerism leads to the generation of central tolerance and donor-specific transplantation tolerance. Achievement of hematopoietic chimerism in the absence of host myeloablative conditioning and development of graft-versus-host disease (GVHD) would allow the widespread application of stem cell transplantation in the clinic. I have developed a strategy to achieve these goals in mice based on a costimulation blockade-based peripheral transplantation tolerance protocol using a donor-specific transfusion (DST) plus a brief course of anti-CD154 monoclonal antibody (mAb). This protocol leads to permanent islet allograft survival, and prolonged skin allograft survival in mice and non-human primates. When applied to stem cell transplantation, administration of a spleen cell transfusion as a DST, anti-CD154 mAb, and allogeneic bone marrow cells permits establishment of hematopoietic chimerism and central tolerance in the absence of GVHD. Hematopoietic chimerism leads to the peripheral deletion of host alloreactive $CD8^+$ T cells, and intrathymic deletion of newly developing alloreactive $CD8^+$ T cells. Deletion of peripheral host alloreactive $CD8^+$ T cells is not sufficient for the establishment of chimerism. MHC-matching between the splenocyte transfusion and bone marrow donors is not required unless supraphysiological levels of host alloreactive $CD8^+$ T cells are present, suggesting that both donor-allo-specific and non-allo-specific mechanisms regulate hematopoietic engraftment. Functionally, establishment of chimerism can be modulated by agents that interfere with peripheral transplantation tolerance. I conclude that it is possible to establish robust allogeneic hematopoietic chimerism in the absence

of host myeloablative conditioning using a peripheral transplantation tolerance protocol consisting of DST, anti-CD154 mAb, and hematopoietic stem cells.

CHAPTER VII

DISCUSSION

Experimental protocols for the induction of allogeneic chimerism have made considerable advances since the landmark experiments by Owen and Medawar (104-107). Currently, the transplantation of hematopoietic stem cells relies on the use of myeloablative conditioning and immunosuppressive drugs. The severe conditioning regime and the detrimental side-effects associated with the procedure limit the utilization of allogeneic chimerism to patients afflicted with fatal autoimmune diseases and malignancies. Attempts to overcome the obstacles involved in stem cell transplantation have led to a new wave of protocols focused on reducing the toxicity of the conditioning treatment and removing the reliance on immunosuppressive drugs. The reduction in morbidity and mortality associated with bone marrow transplantation would make allogeneic chimerism a realistic clinical option for non-malignant tumors, autoimmune diseases and organ transplantation.

Costimulation blockade has been found to induce potent non-responsiveness in donor-specific lymphocytes both *in vitro* and *in vivo*. The use of anti-CD154 mAb to block the costimulatory signals has proven to be effective in prolonging allogeneic grafts such as pancreatic islets, heart and skin (134-136;138;169-171). Based on these results, the next step was to determine if anti-CD154 mAb could also enhance the engraftment of allogeneic bone marrow cells and the induction of a chimeric state in the host. It was

hypothesized that the induction of tolerance using anti-CD154 mAb would significantly reduce the toxicity of the conditioning regimen required for stem cell transplantation.

The progress of this thesis was determined by the level of toxicity of host conditioning regimens required to establish allogeneic chimerism using anti-CD154 mAb. The initial protocol involved the requirement for sub-lethal irradiation, but enhancements in the protocol led to further reduction in radiation requirements until eventually none was needed to induce chimerism. Further analysis was performed to determine the mechanism involved in generating and maintaining the chimeric state without any myeloablative conditioning.

The results from my earliest work demonstrated that it was possible to generate hematopoietic chimerism in mice transplanted with fully allogeneic bone marrow using the combination of sub-lethal irradiation and a brief course of anti-CD154 mAb comprising as few as 2 injections. Chimeric mice treated according to this early protocol showed no evidence of GVHD despite the presence of a peripheral lymphoid compartment almost entirely of donor origin. Chimeric mice also accepted donor-origin skin and islet allografts.

The minimum dose of radiation required to generate chimerism differed between the BALB/c and NOD/Lt strains. Chimerism could be generated with as little as 4 Gy in BALB/c mice, whereas 6 Gy appeared to be the threshold in NOD/Lt mice. These differences may reflect intrinsic differences between these strains with respect to overall radioresistance. The thresholds for chimerism in BALB/c and NOD/Lt mice were roughly proportional to the respective lethal radiation doses of 7 Gy and 10 Gy. The radiation

doses required for chimerism may simply be those that achieve adequate, but not lethal, degrees of elimination of host hematopoietic cells.

