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# Regulatory mechanisms in transplantation tolerance: the induction of tolerance by adoptive transfer in MHC-inbred miniature swine

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**REGULATORY MECHANISMS IN TRANSPLANTATION TOLERANCE: THE  
INDUCTION OF TOLERANCE BY ADOPTIVE TRANSFER IN MHC-INBRED  
MINIATURE SWINE**

by

**RAMYAR TORABI**

B.A, Providence College, 2008

Submitted in partial fulfillment of the  
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Approved by

First Reader

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Theresa A. Davies, Ph.D.  
Director, M.S. in Oral Health Sciences Program  
Adjunct Assistant Professor of Biochemistry

Second Reader

---

Kazuhiko Yamada, M.D., Ph. D.  
Associate Professor of Surgery  
Harvard Medical School

## DEDICATORY PAGE

I want to give special thanks to Dr. Kazuhiko Yamada for giving me the opportunity to participate in his laboratory. I am grateful for all the projects he let me work on throughout the year and also thank him for allowing me to report our lab's findings in my thesis. The current paper being presented is our lab's work and has been accepted for publication in American Journal of Transplantation. The title of the article is The Induction of Tolerance of Renal Allografts by Adoptive Transfer in Miniature Swine and Dr. Yamada is the senior author. This is cited in the references as the article with the authors Okumi et al.

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**RAMYAR TORABI**

Boston University School of Medicine, 2013

Major Professor: Theresa A. Davies, Ph.D., Director, M.S. in Oral Health Sciences and  
Adjunct Assistant Professor of Biochemistry

**ABSTRACT**

Organ transplantation has been the standard of care for end-stage organ failure. While one-year renal allograft survival has increased to over 90% in the past couple of decades with the use of new immunosuppressants, the long term survival of kidney allografts have remained the same due to unchanged rate of chronic rejection. Even with acceptance of renal allografts, patients require lifelong use of immunosuppressants. The induction of transplantation tolerance may allow for patients to receive transplants without having to deal with the side effects of immunosuppression. Using a unique miniature swine model, we have demonstrated uniform induction of tolerance in recipients of MHC class-I mismatched renal allografts with 12 days of high Cyclosporine A. Transplantation tolerance in this model is induced and maintained by regulatory T cells. In our study, we used this model to attempt to induce tolerance in naïve recipients

through adoptive transfer of donor primed or unprimed peripheral regulatory cells and/or kidneys from long-term tolerant donor-matched swine. SLA<sup>dd</sup> miniature swine received 150 rads of whole body irradiation and class I-mismatched SLA<sup>gg</sup> kidneys from naïve pigs with or without co-transplanted kidneys and/or cells from long-term tolerant SLA<sup>dd</sup> recipients of SLA<sup>gg</sup> kidneys. Naïve kidneys transplanted without a long-term tolerant kidney were acutely rejected. Recipients of naïve kidneys co-transplanted with donor primed cells and kidney grafts from long-term tolerant animals had extended survival times for the naïve renal grafts: 2 out of 3 animals in this group became long-term tolerant and the remaining had an extended graft survival (28 days).

These studies demonstrate the first successful adoptive transfer of tolerance in large animals. The data suggest that the tolerated kidneys and cells have regulatory effects, likely due to regulatory T cells that can induce and maintain tolerance. The potency and efficacy of regulatory T cells has been shown in our study and could potentially be exploited to provide for therapies to induce and maintain tolerance.

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## ABBREVIATIONS

ABC	avidin-biotin horseradish-peroxidase complex
APC	antigen presenting cell
CD	cluster of differentiation
CML	cell-mediated lympholysis
CTL	Cytotoxic T lymphocytes
CyA	Cyclosporine A
DNA	deoxyribonucleic acid
DST	donor-specific transfusion
E:T	effector:target ratio
FDA	Food and Drug Administration
Foxp3	Forkhead box P3
GVHD	graft-versus-host-disease
GVL	graft-versus-leukemia
HBSS	Hank's balanced salt solution
HLA	human leukocyte antigen
Ig	immunoglobulin
IL	interleukin
iTreg	inducible regulatory T cells
LTT	long-term tolerant
MGH	Massachusetts General Hospital
MHC	major histocompatibility complex

MHC I	major histocompatibility complex class one
MHC II	major histocompatibility complex class two
MMF	mycophenolate mofetil
MPA	mycophenolic acid
nTreg	natural regulatory T cells
OKT3	muromonab-CD3
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PCNA	proliferating cell nuclear antigen
SLA	swine leukocyte antigen
SRTR	Scientific Registry of Transplant Recipients
Tregs	regulatory T cells
WBI	whole body irradiation
WHO-ONT	World Health Organization-Organización Nacional de Transplantes

## INTRODUCTION

“Organ transplantation is the standard of care for end-stage kidney failure, in terms of survival, quality of life and cost effectiveness compared with other replacement therapies and remains the only available treatment for persons with end stage failure of other solid organs” (“Pages-Facts,” 2013).

Patients throughout the world have lifesaving transplants and patients with kidney failure have a better life expectancy and quality of life compared with dialysis (Bishop et al., 2011). In 2010 alone, there were 106,900 solid organs transplanted worldwide and 73,180 of the transplants were kidney transplants. Those 2010 data are based on the Global Observatory on Donation and Transplantation Data (GODT) data, produced by the WHO-ONT collaboration. While most solid organs are procured from the deceased, kidney transplants can come from appropriately matched living donors with minor risks (“Pages-Facts,” 2013).

Since 1954 when Murray et al. completed the first successful kidney transplant between identical twins, there has been considerable advancement in the science behind kidney transplantation (Murray & Holden, 1954). Although transplantation biology was in its primitive state, it was known from animal models that transplantation between two different individuals (allotransplantation) would lead to graft rejection. For this reason, Murray chose to perform his first procedure with identical twins. Since the first transplant performed by Murray, extensive research has been performed to elucidate the mechanisms of rejection, leading to the identification of the Major Histocompatibility Complex (MHC) loci, which in humans are the Human Leukocyte Antigen (HLA). The HLA genes are highly polymorphic and encode MHC molecules that allow antigen

presenting cells (APCs) to display fragments of antigens to the immune system. The greater number of differences between the donor and recipient at the HLA loci, the greater the probability of rejection is (Nankivell & Alexander, 2010). An overview on MHC and HLA is provided in the transplantation and rejection section.

Since the beginning of transplantation, the ultimate goal has been to prevent rejection. Attempts to prevent rejection with immunosuppressive drugs began in the late 1950's to early 1960's. At that time azathioprine (anti-proliferative agent) and corticosteroids were the main therapeutics used and acute rejection was an ordinary occurrence (Murray, Merrill, Harrison, Wilson, & Dammin, 1963; Nankivell & Alexander, 2010). In the early 1980's, a major breakthrough came via availability of cyclosporine and the OKT3 monoclonal antibody (anti-CD3) which improved one-year survival of renal allografts from 60 percent to over 80 percent. Since that time, there have been enhancements in immunosuppressive agents that have dramatically increased one year survival rates (Pascual, Theruvath, Kawai, Tolloff-Rubin, & Cosimi, 2002).

The current clinical immunosuppressive regimens in the United States includes a triple drug therapy of corticosteroids (e.g. Prednisone), tacrolimus, and MMF/MPA (Mycophenolate mofetil/Mycophenolic acid) as maintenance therapy (Matas et al., 2013). From the 2011 OPTN/SRTR reports, tacrolimus has replaced cyclosporine as the main calcineurin inhibitor used and MMF acts as the main anti-proliferative agent. The rationale is to target multiple layers of a complex immune response that may activate lymphocytes and cause a rejection crisis. Corticosteroids are used to suppress the immune system systemically and non-specifically. Calcineurin inhibitors are used to

prevent the expansion of T cells and anti-proliferative agents prevent DNA synthesis and division of T cells (Abboudi & Macphee, 2012). Over the past couple of decades, these new immunosuppressive drugs have improved one-year survival of kidney allografts to over 90 percent (Lamb, Lodhi, & Meier-Kriesche, 2011). Preventing acute rejection (rejection within 1 year of transplant) was a major problem that has largely been overcome with more effective immunosuppression protocols. Until recently, most research has been focused on preventing acute rejection. While this issue has been resolved in the vast majority of cases, the maintenance and long term survival of the kidney allograft remains a problem (Gaston, 2011). It was thought by many that solving the issue of acute rejection would translate into decreased late allograft loss (Pascual et al., 2002). From 1989 until 2008 the 5 year survival rate of kidney allografts has remained at approximately 80% (Lamb et al., 2011). Acceptance of the donor kidney has been achieved in the induction phase of tolerance (first year), but chronic rejection (rejection after 3 years of transplant acceptance) remains an issue. Kidney transplants from deceased donors have a 5 year survival rate of approximately only 50% relative to those receiving a kidney from a living donor (Leventhal, Miller, Abecassis, Tollerud, & Ildstad, 2013). Despite recent advances in immunosuppressive therapies, the half-life of renal allografts is still only about 8 years (Janeway, Murphy, Travers, & Walport, 2008). Therefore, most recipients of renal allografts will ultimately reject their grafts.

Even with lifelong acceptance of allografts, recipients of transplants require the use of immunosuppressants for the remainder of their lives to prevent graft rejection. With the lifelong use of immunosuppression comes a variety of side effects, including

infection, cancer, diabetes, heart disease, and nephrotoxicity. Even with immunosuppression to maintain acceptance of the graft, there is often ultimately kidney graft loss (Scandling, Busque, Shizuru, Engleman, & Strober, 2011). There are many researchers attempting to find new immunosuppressive medications that have fewer side effects and still prevent graft rejection. The triple drug immunosuppression was designed to have greater potency in accepting allografts, while reducing some of the side effects. The ideal clinical goal is to prevent rejection without the use of immunosuppression. This would require a development of lifelong tolerance and eliminate or substantially reduce the deleterious side effects. In order to devise a protocol that will give patients lifelong tolerance, a more extensive understanding of the basis of transplant rejection is necessary.