The costimulatory blockade produced by treatment with anti-CD154 mAb exerted two important effects. First, it lowered the dose of radiation required to achieve chimerism in both of the strain combinations tested. The effect was to some extent dependent on the overall dose of anti-CD154 mAb administered, but the data indicate clearly that prolonged treatment is not required. Administration of antibody on the day of transplantation, just before the transfusion of cells, and again three days later was sufficient to lead to hematopoietic chimerism.

The second major effect of costimulatory blockade was on the occurrence of GVHD. Treatment with anti-CD154 mAb was clearly associated with prevention of GVHD in successful chimeras. With few exceptions, chimeras generated using irradiation alone showed clinical characteristics of GVHD. In contrast, chimeras generated with the same dose of radiation but with the addition of anti-CD154 mAb showed no evidence of this common complication, either acutely or chronically, for up to ~11 months after bone marrow transplantation. These observations are consistent with previous reports documenting that treatment with anti-CD154 mAb can prevent GVHD in a model system based on the transfer of alloreactive T cells (122;123;172). The work presented in this thesis and from other laboratories generating chimeras using anti-CD154 mAb (141-143) extend these earlier reports by demonstrating that donor T cells that continue to develop in a fully reconstituted bone marrow allograft recipient are tolerant of the host in the absence of additional costimulatory blockade.

The ability to generate fully allogeneic chimeric mice free of GVHD provided us with the opportunity to perform additional studies in the areas of transplantation and autoimmunity. As expected, based on the work of Owen (104) and many others (173-175), allogeneic chimeric mice were able to accept donor origin allografts while retaining the ability to reject third party grafts. Because skin contains a large number of professional antigen presenting cells, skin allografts represent a robust stimulus for rejection. Our observation of prolonged skin allograft survival in mice treated with irradiation and anti-CD154 mAb suggests that the tolerant state induced by our protocol is robust and may support the transplantation of other vascular organs. It is important to point out that treatment with anti-CD154 mAb was required for uniform donor skin graft tolerance in chimeric mice. The survival of skin allografts placed on animals that had been rendered chimeric by irradiation alone tended to be short, and in those cases where skin grafts were not immediately rejected, GVHD appeared to be present. Several mice with intact skin allografts died of GVHD.

In the case of type 1 diabetes, which occurs in the NOD mouse, the tissue affected by autoimmunity is destroyed. Replacement of the affected tissue in these instances requires procedures that overcome not only allorejection but also recurrent autoimmune destruction of the transplanted tissue. Diabetic NOD mice reject syngeneic islet grafts (149), and only *lethal* irradiation followed by allogeneic bone marrow transplantation has prevented recurrent autoimmunity in donor-origin islet allografts (101;103). The work performed in this thesis now demonstrate that it is possible to cure autoimmune diabetes in NOD/Lt mice by first generating hematopoietic chimerism using sub-lethal irradiation

plus a brief course of anti-CD154 mAb and then transplanting donor-specific islets of Langerhans. These grafts restored normoglycemia, and none failed during the period of experimental observation. The data suggest that the generation of allogeneic hematopoietic chimerism based on sub-lethal irradiation and costimulatory blockade can prevent both allograft rejection and recurrent autoimmunity.

In our continued effort to reduce the conditioning toxicity required for allogeneic stem cell engraftment, we attempted to decrease the radiation requirement by increasing the number of donor bone marrow cells transplanted into the host. BALB/c mice transplanted with 10 times the normal dosage of C57BL/6 bone marrow cells did not become chimeric when the radiation dosage was reduced by 2 Gy from the standard protocol discussed above. Recently, other laboratories have been able to generate allogeneic chimeras by using anti-CD154 mAb and very high (2×10^8) numbers of bone marrow cells (142;143). A possible explanation is that one group used B10.A and C57BL/6 as donor and host, respectively. It could be argued that even though these mice express different MHC haplotypes, these strains of mice share many non-MHC background genes due to their common derivation from a C57BL ancestor (176). Similarity in their non-MHC genes may have facilitated allogeneic hematopoietic cell engraftment. The other group used fully-disparate strains of mice as donor and host, but their protocol involved 8 doses of anti-CD154 mAb and bone marrow cells over a time-period of 3 months. It may be that the constant dosage of transplanted donor bone marrow and the precise regimen of costimulatory blockade exert effects on the extent and durability of chimerism achievable using this approach.