## **BACKGROUND**

### **Transplantation and Rejection**

Rejection of transplanted organs is caused by the immune system's response to alloantigens on a donor graft. An alloantigen is a protein or a carbohydrate that is different between individuals of the same species. Due to this variance, the recipient may see these antigens as foreign and mount an immune response. The major histocompatibility complex (MHC) molecules are highly polymorphic; most rejection is caused by differences in the MHC class I antigens. However, even with perfect matching, rejection may occur because of genetic differences at the minor histocompatibility level. While genetic differences at the minor loci provide a slower

rejection, it is still ultimately rejection. Unless patients are identical twins, the differences at major and minor histocompatibility complexes create the necessity for immunosuppression to avoid rejection (Janeway et al., 2008).

The MHC is also known as the HLA in humans and the Swine Leukocyte Antigen (SLA) in pigs. T cells encounter antigens as peptides displayed by the two major MHC molecules, MHC class I and MHC class II. MHC I and MHC II glycoproteins present peptides on the surface of their cells so the immune system can mount a response if needed. MHC I presents endogenous antigens such as viruses, are recognized by CD8 cytotoxic T lymphocytes (CTLs); all nucleated cells express MHC I. On the other hand, MHC II presents exogenous antigens such as bacteria. These antigens are then recognized by CD4 helper T cells. Only APCs (dendritic cells, macrophages, and B cells) can express MHC II. MHC genes are polygenic, meaning there are several genes for class I and class II molecules for each individual. To further complicate matters, the MHC molecules are the most polymorphic genes known. For example, for HLA-B there are more than 700 alleles. Due to the number of alleles seen in the population, most individuals are heterozygous at the MHC loci. Each person expresses different MHC I and MHC II molecules and has various haplotype combinations. The variance in individuals provides for alloreactivity and rejection in transplants (Janeway et al., 2008).

The current problem is the long term survival of grafts. The rejection is seen to occur via two major pathways. One path is by direct presentation of donor antigens by donor APCs that results in a rejection crisis. The APCs present antigens to lymph nodes, T cells expand and then attack the donor graft. In the indirect pathway, host APCs

present donor antigens leading to rejection. The alloantigens presented are from the disparate MHC molecules on the donor graft (Janeway et al., 2008).

Another problem seen in transplantation, specifically with bone marrow, is graft-versus-host disease (GVHD). The mismatch of class I MHC or class II MHC antigens cause the reaction. The minor histocompatibility complex also can create the reaction, although usually less severe. In this process, T cells from the donor develop and mature; these T cells recognize the recipient as foreign using the recipient's immune system. One interesting phenomenon related to GVHD is graft vs. leukemia (GVL), which can occur in leukemia patients that receive bone marrow transplantation. In this case, the donor bone marrow kills off the leukemic cells. In bone marrow transplantation for the treatment of leukemia, the goal is to reduce GVHD effects while enhancing the GVL effects (Janeway et al., 2008). GVHD and rejection have greater complexities that must be studied further to advance transplantation. At the same time, advancements in the understanding of these two processes have allowed for improvements in protocols to achieve lifelong tolerance.

## **Tolerance**

In transplantation, the goal is to prevent rejection, and with bone marrow, GVHD as well. To prevent these outcomes, the major goal since the first induction of tolerance in mice in 1953 has been to establish tolerance for organ transplants. For a long time tolerance was simply defined as the “specific absence of an immune response”. Over the past sixty years the field of transplantation biology, and more specifically tolerance, has

rapidly evolved. Much is now known about a positive regulatory immunological response involved in tolerance. Due to the complicated nature of tolerance, the concept is now referred as “operational transplant tolerance”. This is defined as “the specific absence of a destructive immune response to a transplanted tissue in the absence of immunosuppression” (Sachs, 2011). The definition includes the involvement of regulatory mechanisms in tolerance and is a state where patients accept transplants without any immunosuppression. Included in the definition is that the recipient has a functional immune system (Bishop et al., 2011).

Recent achievements include the use of bone marrow or stem cell transplantation before kidney transplantation to induce tolerance in HLA-matched and HLA-mismatched humans. Mixed hematopoietic chimerism has been used in HLA-matched and mismatched humans to achieve tolerance via a non-myeloablative regimen. The mixed chimerism approach allows the donor cells to take up most of the bone marrow of the recipient, but not deplete the recipient bone marrow. This allows the patient to become tolerant to donor while retaining self. In addition, the non-myeloblative approach allows for more widespread applicability in the field of transplantation due to the sub lethal amounts of radiation that are necessary. Non-myeloablative regimens are of reduced intensity and do not completely deplete a recipient’s immune system before transplantation. This allows for bone marrow transplantation in the absence of malignancy. Previously, the ablative approach would only allow for this approach in patients with malignant diseases (Leventhal et al., 2013). The first approach to using a non-myeloablative mixed chimerism strategy employed a protocol originally studied in

mice and primates, that was recently transitioned to the clinic at Massachusetts General Hospital (MGH) ("A step closer to effective transplant tolerance?," 2012). The first six patients in the trial received bone marrow and kidneys from HLA identical siblings and became tolerant to their renal transplant via a mixed chimera approach (Fudaba et al., 2006). Studies performed at Northwestern and Stanford using different, non-lethal preparatory regimens have similarly led to transplantation tolerance in conditioned HLA-matched individuals. At MGH, the next trial involved HLA-mismatched transplants and four of the first five patients became tolerant (Kawai et al., 2008). Over two trials, seven of ten patients have been weaned off immunosuppressants without signs of rejection. Thus, this protocol provides promise for the future of tolerance (Kawai, Cosimi, & Sachs, 2011).

A further understanding of tolerance will also lead to a greater insight of the immune system's self/non-self discrimination mechanisms. In addition, with greater understanding of tolerance there is a chance to provide patients with transplants while avoiding complications and adverse effects associated with immunosuppressants for life. Currently, all clinical trials that are able to induce renal allograft tolerance are from living donors and the recipient must undergo a conditioning protocol a few days prior to transplant. The goal is to find a course of treatment where tolerance can be induced after transplant, so that cadaver donors can be used (Sachs, 2011). There are protocols currently under investigation that use a delayed tolerance protocol. This will allow a patient to become tolerant after a transplant (Kawai et al., 2011). By defining the mechanisms underlying tolerance and the interplay of various components of the immune

system, tolerance strategies could be improved and offered to a larger number of transplant patients.

### **Mechanisms of Tolerance**

Tolerance involves both central and peripheral mechanisms. While historically there has been a distinction between central (thymic-dependent) and peripheral (extrathymic) mechanisms, there seems to be much less of a distinction between the two now. Central and peripheral mechanisms work together to induce and maintain tolerance. Using the mixed chimerism protocol discussed above, initial bone marrow transplantation allows for proliferation of donor stem cells in a host bone marrow compartment. With current conditioning, the host bone marrow is depleted with a non-myeloablative protocol. After populating the host compartment, the donor cells along with host cells produce a mixed chimera environment in the recipient bone marrow. APCs will then present these cells from the bone marrow to the thymus to attain tolerance by elimination of host and donor reactive T cells. This is achieved via negative selection to discriminate between self and non-self. The thymus sees the presented donor and host cells as self and will delete T cells that will react against these cells. Thus, the donor and recipient are seen as self by the immune system. While this is the basis for central tolerance, there are undoubtedly cells that escape the thymus and thus peripheral tolerance mechanisms are necessary. Maintenance of tolerance is believed to be produced by regulatory cells within the kidney graft and in the periphery which will be discussed later (Al-Adra & Anderson, 2011).

There are thought to be four main mechanisms of tolerance that work together in induction and maintenance phases (Al-Adra & Anderson, 2011). While these mechanisms also can apply to B cell tolerance, the focus in this thesis will be on T cells because of their pivotal role in transplant rejection.

### ***Deletional Tolerance***

The immune system's major mechanism for establishing tolerance to self occurs in the thymus. During development, thymic epithelial cells present self-antigens to T cell receptors, so that T cells can play the important role of discriminating between self and non-self. Thymocytes and APC progenitors are transported from the bone marrow to the thymus to mature. In the thymus, thymocytes can become double negative ( $CD3^+CD4^-CD8^-$ ); CD3 is a marker for all T cells, CD4 for helper and regulatory T cells (Tregs), and CD8 for cytotoxic T lymphocyte. APCs also undergo maturation in the thymus. Thymocytes are evaluated in the thymus in a series of steps. The double negative T cells first may rearrange their T cell receptor (TCR) and become double positive cells ( $CD3^+CD4^+CD8^+$ ). Through a series of low intermediate affinity interactions between the rearranged T cell receptors on the thymocytes and self MHC/peptides on the thymic epithelial cells, only the immature thymocytes with a functional TCR will be selected (positive selection). Positively selected thymocytes then mature to either the T helper phenotype ( $CD3^+CD4^+CD8^-$ ) or cytotoxic T cell phenotype ( $CD3^+CD4^-CD8^+$ ). Negative selection ensures the elimination of autoreactive T-cell clones, by deleting T-cell populations that bind T cell receptors with high affinity. After positive and negative

selection in the thymus, only T cells with low intermediate binding affinity will be released into the periphery (Sykes, 2007).

In mixed chimeras, donor and recipient thymocytes and APC progenitors travel from the bone marrow to the thymus and undergo the process of selection and maturation as described above. The difference in mixed chimeras as compared to normal immune system development is in the process of positive selection; in this situation selection occurs in the context of host thymic epithelial cells. The MHC is of host origin and will select for donor and recipient single positive T cells of appropriate low intermediate affinity interactions. Donor and recipient single or double positive T cells that interact with high affinity to donor or recipient APCs or thymic epithelial cells presenting self MHC/peptide will be deleted by negative selection. Thus, the only T cells allowed out of the thymus will be cells that are tolerant of both donor and host (Sykes, 2007).