In an effort to reduce the conditioning toxicity without using large doses of donor bone marrow cells, we established a new protocol based on the induction of peripheral transplantation tolerance. Our lab has previously documented that DST and anti-CD154 mAb can induce peripheral tolerance in mice by the prolonged survival of skin allografts (134;138;171) and the indefinite survival of pancreatic islets (135;136;169). The peripheral tolerance protocol was shown to delete alloreactive $CD8^+$ (139;140) and $CD4^+$ T cells (unpublished observation). Based on these data, we hypothesized that DST and anti-CD154 mAb could also prolong the survival of donor bone marrow cells by peripheral tolerance to allow for their engraftment. The deletion of the alloreactive T cells before bone marrow transplantation was predicted to drastically reduce the radiation requirements for allogeneic chimerism.

In BALB/c mice, at least 4 Gy of irradiation was required for bone marrow engraftment, but the introduction of DST into the protocol allowed for the generation of mixed chimeras at only 1 Gy. Further experiments using two other strains of mice, B10.BR and CBA/J, and still using C57BL/6 as donor demonstrated that this protocol was not strain-specific and could be used to turn various strains of mice chimeric using only 1 Gy of irradiation.

We next attempted to employ this protocol in the NOD mice in an effort to address its clinical feasibility in the autoimmune setting. Unfortunately, the addition of DST into our protocol did not reduce the radiation requirement necessary for allogeneic stem cell engraftment. To some extent, the outcome is not surprising. Our lab has previously shown that costimulatory blockade also fails to prolong skin allografts in non-

diabetic NOD mice (149). It was initially hypothesized that this outcome was due to a generalized defect in the response of NOD mice to tolerance induction resulting from their autoimmunity. This concept seemed attractive as there are multiple immune defects that could be involved in both the autoimmune phenotype and resistance to transplantation tolerance phenotype. But recent data suggest that the two phenotypes can be genetically separated (177;178).

The promising results using low-dose irradiation with DST and anti-CD154 mAb to induce allogeneic chimerism prompted another examination of the protocol. A major goal of the stem cell transplantation field is to induce engraftment using the least toxic conditioning regimen. In terms of radiation requirement, the ultimate goal would be to not use any radiation. Recently, a few publications have demonstrated that mixed hematopoietic chimerism can be obtained in the absence of host conditioning (142;143;179). However, these protocols require supraphysiological doses of bone marrow cells or the introduction of cytotoxic drugs. The data in this thesis document that, even in the absence of any host conditioning, permanent mixed hematopoietic chimerism and central tolerance are achieved using our peripheral transplantation tolerance protocol to facilitate the engraftment of hematopoietic stem cells.

It has been shown that peripheral costimulation blockade-based protocols work to varying degrees depending on the host strain (180). To confirm that the ability of DST plus anti-CD154 mAb to facilitate hematopoietic cell engraftment was not unique to the BALB/c strain, we documented the ability of our protocol to establish hematopoietic cell engraftment in the absence of host conditioning in two additional strains. Surprising, the

B10.BR mice were also found to turn chimeric with only C57BL/6 bone marrow and anti-CD154 mAb; the BALB/c and CBA/J strains displayed no donor cells as expected. As discussed previously, it could be argued that the B10.BR and C57BL/6 mice share many non-MHC background genes due to their common C57BL ancestor (176). It is known that minor MHC antigens are important determinants of hematopoietic graft rejection, particularly by the innate immune system (181-183). Results showing that the level of chimerism in B10.BR mice (treated with only anti-CD154 mAb and bone marrow) was much lower using a completely mismatched donor (DBA/2) than the C57BL/6 donors support this theory. But the fact that they were able to even become chimeric with DBA/2 donors suggest that B10.BR mice might be more susceptible to chimerism induction than other strains. These results highlight the importance of using the most diverse combination of strains to meet the strictest experimental test for transplantation tolerance induction.

Of interest was the time interval required between induction of tolerance by injection of DST plus anti-CD154 mAb and the susceptibility of the host to hematopoietic cell engraftment. Previous reports from our lab have shown that alloreactive $CD8^+$ T cells disappear from the circulation within 3 days after DST and anti-CD154 mAb treatment (139;140). This suggested that within 3 days of tolerance induction, hosts should be susceptible to engraftment with hematopoietic stem cells. However, we observed that 7 days was the minimum time between tolerance induction and bone marrow injection that permitted establishment of chimerism.