Mature T cells can also be deleted in the periphery by various mechanisms if they escape central deletion. One such mechanism is deletion of tissue specific cytotoxic lymphocytes in the lymph nodes via self-antigen cross presentation by dendritic cells. In addition, cytotoxic T cells may be deleted in the periphery because of exhaustion. Exhaustion is repeated exposure to an immense antigen load that results in CD8 cell deletion (Sykes, 2007). Although deletion is the major mechanism to inducing tolerance, there are many other mechanisms that play a role in inducing and maintaining tolerance.

### ***Anergy***

Anergy describes T cells incapable of expanding and producing cytokines in response to antigens that they can identify. Mature T cells will undergo activation if they are stimulated by an antigen they can recognize. In order for T cells to be activated they must be stimulated at both the T cell receptor and co-stimulatory receptors. Without stimulation of the co-stimulatory molecules, the T cell will remain in a quiescent, anergic state; this observation has inspired a great deal of research into methods for blocking co-stimulation and subsequent T cell anergy. In some cases, anergy can also occur if T cells encounter low affinity MHC/peptide complexes on APCs. In tolerogenic states, specific APCs have been shown to induce anergy by producing cytokines that suppress T cell activity. Anergic T cells may suppress other cells, including Tregs. Tregs can function to put cells into an anergic state. Anergy can occur in the periphery and intrathymically through interaction of T cell receptors with thymic stromal cells or hematopoietic cells (Sykes, 2007).

### ***Regulation (Suppression)***

The immune system can play an active role in regulating itself and T cell reactivity through regulation and suppression. There has been a great deal of research devoted to elucidating this mechanism for maintaining tolerance. This work has revealed a great deal about the intricacies of the suppressive and regulatory mechanisms of the immune system, especially as they relate to transplantation biology and tolerance. Some of these mechanisms include the production of inhibitory cytokines and killing of APCs

by cytotoxic T lymphocytes. Also, the thymus can produce Tregs that play a role in inducing and maintaining tolerance. In short, the Tregs play a role in suppressing a rejection of donor graft when tolerance is achieved. Recent work in this field has demonstrated the importance of this cell subtype in maintaining tolerance. The suppressive functions of these cells include the secretion of cytokines and inhibition of the immune responses towards donor grafts. There will be a more detailed discussion of Tregs and their applicability to the clinic later in this thesis. Due to their suppressive nature, Tregs have been studied extensively in order to find a way to use them as a tool to achieve tolerance in transplantation (Sykes, 2007).

### ***Ignorance***

In some situations, recipient cells may also just ignore the donor graft antigens. Receptors are able to identify donor antigens, but may fail to mount an immune response. A couple of explanations for this observation are that (i) T cells are not able to expand and transport to the tissue with the antigen and (ii) there have been certain ‘nonprofessional APCs’ that are unable to activate T cells (Sykes, 2007). In this state tolerance can be achieved, but is in a metastable state. If a more immunogenic environment is provided, such as by adding IL-2, rejection occurs (Li, Strom, Turka, & Wells, 2001). Further knowledge of the interplay between all these mechanisms will allow for attaining the ultimate goal of achieving lifelong tolerance in transplant patients. The small animal model provides an excellent opportunity to attain a more complex understanding of the mechanisms and interactions involved in tolerance.

### **Small Animal Model- Mouse**

In the past three decades studies in rodent models have facilitated progress in immunology and biology of transplantation. The small animal models have allowed for experimentation to further the understanding of tolerance. Mice provide a model with many genetic similarities to humans. Before experimentation is done in large animals and then humans, the mouse model provides a simple model to determine if these experiments will be feasible (Russell, 2002). The genetic similarities allow for manipulation of genes and other components to mimic certain human conditions and human diseases. In both species, the paths that control normal and pathological conditions are mostly preserved (Rivera & Tessarollo, 2008). The close relationship to humans, ease of manipulation, and abundance of small animal models provides great advantages for research.

Studies in rodents have progressed dramatically with outstanding results. The first studies to induce tolerance with mixed chimerism in mice used lethal irradiation of the recipient mouse. The donor bone marrow and recipient's T cell depletion resulted in tolerance and mixed chimeras for the remainder of the mice's lives. An initial study of hematologic malignancies in mice to achieve the graft versus leukemia effects without graft versus host disease using mixed chimerism and donor leukocyte infusion has been modified and transferred to the clinic to treat patients with lymphomas and leukemia (Fudaba et al., 2006; Mapara, Kim, Marx, & Sykes, 2003; Mapara et al., 2002). While tolerance was achieved, a conditioning regimen in humans that involves lethal irradiation and chemotherapy is toxic and could only be used in malignant cases. Thus, a less toxic

protocol was necessary for organ transplantation in non-malignant cases. Conditioning needed to be enough to kill alloreactive T cells centrally in the thymus and in the periphery, while trying to reduce the side effects of irradiation. In a mouse study, a low amount of total body irradiation (300 rads) was used to eradicate the T cells in the periphery; thymic irradiation (700 rads) was added to rid the thymus of the reactive T cells centrally, followed by donor bone marrow infusion and short term treatment with T cell depleting antibodies. The result was tolerance via central deletion by generating mixed chimerism in MHC mismatched mice. In an attempt to separate GVL from GVHD, successful protocols have been devised for HLA-matched patients with multiple myeloma and afterword for HLA-mismatched patients without other life-threatening diseases (Sachs, Sykes, Kawai, & Cosimi, 2011).

While there have been numerous studies displaying tolerance in mouse models, many of these protocols have not been successfully transferred to large animal models and humans. Large animals and humans have greater complexities that cannot be recreated in a mouse model. This is especially the case when one considers the immune system and transplant tolerance. A limited number of tolerance inducing protocols have been transferred from rodents to nonhuman primates and even fewer to humans. One possible reason for this is the T memory response that is seen in nonhuman primates, but not rodents. The T memory responses are thought to be the cause of resistance to tolerance by mixed chimerism (Kawai et al., 2011). Thus, it is evident that there are major differences in the biology of mice, large animals, and nonhuman primates. The developmental and genetic differences of these animals provides for significant variation

in the way their immune systems function, as well as how their bodies respond to drugs (Sachs et al., 2011). Therefore, experiments performed in small animal models need to be reproduced in the large animal models before being applied to humans.

### **Large Animal models**

Before protocols can be applied in the clinic, they should be demonstrated in large animal models, such as miniature swine and nonhuman primates, to test protocols for safety and efficacy. Using these models, it is possible to refine protocols and identify problems that may affect patients. Miniature swine have many of the same immunological properties and genetics as humans. Nonhuman primates are even closer genetically with even more similar immune interactions. One biological difference that has been seen in large animals and not in rodents is the expression of class II antigens continuously on vascular endothelium of organs. This is only one difference that has been seen in swine and humans as compared to rodents that may play a role in determining successful transplants (Yamada et al., 1997).

With the mouse model as a basis for preliminary studies in 1995, our lab used a non-myeloablative protocol to induce renal allograft tolerance in cynomolgous monkeys. Mixed chimerism and tolerance was achieved in MHC-mismatched nonhuman primates. The conditioning regimen included anti-thymocyte globulin as a T-cell depleting agent, total body irradiation of 300 or 150 rads, thymic irradiation of 700 rads, 30 days of Cyclosporine A and donor bone marrow infusion. In the study, mixed chimerism was observed, as well as long term renal allograft tolerance. The monkeys became tolerant,

were weaned of immunosuppression, and accepted the transplanted grafts indefinitely (Kawai et al., 1995).

In addition, a similar conditioning and mixed chimerism approach has been used in the miniature swine model in our laboratory. Miniature swine were conditioned with a non-myeloablative protocol that included whole body irradiation, thymic irradiation, CD3-immunotoxin treatment to reduce T cells, 30 days of Cyclosporine A, and infusion of donor bone marrow or peripheral blood stem cells. These SLA matched recipients developed mixed chimerism and long-term tolerance. This was the first time tolerance was successfully induced via mixed chimerism in a swine model (Huang et al., 2000).

Previous work from this lab has also demonstrated that the thymus plays an essential role in the induction of transplantation tolerance. In class I mismatched swine, thymectomized swine displayed rejection of their transplants, while those with an intact thymus were shown to induce and maintain tolerance. This supports the theory of the thymus and central deletion being vital to inducing tolerance. While central tolerance via the thymus is critical, there are also peripheral mechanisms that work in synergy with the central mechanisms (Yamada et al., 1997). In another study of MHC I disparate swine, the thymus' presence was shown to be critical in inducing tolerance until day 42. Animals that underwent thymectomy on days +8 and +21 post transplant did not show stable graft function, while animals that were thymectomized on POD 42 or later became uniformly tolerant. Thus, the thymus is not required to maintain tolerance, but is required to induce central and peripheral tolerance (Vagefi et al., 2004).

Our lab has developed a tolerant renal transplant model in Class I mismatched miniature swine. By using Cyclosporine A for a twelve day period at 10-13 mg/kg daily, long term tolerance was achieved in 100% of two-haplotype class I mismatched, class II matched, renal allografts (for example (class I<sup>dd</sup> to class I<sup>gg</sup>)). Kidney transplants without Cyclosporine A in this same model were uniformly rejected by two weeks. After tolerance was achieved, systemic tolerance was tested by removing the primary graft and replacing the graft with a second donor-matched kidney graft. Tolerance was confirmed as the swine were able to accept the second kidney without immunosuppression. Cyclosporine A is used in this model to suppress helper T cells that are class I specific. Consistent with this, graft acceptors do not class switch from IgM to IgG antibody production due to the absence of T helper cells (Rosengard et al., 1992).

In MHC class I mismatches, the indirect pathway of antigen presentation must be involved because MHC I antigens have to be presented by host MHC II. Cyclosporine A is effective in preventing the indirect pathway for antigen presentation. Fully mismatched transplants refers to disparities at both the MHC I and MHC II loci. Use of Cyclosporine A did prolong survival in this model, but was not able to maintain tolerance in fully-mismatched barriers. The fully mismatched transplants invoke the indirect and direct pathways of antigen presentation. Thus, Cyclosporine A is less effective when the direct and indirect pathways of allorecognition are involved. In this model, regulatory mechanisms have been seen to induce and maintain tolerance (Rosengard et al., 1992).

Our lab was able to induce tolerance in miniature swine across full-MHC barriers. This was done by using 0.15-0.30 mg/kg daily of tacrolimus (FK 506) for 12 days to

induce tolerance across two-haplotype fully mismatched MHC barriers. Examples of this would be a SLA<sup>dd</sup> (Class I<sup>dd</sup> Class II<sup>dd</sup>) kidney transplanted into a SLA<sup>cc</sup> (Class I<sup>cc</sup> Class II<sup>cc</sup>) animal. In this model, analysis indicated the presence of regulatory mechanisms and cells maintaining tolerance (Utsugi et al., 2001). Currently, we use these two models in MHC class I mismatched and MHC fully mismatched experimental transplants.

### **Why our model?**

The 12-day course of Cyclosporine A tolerance model has been extensively used to study the mechanisms of transplantation tolerance in large animals. In a previous study, we isolated peripheral blood lymphocytes (PBL) from tolerant animals and primed them *in vitro* with donor antigen. Using cell-mediated lympholysis assays (CML), these primed PBLs were added to cultures of naïve recipient-matched PBLs stimulated with donor-matched targets. The *in vitro* results showed suppression of anti-donor cytotoxic T lymphocytes by PBLs from the tolerant animals. Conversely, when donor primed tolerant PBLs were added to cultures containing naïve recipient-matched PBLs and third party target cells, suppression was not observed. Similarly, PBLs from naïve animals primed with donor Ag that were added to cultures of PBLs from tolerant animals stimulated with third-party antigen did not suppress proliferation. Additionally, the suppression was dose dependent, sensitive to radiation, needed cell-to-cell contact, and was not reversed by administration of exogenous IL-2 (Ierino, Yamada, Hatch, Rembert, & Sachs, 1999; Ierino, Yamada, Lorf, Arn, & Sachs, 1998). These studies provide data in support of regulatory mechanisms and peripheral regulatory cells being essential in

inducing and maintaining tolerance to renal allografts. At the same time, these studies only provided *in vitro* evidence for the role of regulation in tolerance. In our study, we investigated the role of Tregs in an *in vivo* model.

## OBJECTIVES

The objective of this study is to provide direct evidence of role of Tregs in transplantation tolerance using an adoptive transfer model.

Specifically, we will determine the interactions involved in maintenance of tolerance.

We hypothesize that T regulatory cells are present in the kidney graft and in the periphery. We hypothesize that the Tregs interact with the peripheral T cells and are vital in maintaining tolerance through suppression.

Using this as our basis we want to:

- Test if a large dose of tolerant cells can have an effect on graft survival.
- Test if long-term tolerated kidney allografts have immunoregulatory effects.
- Test if we can adoptively transfer tolerance with donor-matched cells and kidneys from long-term tolerant animals.

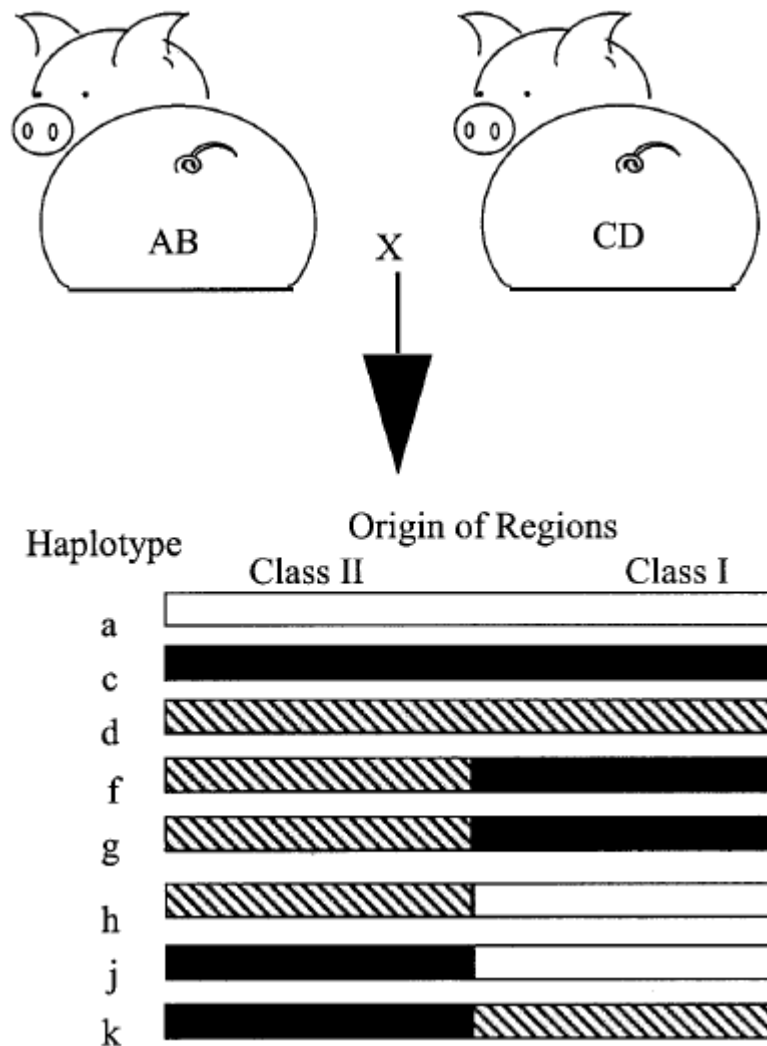
We hope these studies will display the importance of Tregs in the induction and maintenance of tolerance in an *in vivo* animal model.

## MATERIALS AND METHODS

### Animals

MHC inbred MGH-miniature swine provides the advantage of studying transplantation biology in a genetically defined model that can mimic transplantation in humans. MHC homozygous and intra-MHC recombinant haplotypes allow us to study that the class I and/or class II loci play in transplantation. This is the only large animal model that can select for MHC I and MHC II genes in order to study their role in transplant rejection (Pennington, Lunney, & Sachs, 1981; Sachs et al., 1976). Swine are one of the only species where inbreeding can allow for genetic experiments in a large animal model. This is due to sexual maturity being at 6 months of age, a large litter size of about 3-10 offspring, and a short gestation time of 3 months. Due to these circumstances, specific MHC lines could be made in a short amount of time. Being the only MHC defined large animal model allows us to study MHC matching's effects on tolerance and rejection. There are currently three homozygous SLA haplotypes and five recombinant haplotypes. See Figure 1 for the origin of haplotypes. Every swine line varies in minor histocompatibility loci, which is usually the case in human transplantation. Thus, most of the transplantation combinations seen in humans can be reproduced in this model. Transplants within a MHC homozygous herd can mimic HLA identical siblings, transplants between herds can represent non-matched siblings, and transplants between heterozygotes can represent one haplotype mismatched siblings. These are only examples, but use of these animals clearly displays a tremendous advantage in studying transplantation biology for humans.

The SLA<sup>dd</sup> animals were selected for further inbreeding to create a fully inbred line and have reached a coefficient of inbreeding of > 94% (Mezrich et al., 2003). These animals have allowed for adoptive transfer protocols for the first time in a large animal model by reducing minor histocompatibility differences that may cause rejection.



**Figure 1:** The origin of haplotypes of MGH inbred miniature swine (Figure taken from Vagefi et al., 2004)

Donor animals used in our study were SLA<sup>gg</sup> (Class I<sup>c</sup>/II<sup>d</sup>) partially inbred MGH miniature swine. Recipient animals were 4-10 months of age from the inbred line of SLA<sup>dd</sup> (Class I<sup>d</sup>/II<sup>d</sup>) MGH miniature swine that were chosen to reduce minor antigen differences.

### **Experimental Groups**

SLA<sup>dd</sup> animals received two-haplotype class I mismatched kidneys (SLA<sup>gg</sup>) with a 12-day course of Cyclosporine A to attain blood levels of 400-800 ng/ml (CyA; Sandimmune, generously provided by Novartis Pharmaceutical Corporation, East Hanover, NJ) (Yamada et al., 1997). All animals became long-term tolerant (LTT) animals. This was shown through acceptance of the first renal graft with stable renal function for at least 90 days. These long-term tolerant animals were then used as adoptive transfer donors for naïve SLA<sup>dd</sup> recipients in Groups A-E. With the exception of one animal in Group A, naïve adoptive-transfer recipients were treated with 150 rads of whole body irradiation (WBI) one-day before receiving class I MHC-mismatched kidney grafts from naïve SLA<sup>gg</sup> pigs. Group A animals received the naïve SLA<sup>gg</sup> kidney grafts and peripheral blood mononuclear cells (PBMCs) from long-term tolerant animals. Group B animals received kidney grafts from long-term tolerant animals. Group C animals received kidney grafts from long-term tolerant animals that were treated with donor-specific transfusion as described below. Group D animals received kidney grafts from long-term tolerant animals without donor-specific transfusion. Group E animals received PBMCs and kidney grafts from long-term tolerant animals with donor-specific

transfusion. Additionally, the long-term tolerant SLA<sup>dd</sup> pigs that were adoptive transfer donors for the animals in groups A-E received a second class I MHC-mismatched graft from a naïve SLA<sup>gg</sup> animal on the same day as the nephrectomy of the tolerated graft. There were three recipients of adoptive transfer in most of the animal group (A,C,D,E) and 6 in group B. Table 1 shows the experimental groups that were used.

**Table 1: Treatment to recipient (SLA<sup>dd</sup>) of naïve kidney (SLA<sup>gg</sup>).**  
DST = donor-specific transfusion; LTT = long-term tolerant; PBMCs = peripheral blood mononuclear cells; WBI = whole body irradiation (Table taken from Okumi et al., 2013. Permission given by Wiley Online Library to use Table.)