The kinetics of susceptibility to engraftment may be related to the ability of splenocyte transfusion to induce a "non-specific" regulatory mechanism that facilitates this process. Speculatively, this could be due to the development of "tolerogenic" dendritic cells and/or the production of regulatory cytokines by an immune system activated in the presence of anti-CD154 mAb. Supporting these possibilities are the observations that dendritic cells that ingest apoptotic cells (184) or are activated in the presence of CD40-CD154 blockade, become tolerogenic cells that suppress immune responses and secrete regulatory cytokines such as TGF-β and IL-10 (85;185). An alternative explanation may be a delayed deletion of host alloreactive T cells. It is known that fully-activated $CD8^+$ T cells migrate to non-lymphoid tissues where they become memory cells (186;187). Incomplete activation in the presence of CD40-CD154 blockade may induce migration and initiate apoptosis of antigen-activated T cells, but these cells may be rescued if re-stimulated with allogeneic bone marrow cells prior to the final stages of apoptosis.

The establishment of central tolerance to alloantigens is thought to require intrathymic and peripheral deletion of host alloreactive T cells (40). However, the conclusion that establishment of hematopoietic chimerism generates central tolerance to alloantigens by intrathymic deletion of alloreactive T cells is derived from indirect data based on deletion of superantigen-reactive specific Vβ subpopulations $CD4^+$ T cells (188). In an effort to study a system that contained a trace population of high-affinity alloreactive T cells that not only circulated, but also developed in a normal microenvironment, we developed a new model based on allo-TCR-transgenic

hematopoietic chimeric recipients (synchimeras) (139). We designed these synchimeras to respond to tolerance induction and maintenance in a manner similar to that observed in normal mice and to overcome the limitations inherent in Vβ analysis and full TCR-transgenic mice.

The results demonstrate that $CD8^+$ alloreactive T cells from a "normal" immune system are peripherally deleted upon chimerism induction using DST plus anti-CD154 mAb and allogeneic bone marrow without any radiation pre-treatment. As expected, DST and anti-CD154 mAb alone induced temporary peripheral tolerance as shown by the fall and then rise of the alloreactive KB5 T cells over time (139). However, the chimeric mice displayed permanent deletion of the alloreactive T cells as shown not only peripherally, but also intrathymically by the absence of $CD8^+CD4^-$ KB5 thymocytes. Of additional interest is our observation that our peripheral tolerance protocol in the absence of bone marrow engraftment leads to transient deletion of peripheral but not intrathymic alloreactive $CD8^+$ T cells. Previous studies of skin allografts on mice treated with DST and anti-CD154 mAb have shown that graft survival is greatly prolonged, but seldom permanent unless the recipient has been thymectomized (138). The inference has been that the failure of graft maintenance is due to the release of new alloreactive thymic emigrants into the periphery (139). The present results strongly suggest that this inference is correct; they demonstrate that the generation of hematopoietic chimerism in effect "thymectomizes" recipients in a donor-specific manner. The data imply that central tolerance is the essential complement to peripheral tolerance induction if allografts are to be truly durable.

Almost all of the protocols involved in allogeneic chimerism induction using anti-CD154 mAb use bone marrow cells as the only source of donor antigens involved in T cell costimulatory blockade. They must use radiation, anti-T cell antibodies or very high doses of bone marrow cells to achieve engraftment. A possible explanation of why these additional measures are required is shown in Figure 6. Bone marrow cells do not efficiently delete allo-reactive T cells in conjunction with anti-CD154 mAb. They eliminate only ~50% of the $DES^{+}CD8^{+}$ T cells in the PBMC, whereas using splenocytes as DST can remove almost all of the alloreactive cells circulating in the host. Additionally, peripheral tolerance experiments in our lab using bone marrow cells as DST are not as efficient as spleen cells as DST with anti-CD154 mAb in prolonging skin allografts (Tom Markees, unpublished). This observation provides further evidence that alloreactive T cells are not entirely deleted when bone marrow cells are the only source of donor antigens in the tolerance protocols.

The results from Table 9 document, as expected from previous studies of peripheral tolerance induction (138), that anti-CD154 mAb monotherapy in the absence of DST is generally ineffective for generating hematopoietic chimerism. Surprisingly, however, we found that a third-party (MHC-disparate) transfusion could be substituted in some (but not all) cases for the standard donor-specific transfusion (DST). The key observation for understanding this counterintuitive result was that an MHC-disparate transfusion was effective only in normal recipients with a physiological (low) percentage of alloreactive T cells. Synchimeric recipients, with their high level of alloreactive T cells, became chimeric only if given a *donor specific* transfusion, presumably to reduce

that number of alloreactive cells. In a normal CBA/J mouse, the number of naturally occurring T cells with this allospecificity is far fewer, and the requirement for donor specificity of the transfusion was less stringent. In their aggregate, the results suggest that the generation of hematopoietic chimerism using DST and anti-CD154 mAb involves both the deletion of host allospecific T cells and an additional, non-allospecific suppressive mechanism.