Group	Treatment to adoptive transfer donor (LTT animal) Day -8 DST 10 ml/kg	Day -1 150 rads WBI	Day -1 Co-administration of PBMCs transfer 2.5 x10 <sup>9</sup> cells/kg	Day 0 Co-transplantation of tolerated kidney graft transfer	Survival (days)
A	(-)	(-)	(+)	(-)	7, 7, 7
B	(+)	(+)	(-)	(+)	8, 8, 9
C	(+)	(+)	(-)	(-)	7, 10, 30
D	(-)	(+)	(-)	(+)	9, 10, 73
E	(+)	(+)	(+)	(+)	4, 10, 46
					28, >150, >150

## Surgery

Details of the surgical procedures have been described elsewhere (Rosengard et al., 1992; Yamada et al., 1997). Briefly, both native kidneys were removed on the day of the primary kidney transplant. Donor kidneys were perfused with Eurocolins and immediately anastomosed to the nephrectomized recipients' aorta and vena cava. The ureter was anastomosed to the recipient bladder. For secondary transplantation, the kidney was transplanted using the same procedure at the same site or 1 cm below the

primary graft anastomoses. Indwelling central venous catheters were placed surgically in the external and internal jugular veins of recipient animals to facilitate frequent blood sampling and the administration of fluid, drugs, blood, and PBMCs.

### **Donor-Specific Transfusion (DST)**

SLA<sup>dd</sup> LTT animals that served as adoptive transfer donors to groups B, C, and E were given an intravenous transfusion of 10 ml/kg (body weight) non-irradiated SLA<sup>gg</sup> whole blood one week prior to leukapheresis. The dose of blood for the DST was based upon rodent allotransplantation models (Bushell, Karim, Kingsley, & Wood, 2003; Niimi et al., 2000).

### **Transfer of Peripheral Blood Mononuclear Cells (PBMCs)**

A total of  $2.5 \times 10^9$  PBMCs/kg (recipient body weight) were collected by leukapheresis using a COBE Spectra machine (COBE BCT Inc., Lakewood, Colorado, USA) from LTT animals and were then infused intravenously to recipient in groups A, B, and E one day prior to transplantation. The appropriate number of tolerant PBMCs was based on studies of the adoptive transfer of tolerant spleen cells in mouse models (Bemelman, Honey, Adams, Cobbold, & Waldmann, 1998; Qin et al., 1993).

### **Transfer of Long-Term Tolerated Kidney Allografts**

Long-term tolerated SLA<sup>gg</sup> kidney grafts were harvested from LTT SLA<sup>dd</sup> animals and transplanted into SLA<sup>dd</sup> recipients in groups C, D, and E at the same time as the

naïve SLA<sup>g8</sup> kidney grafts. Following donation of the tolerated kidney allograft, LTT animals received a second naïve kidney graft from a SLA<sup>g8</sup> animal.

### **Histology of Long-Term Tolerated Kidney Allografts**

Immunohistochemical analysis of frozen sections of long-term tolerated kidney grafts was performed using the standard avidin-biotin horseradish-peroxidase complex (ABC) technique to detect the phenotype of graft infiltrating cells. For the detection of proliferating cell nuclear antigen (PCNA), sections of 10% buffered formalin-fixed, paraffin-embedded tissue blocks were stained using the ABC technique (Shimizu, Yamada, Meehan, Sachs, & Colvin, 2000).

### **Quantitative Analysis of Tregs in kidney grafts**

Two LTT kidneys and one rejected kidney from Group B were double stained for CD25 (FITC) and Foxp3 (Bio-PEAV) to assess the Treg cell population. The percent of CD25 and Foxp3 double positive cells out of CD25 cells at 100x magnification were viewed with a standard deviation of  $\pm 9$ .

### **Monitoring of Rejection**

Rejection of kidney grafts was monitored by plasma creatinine levels and by histological examination of kidney biopsies. Renal open-wedge biopsies were performed through a flank incision. Tissues were stained using hematoxylin-eosin and periodic acid-Schiff, and coded slides were examined by light microscopy by a pathologist so that

the analysis would be blinded. Graft rejection was scored according to a standardized grading system of pathological specimens (Colvin, 1996).

### **Preparation of Peripheral Blood Mononuclear Cells (PBMCs)**

For the separation of PBMCs, freshly heparinized whole blood was diluted 1:2 with Hank's balanced salt solution (HBSS, Invitrogen) and the mononuclear cells were obtained by gradient centrifugation using Histopaque (Sigma). The mononuclear cells were washed once with HBSS, and contaminating red cells were lysed with ammonium chloride potassium buffer (BioWhitaker). Cells were then washed with HBSS and resuspended in tissue culture medium. All cell suspensions were kept at 4°C until used in cellular assays.

### **Cell-Mediated Lympholysis Assays**

The procedure for CML assays has been described previously (Yamada et al., 1997). Briefly describing the assay, lymphocyte cultures containing  $4 \times 10^6$  responder and  $4 \times 10^6$  stimulator PBLs (irradiated with 2,500 rads) were incubated for 6 days at 37° C in 7.5% CO<sub>2</sub> and 100% humidity. Bulk cultures were harvested and effectors tested for cytotoxic activity on <sup>51</sup>Cr (Perkin Elmer) labeled targets generated from lymphocytes stimulated for 24 hours with a 1:500 dilution of phytohemagglutinin (PHA, M-Form; Invitrogen) previously titrated to give optimal proliferation. Effector cells were incubated for 5.5 hours with a negative control target (i.e., target PBL matched to the effectors) and targets matched to the stimulators which included donor-matched PBL

(SLA<sup>gg</sup>: Class I<sup>cc</sup>/Class II<sup>dd</sup>) and third party stimulators (SLA<sup>aa</sup>: Class I<sup>aa</sup>/Class II<sup>aa</sup>).

Effector:Target ratios of 100:1, 50:1, 25:1, and 12.5:1 were tested. Supernatants were harvested using the Skatron collection system (Skatron, Sterling, VA). <sup>51</sup>Cr release was determined on a gamma counter. The results were expressed as:

$$\% \text{ specific lysis} = \frac{\text{Experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{Maximum release (cpm)} - \text{spontaneous release (cpm)}} \times 100\%$$

### **Co-Culture Assays**

Peripheral regulatory mechanisms were investigated by *in vitro* co-culture assays (Ierino et al., 1999). The primary culture was set up as in CML assays. These primed cells were then harvested and rested overnight at 4°C. The resulting effector cells were co-incubated with naïve SLA matched PBMCs and irradiated donor-type or third party PBMCs for an additional 5 days.

### **Antibodies and Flow Cytometry**

The presence of anti-donor class I (MHC class I<sup>c</sup>) IgM and IgG in the serum of recipient swine was detected by indirect flow cytometry Fluorescence-activated cell sorting (FACS) that was performed using a Becton Dickinson FACScan microfluorometer (Sunnyvale, CA) and recombinant SLA PBMCs to determine the SLA-binding specificity of the antibody as described in a previous paper (Griesemer et al., 2008).

## RESULTS

### **Adoptive transfer of a high-dose of tolerant cells had a minimal outcome on graft survival**

#### *Infusion of cells from long-term tolerant animals was not enough to increase graft survival of naïve kidneys in a naïve recipient across a class I mismatch*

We tested if adoptive transfer of a high or “mega”-dose of PBMCs ( $2.5 \times 10^9$  unprimed PBMCs/recipient kg) from a long-term tolerant animal one day before kidney transplant could induce acceptance of a class I mismatched kidney in a naïve animal without any additional treatment (Group A, Table 1). The kidney was rejected by day 7, which is similar to rejection of class I mismatched kidneys transplanted without immunosuppression (Giangrande et al., 1997). The infusion of PBMCs from the tolerant animal did not increase the graft survival in the naïve recipient (Figure 3A). Thus, other changes would be necessary in the recipient or adoptive donor in order to increase graft survival.

#### *Donor-specific transfusion increased the in vitro suppressive effects of Tregs from long-term tolerant pigs*

We hypothesized that DST can increase the number and/or potency of Tregs in vivo in LTT animals (Abe et al., 2009; Bushell et al., 2003). We thought that these Tregs from long-term tolerant animals given as a high-dose of PBMCs to naïve recipients could increase graft survival. We first tested if donor-specific transfusion in a LTT animal would increase the suppression of the normal immunologic response to donor stimulation

*in vitro* using co-culture assays. After administration of 800 mL (10mL/kg) of donor-matched, non-irradiated whole blood to LTT animals, we measured plasma creatinine twice weekly to assess kidney function. CML and co-culture assay was setup before the DST and 1 week after the DST.

In all of the animals, plasma creatinine was within the normal range before and after the DST, indicating that DST did not have an effect on kidney function. More importantly, the inhibitory effects of Tregs increased after DST. The anti-donor CTL was 16% and 20% (at 100:1 of E:T ratio) before DST, and was gone one week after DST (Figure 2A). A co-culture assay showed cells from the long term tolerant animal primed by donor antigens suppressed the naïve cytotoxic T lymphocyte response against donor-type cells 70% and 80% before the DST and completely after the DST (Figure 2B).

### ***The effect of donor-specific transfusion on regulatory cells in vivo was minimal***

Group B included 6 LTT donors primed with DST 8 days before adoptive transfer of cells in order to increase the number and/or potency of Tregs. Recipients also received 150 rad of WBI in an effort to decrease precursor alloreactive T cells (Table 1, Group B). 3 of the 6 recipients also received high-dose of PBMCs ( $2.5 \times 10^9$  unprimed PBMCs/recipient kg) from a LTT animal one day before kidney transplantation. The 3 animals who received only 150 rads of WBI rejected their kidney grafts by day 9 (Figure 3B red lines). Therefore, the donor-specific transfusion by itself could not increase survival of kidney grafts. Two of the three animals that received PBMCs from LTT animals rejected in a manner similar to those without PBMCs from LTT animals (Figure

3B red) or Group A animals. The remaining animal had renal graft function until day 30, although creatinine levels fluctuated (Figure 3B green).

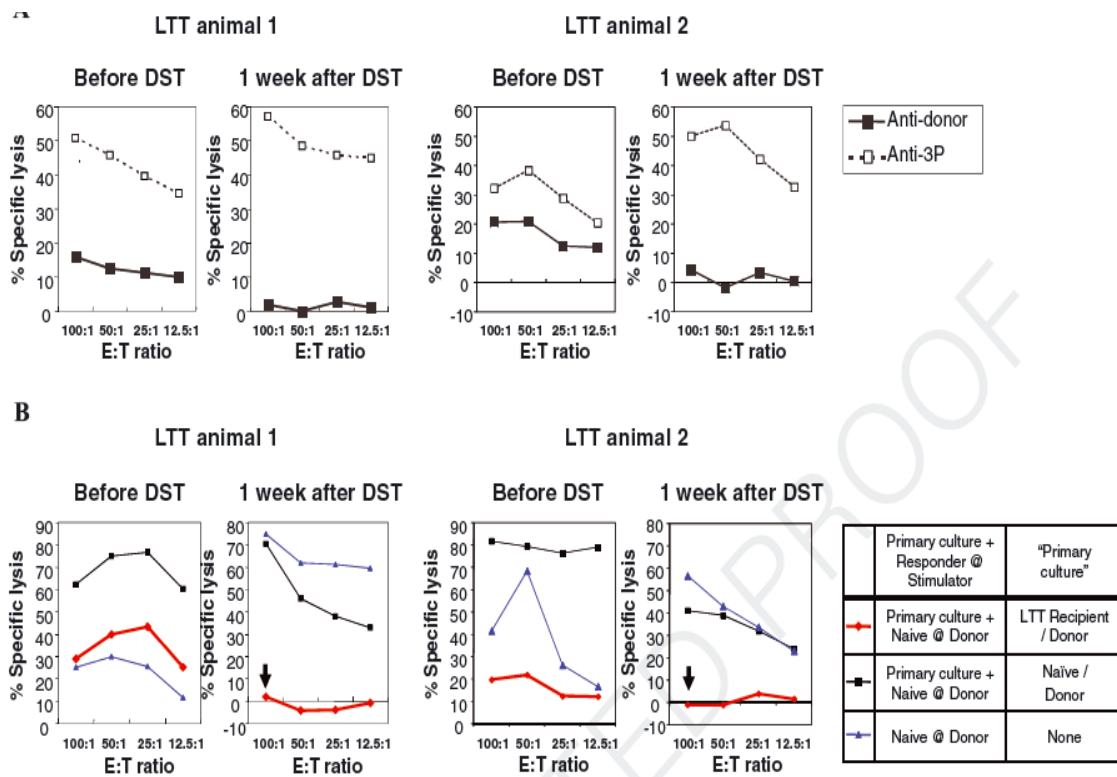
### **Adoptive Transfer of long-term tolerated kidney allografts had immunoregulatory effects**

#### ***Histology showed Foxp3<sup>+</sup>/CD 25<sup>+</sup> cells in the LTT kidneys***

LTT kidneys in class I mismatched Cyclosporine A model showed focal mononuclear cell infiltrates around vessels (Figure 4A). These cells have been shown to be CD4<sup>+</sup>/CD8<sup>+</sup> cells, while cells in rejection are only CD4<sup>+</sup> (Giangrande et al., 1997). Due to previous findings of IL-10 in the kidney infiltrate of tolerant animals (Blanco et al., 1995), we saw if the infiltrate included Foxp3<sup>+</sup> Treg cells. Tregs express the transcription factor Foxp3, which gives Tregs their suppressive abilities (Bishop et al., 2011).

Most of the infiltrate from the LTT grafts were CD25<sup>+</sup> T cells (Figure 4B), but not PCNA positive (Figure 4c). CD25 is displayed on alloactivated effector T cells and Tregs. Additional cell-staining showed that one third of the infiltrate was also Foxp3<sup>+</sup> (Figure 4d) and that some of these Foxp3<sup>+</sup> cells were also CD25<sup>+</sup> (Figure 4E). These Foxp3<sup>+</sup> CD25<sup>+</sup> cells confirmed the presence of Tregs in the long-term tolerated kidney grafts in the adoptive transfer model. Foxp3 is a transcription factor that is expressed exclusively in Tregs and not in activated effector T cells. In addition, as a control samples were evaluated from the class I mismatched kidney transplant recipient that rejected in a cellular manner (30-day survivor in Group B). The kidney was stained with

anti-pig CD25 and Foxp3 antibodies (Figure 4F). A large number of CD25 positive cells were found in the graft, but most were Foxp3 negative (red). Phenotypic analysis of the cell populations in the kidneys of the two LTT animals showed  $45.8 \pm 10.6\%$  of CD25<sup>+</sup> cells also were Foxp3<sup>+</sup>, as compared to the 30 day survivor graft with  $10.3 \pm 6.2\%$  of CD25<sup>+</sup> cells being Foxp3<sup>+</sup>.



**Figure 2: CML and co-culture CMLs demonstrated that DST in LTT animals increased suppression of the anti-donor response *in vitro*.** CTL and co-culture CTL assays were set up 1 week before and 1 week after the DST. Recipient responses to donor stimulation are shown with solid lines and boxes; third party stimulation are shown with dotted lines and striped boxes. (A) While the anti-donor CTLs were 16% and 20%, respectively before the DST, there were no anti-donor CTL responses 1 week after the DST. (B) Cells from the LTT animal primed by donor antigens specifically suppressed the naïve CTL responses against donor-type cells 70% and 80%, respectively before the DST, and completely (red line with an arrow) after the DST.

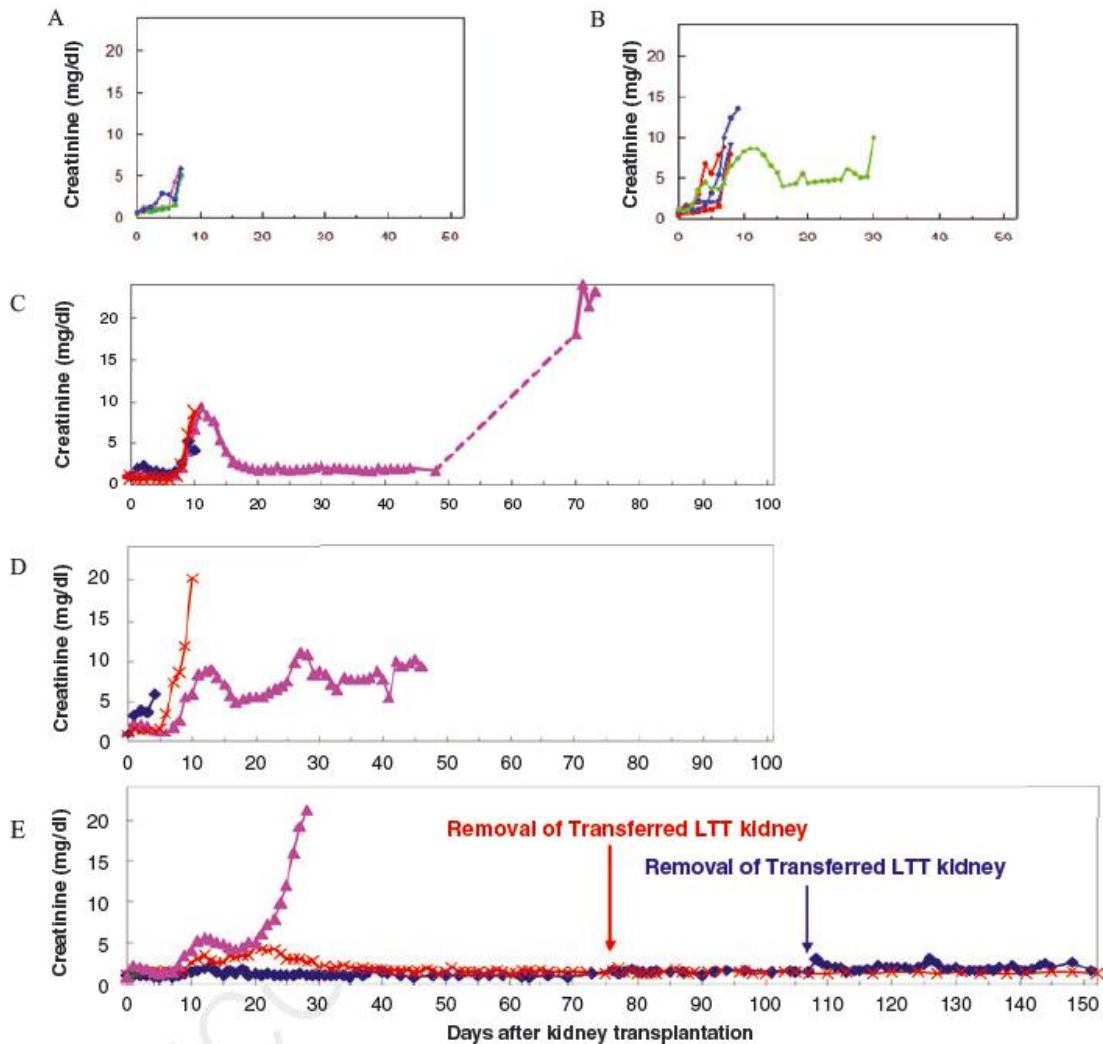
(Figure taken from Okumi et al., 2013. Permission given by Wiley Online Library to use Figure.)

### ***Long-term tolerated kidney grafts have regulatory effects***

Using groups C and D, we evaluated if transfer of LTT kidneys with the graft infiltrate, including Tregs, would increase survival in naïve recipients. Six SLA<sup>dd</sup> animals were treated with 150 rads WBI one day before kidney transplant. Each of these animals had a SLA<sup>gg</sup> tolerant kidney from a LTT SLA<sup>dd</sup> pig cotransplanted with a naïve SLA<sup>gg</sup> kidney. Three of the LTT donors were given donor-specific transfusion one week before transplant like Group B (Group C). The other three donors were not given DST and made up Group D.