Recent publications have reported that immunoregulatory cells can be generated by blockade of the CD40:CD154 costimulatory pathway *in vivo* or *ex vivo* (189;190). These cells are able to inhibit both naïve and primed alloresponses. So, in addition to the deletion of high-affinity $CD8^+$ alloreactive T cells, our DST plus anti-CD154 mAb and bone marrow protocol may also generate potent immunoregulatory cells to help facilitate in inducing allogeneic chimerism. These cells may help regulate lower affinity alloreactive T cells that may have escaped deletion. It has been demonstrated that antigen-activated $CD4^+$ T cells can enter a functionally unresponsive state (145;191) and that these anergic/regulatory cells may be mediators of tolerance (192;193). More recently, it has been shown that the suppressive effect of $CD25^+$ regulatory cells is not antigen-specific (194;195). So, it is possible that these regulatory cells are the reason for the non-donor-specific induction of chimerism seen in the CBA/J mice in Table 13. But the regulatory cells may not have been able to overcome the higher than normal level of high-affinity alloreactive T cells present in the KB5 synchimera. Also, the addition of depleting anti-CD25 mAb into the standard chimerism protocol in BALB/c mice using C57BL/6 DST and bone marrow yielded no adverse affect on chimerism induction.

These results seem to imply that regulatory cells alone cannot lead to the establishment of allogeneic chimerism using a non-myeloablative protocol.

In order to understand further the mechanisms involved in the induction of allogeneic chimerism using our protocol, we used depleting and blocking antibodies to determine both the deletion and presence of specific cell populations required for the process. Previous publications from our lab has shown that skin allograft survival using DST and anti-CD154 mAb depended mostly on the deletion of $CD8^+$ alloreactive T cells and the presence of $CD4^+$ T cells (138;140). So, we hypothesized that the same conditions would be required for the engraftment of donor bone marrow cells using DST and anti-CD154 mAb.

Interestingly, the replacement of DST with depleting anti-CD8 mAb, which prolonged skin allograft survival (140), did not fully facilitate the induction of chimerism. The fact that 2 mice did display donor-origin cells in their PBMC led us to believe that the deletion of alloreactive $CD8^+$ T cells is a major step required in the process but not the sole requirement. Therefore, we next tested the possible role of NK cells, which have been shown to participate in the rejection of allogeneic bone marrow cells in lethally irradiated mice (196-199). In an effort to deplete NK cells (and also activated $CD8^+$ T cells), we decided to use anti-CD122 mAb (TM-β1). CD122 is preferentially expressed on a subpopulation of $CD8^+$ T cells, activated macrophages and almost all NK cells (200-202). Anti-CD122 mAb has been shown to selectively eliminate natural killer (NK) cells and also significantly reduce cytotoxic T cell (CTL) activity. By substituting DST with anti-CD122 mAb in our protocol, we were able to establish allogeneic chimerism in all of

the BALB/c mice at levels of donor-origin cells similar to that observed in the control group. Because CD154 is also expressed on NK cells, we hypothesize that engraftment of allogeneic bone marrow cells in non-myeloablated hosts requires not only the deletion of alloreactive $CD8^+$ T cells, but also inactivation of host NK cells. But the possibility exists that anti-CD122 mAb is affecting activated macrophages, as they too also express CD122, to regulate stem cell engraftment. In order to further explore the role NK cells may have in chimerism induction without myeloablation, experiments need to be done in C57BL/6 mice as hosts using depleting anti-NK1.1 mAb in place of anti-CD122 mAb. If the deletion or inactivation of NK cells is required, then the use of anti-NK1.1 mAb plus anti-CD8 mAb without DST should produce chimeric mice with high frequency.

As another possible interpretation, the anti-CD8 mAb injected into the host may remove not only the indigenous population of $CD8^+$ cells, but also the population from the donor bone marrow that was introduced into the host. Several laboratories have reported the existence of "facilitator" cells, a rare $CD8^+$ bone marrow subpopulation, from the donor that can enhance allogeneic hematopoietic stem cell engraftment (120;121;203;204). The circulating anti-CD8 mAb in the host may be deleting these donor facilitating cells required for stem cell engraftment in a non-myeloablated host.