Two animals in Group C and two animals in Group D rejected grafts by 10 days post-transplant. One pig in group C did maintain stable renal function for at least 50 days and lost the graft at day 73 (Figure 3C). Graft life was decreased when adoptive transfer donors did not receive DST. One animal in Group D had renal function for 46 days but it was unstable throughout (Figure 3D). Kidney biopsies from the two recipients who had increased graft survival showed differences between the naïve kidney grafts and LTT kidney grafts. Histology of kidney graft biopsies taken at day 22 post-transplant in the 73 day Group C survivor is in Figures 5A and 5B. At day 22 the naïve kidney graft was rejected (Figure 5A) and the LTT kidney graft had minimal cellular infiltrate (Figure 5B). This shows that pre-existing Foxp3<sup>+</sup>CD25<sup>+</sup> cells in the kidney graft had a protective role. Moreover, although the naïve kidney graft was rejected, no anti-donor (SLA<sup>gg</sup>) class I IgG was seen in either graft. Also, the CML for the 73 day survivor showed donor specific hyporesponsiveness at day 46, while the 46 day Group D survivor had a high

anti-donor CML response on day 36. This data indicated that the tolerant kidney had a role in regulating the local and systemic immunologic responses.



**Figure 3: Plasma Creatinine levels of recipients of class I mismatched kidneys with various treatments.** Plasma creatinine levels (mg/dL) of recipients of class I mismatched kidneys treated with (A) infusion of tolerant PBMCs alone (Group A), (B) DST and a 150 rad WBI with/without from LTT animals (Group B), (C) transfer of tolerated kidney grafts from LTT animals treated with DST and a 150 rad WBI (Group C), (D) transfer tolerated kidney grafts from LTT animals with a 150 rad WBI, without DST (Group D) and (E) transfer of both PBMCs and tolerated kidneys from LTT animals treated with DST and a 150 rad WBI (Group E).

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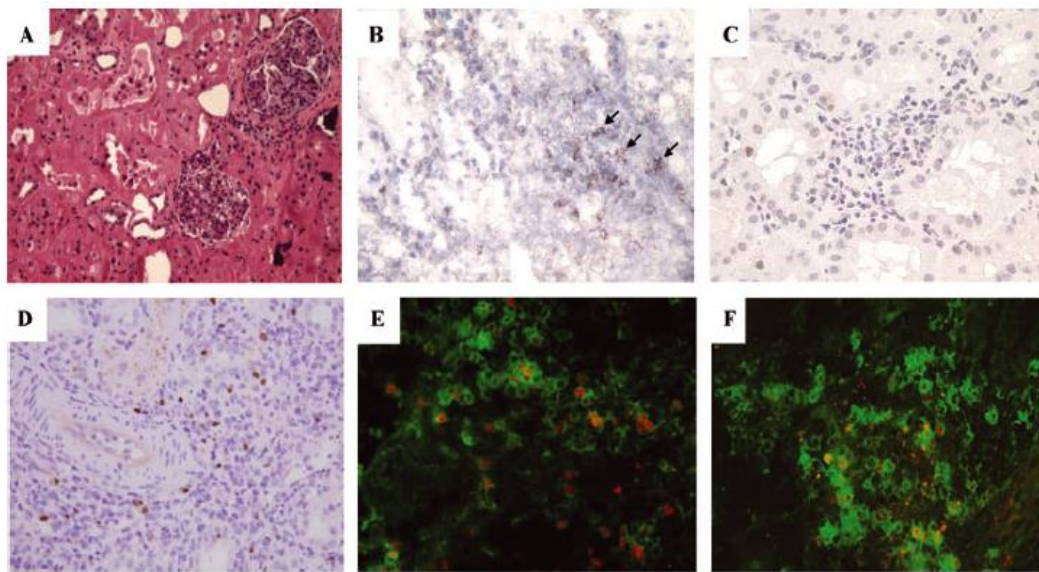
### **Inducing tolerance of naïve class I mismatched kidneys using adoptive transfer of donor-kidney matched cells and kidneys form long-term tolerant animals (Group E)**

To determine if using DST-priming of donor and subsequent transfer of PBMCs with tolerated kidney grafts from LTT animals could increase kidney graft survival or induce tolerance in recipients that had 150 rads WBI (Group E), additional studies were done. This protocol is detailed in Figure 6. The three animals were co-transplanted with naïve and tolerated class I mismatched kidneys. Two of three animals underwent graftectomy of the tolerant grafts from LTT donors to evaluate graft function of SLA<sup>gg</sup> kidney grafts from naïve animals on days 76 and 107 respectively. One animal out of the three rejected both grafts by day 28 (Figure 3E). Histology of the naïve graft showed severe diffuse interstitial hemorrhages and mononuclear cell infiltrates (Figure 5C) on day 28. Biopsies taken from the LTT graft showed diffuse mononuclear cell infiltrates and glomerular changes, but with mild interstitial hemorrhages (Figure 5D). Like Groups C and D, the tolerated kidney graft's pre-existing intra-graft passenger leukocytes may have played a protective role.

The other two animals maintained stable renal function, although there were brief spikes of creatinine (Figure 3E). Histology of the naïve and LTT kidney grafts on day 60 displayed minimal mononuclear cell infiltrates without chronic vasculopathy (Figure 5E and 5F). To assess the function of the naïve SLA<sup>gg</sup> kidney grafts, we removed the kidney grafts from the LTT animals on days 76 and 107 respectively. Both animals were able to maintain normal kidney function with the naïve kidney graft for over 90 additional days (Figure 3E) without histological evidence of rejection (Figure 5G). The 2 animals

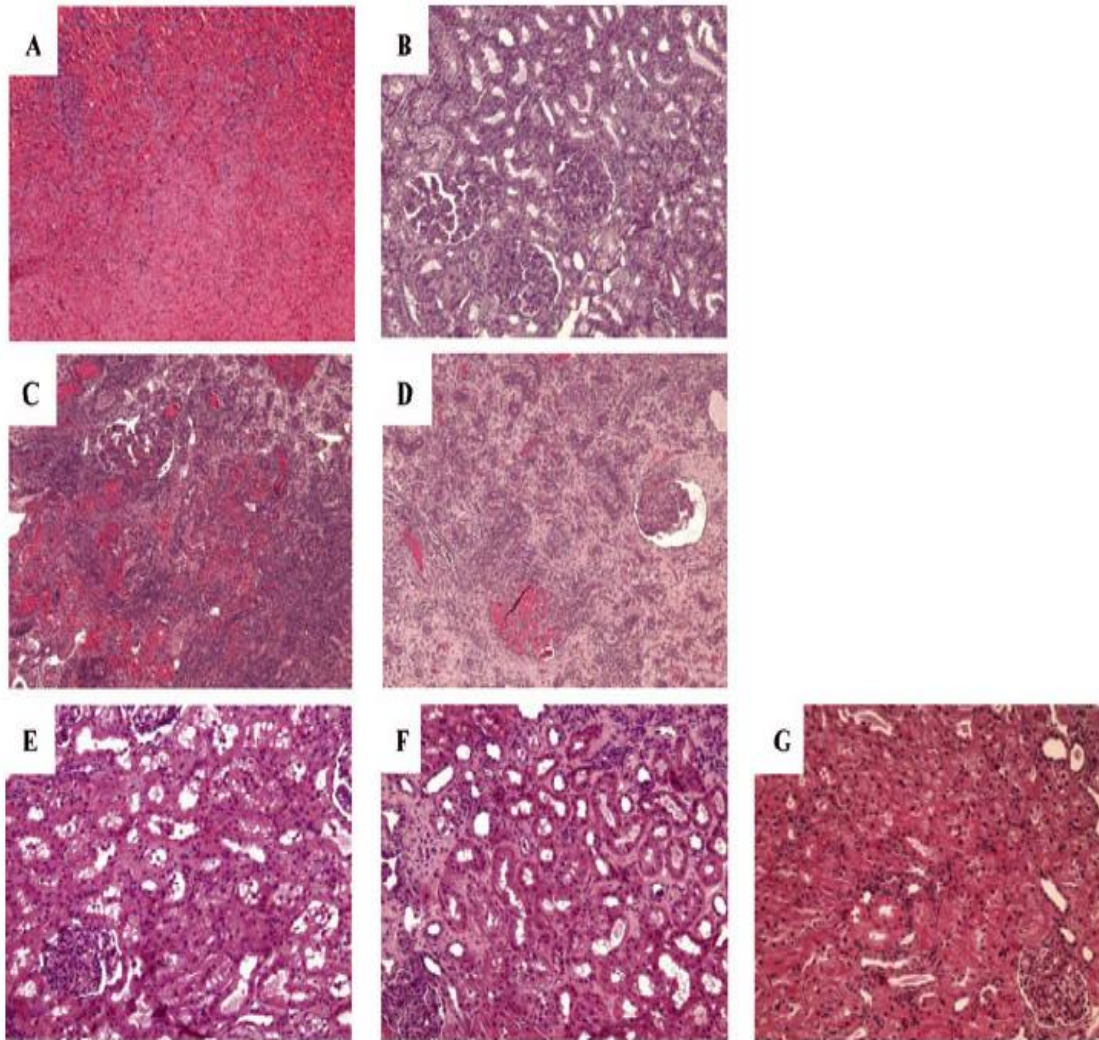
accepted class I MHC mismatched grafts long-term demonstrating that co-transplantation of cells and kidneys from tolerant animals can induce and maintain systemic tolerance in a large animal model.

CML assays showed donor-specific unresponsiveness in the two long term acceptors at 30 and 60 days post-transplant. In addition, cells from the animals remained unresponsive to donor stimuli for 75 days after removing the LTT kidney graft (Figure 7A). Lastly, co-culture assays to test for regulatory mechanisms displayed 100% inhibition of naïve CTL response to donor antigens.