$CD4^+$ T cells have been proposed to be important in skin allograft survival using DST and anti-CD154 mAb by providing helper factors to induce alloantigen-activated $CD8^+$ T cell to undergo activation-induced cell death (138;139). In contrast, the addition of anti-CD4 mAb to our chimerism protocol did not prevent hematopoietic engraftment, but it did lower the percentage of donor-origin cells in the PBMC when compared to

controls. This observation supports the previous report that anti-CD4 mAb does not prevent the establishment of hematopoietic chimerism in mice treated with anti-CD154 mAb plus busulfan (179). Finally, as expected (142;143), increasing the dose of bone marrow cells or adding minimal myeloablative conditioning to our protocol increased both the frequency of mice engrafted as well as the level of engraftment.

An issue relevant to the utility of "multi-stage" transplantation tolerance induction procedures in clinical medicine is the stringency with which the components of the therapy need to be timed. We observed that our procedures were successful if initiated 1-2 weeks before bone marrow transplantation, but not 5 or fewer days before transplantation. This observation has clinical implications as extended procedures are usually not desirable for a number of reasons, such as the use of cadaver donors. Further research must be conducted to determine the reason for the timing requirement. Another important clinical issue is the surprising discovery that the priming transfusion given after the first injection of anti-CD154 mAb need not be MHC-matched with the eventual bone marrow donor. The clinical implication is obvious, in that the complex orchestration of obtaining and delivering to a recipient a DST and bone marrow and an organ for transplantation can be greatly simplified. But the supposedly non-specific tolerance seen after DST plus anti-CD154 mAb treatment holds some cause for caution because of the possibility of tolerance toward a pathogen. This concern has been examined by Welsh *et al.* (205) who found that mice treated with DST and anti-CD154 mAb are able to clear LCMV infection successfully, suggesting that the treatment does not have detrimental affects on the host's viral immunity. But viral infections, both acute and chronic, have

been found to prevent allogeneic chimerism induction (206;207); whereas, prior viral infection that has been cleared does not have any effect (207). So, in the clinical setting precaution must be employed to avoid exposure to viral pathogens for the safety and successful treatment of the patient. Another clinical concern is the thrombotic events seen in human trials using anti-CD154 mAb. In an effort to address this issue, one of the highlights of the protocol discussed in this study is that the regimen used for chimerism and tolerance induction is very short without requiring any further manipulations (*i.e.* only 4 doses of anti-CD154 mAb).

The use of immunosuppressive drugs is a standard therapy in the clinic when dealing with any form of transplantation, including stem cell transplantation. Translation of the chimerism protocol presented in this study to human patients will undoubtedly include some form of immunosuppression to ensure their safety and prevent loss of the organ graft in case tolerance is not achieved. The effects of immunosuppressive drugs on the efficacy of this protocol are unknown, but a recent study using costimulatory blockade and low-dose irradiation has shown that calcineurin inhibitors prevent chimerism and tolerance induction; whereas compatible immunosuppressive drugs, such as rapamycin and mycophenolate mofetil, may facilitate in allogeneic stem cell engraftment. Therefore, serious consideration should be made in the selection of immunosuppressive drugs when used in conjunction with the chimerism protocol discussed in this study.

In conclusion, I have developed an allogeneic chimerism protocol that incorporates a technique that has been used in my lab to induce peripheral tolerance to

condition the host to accept donor hematopoietic stem cells. But most importantly, the induction of chimerism does not require any radiation pre-treatment or large number of bone marrow cells. In addition, by using the new synchimera model system, we are able for the first time to analyze the mechanism necessary for the induction and maintenance of allogeneic chimerism in a "normal" immune system. The key element to this system is having a small trace population of self-renewing $CD8^+$ alloreactive T cells within a normal micro environment. The data presented here document directly that the generation of allogeneic chimeras involves the peripheral deletion of high-affinity $CD8^+$ alloreactive T cells by DST and costimulatory blockade, and supports the theory that chimerism induces continued elimination of alloreactive cells by negative selection in the thymus. The results further suggest that there is commonality to the generation of peripheral and central tolerance and that the maintenance of transplantation tolerance will require either physical thymectomy or its biological equivalent—central tolerance induction. As a final note, the protocol I have developed does not require host myeloablative conditioning, appear not to lead to GVHD, and may not necessarily require MHC matching of transfusion and bone marrow—characteristics that make these procedures highly attractive for translation to clinical medicine.

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