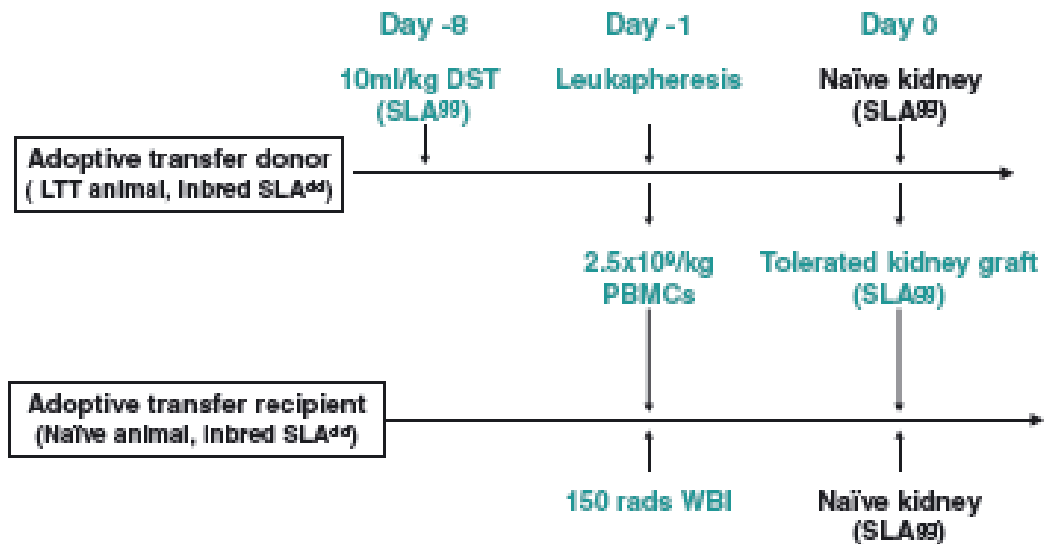
**Figure 4: Histological analysis of representative long-term tolerated kidney allografts.** (A) Minimal cell infiltrate was seen in the tolerated kidney graft (H&E, x200). (B) Several of the cells in the graft were CD25 positive (black arrows); (C) most cells were PCNA negative (x400). (D) Foxp3 positive cells, shown with arrows, were found in the tolerated kidney graft (x400). (E) Some cells in the infiltrate were CD25 (green)/Foxp3 (red) double-positive. (F) As controls, many CD25 positive (green) cells were found within a class I mismatched kidney graft that was rejected in a cellular manner, but most of them were FoxP3 negative (red).

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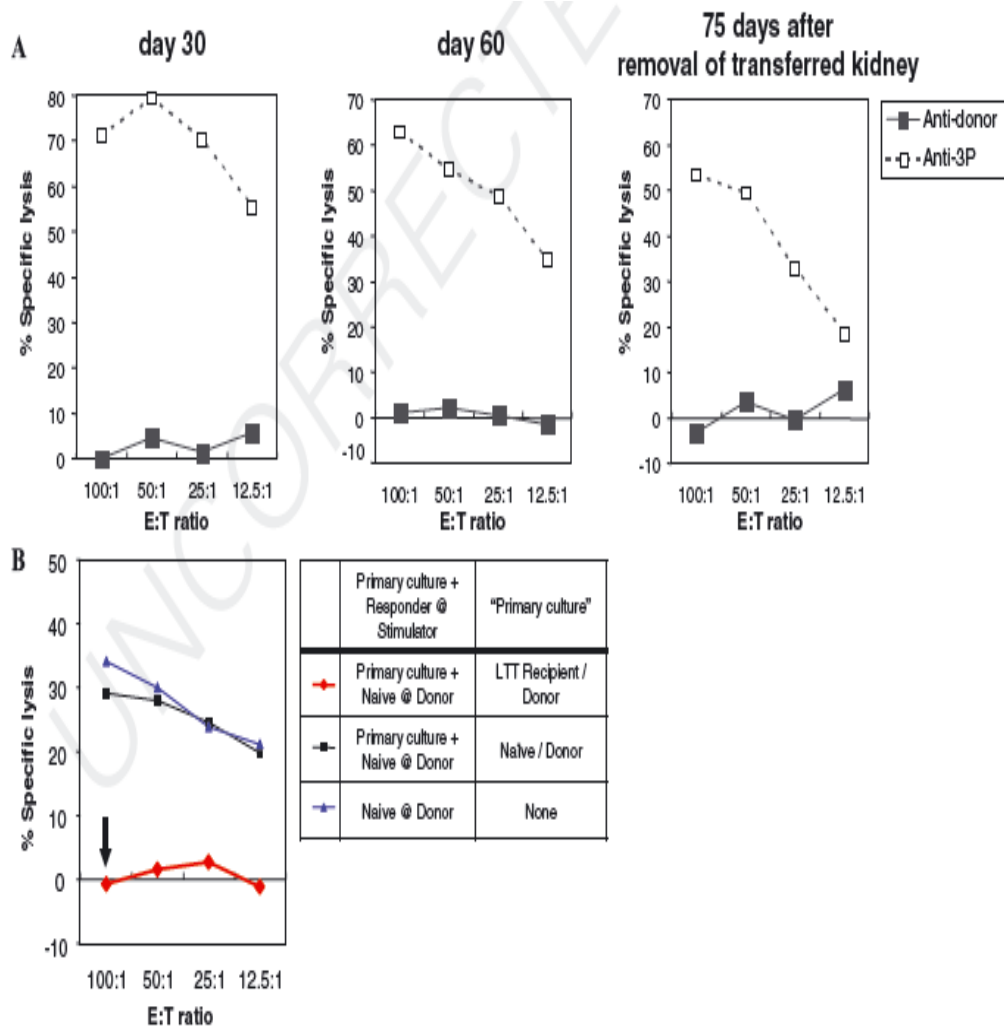
**Figure 5: Histological analysis of renal allografts.** Biopsy specimens on day 22 from the 73-day survivor in Group C showed severe rejection in the kidney from a naïve animal (A, H&E x100), but only minimal cell infiltrate in the kidney from an LTT animal (B, PAS x100). Day 28 biopsy specimens from the 28-day survivor in Group E showed severe diffuse interstitial hemorrhage and cellular infiltrate in the kidney from a naïve animal (C, H&E x100) and glomerular changes but much less interstitial hemorrhage in the kidney graft from an LTT animal (D, H&E x100). Representative histological findings on day 60 from long-term acceptors in Group E showed minimal mononuclear cell infiltrate without chronic vasculopathy in the kidney grafts from both the naïve animal (E, H&E x200) and the LTT animal (F, H&E x200). Biopsies of the naïve graft in long-term acceptors in Group E 21 days after the removal of the tolerated graft showed minimal cell infiltrates with no evidence of glomerulitis or vasculitis (G, H&E x200).

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**Figure 6: Experimental protocol for Group E.** The adoptive transfer donors (LTT animals) received 10 mL DST/kg body weight 1 week before leukapheresis. The adoptive transfer recipients were treated with 150 rads WBI and given  $2.5 \times 10^9$  PBMCs/kg (recipient body weight) from the adoptive transfer donor 1 day before kidney transplantation. The recipient then received both a class I mismatched kidney from a naïve SLA<sup>ss</sup> pig and a long-term tolerated kidney allograft (SLA<sup>ss</sup>) from the adoptive transfer donor without further immunosuppression. In order to assess graft function of SLA<sup>ss</sup> kidneys grafts from naïve animals, two of three animals underwent graftectomy of the tolerant grafts from LTT donors on days 76 and 107, respectively.

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**Figure 7: Representative results of CML and co-culture CML assays for the long-term acceptor animals in Group E.** (A) CML assays 30 and 60 days after primary transplant, as well as 75 days after removal of the LTT kidney showed donor-specific unresponsiveness. Recipient responses to donor stimulation are shown with solid lines and boxes, while responses to third party stimulation are shown with dotted lines and striped boxes. (B) Coculture assay performed at 126 days showed that recipient PBMCs inhibited naïve CTL responses against donor antigens nearly 100% (red line with an arrow) when primed with donor antigen.

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## DISCUSSION

Tregs are vital in maintaining immunological balance. The two main kinds of Tregs are the natural Tregs (nTreg) that are thymus derived and the inducible Tregs (iTreg) that are derived from naïve T cells in the periphery in a tolerogenic environment. The iTregs must be stimulated by donor antigen to expand. Both iTregs and nTregs are CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> and play a role in tolerance. Foxp3 is the transcription factor that determines what happens to Tregs and must be expressed for Tregs to exert their regulatory effects. The environment and the types of signals that a T cell receives will determine its lineage (Burrell, Nakayama, Xu, Brinkman, & Bromberg, 2012).

Importantly, Tregs have been found to play important roles in the induction and maintenance of tolerance. The presence of Tregs has been documented in kidney, cardiac, and skin grafts, as well as in the periphery. Tregs have been shown to suppress helper T cell and effector T cell function as well as modulate APCs so they can not present graft antigen and endothelial cell status that can trigger inflammation (Burrell et al., 2012). Tregs have been shown to be regulatory by generating cytokines like IL-10. Nevertheless, a great deal of work remains to be done to fully understand the role of Tregs in tolerance.

We based our study on the fact that Tregs have been shown to suppress rejection of anti-donor cytotoxic T cells *in vitro* (Ierino et al., 1999; Ierino et al., 1998). Our study has displayed the importance of Tregs that are present in the peripheral blood and part of the infiltrate of kidney grafts. We were able to induce systemic tolerance of naïve class I mismatched kidneys by adoptive transfer of donor primed PBMCs and long-term tolerant

kidneys using only 150 rads of WBI on recipient swine. This is the first time adoptive transfer of tolerance has been induced and maintained in large animals to our understanding.

In our study, through CML and co-culture assays, we showed the beneficial effects of donor-specific transfusion in LTT animals by increasing the suppressive effects of the Tregs. We used DST to increase Tregs in our adoptive transfer donors. One of three animals in group C which received a kidney from a LTT donor that was DST primed survived for 73 days, compared to the longest survival in Group D of 46 days without DST priming. The rest of the animals rejected, thus showing a minimal effect of DST alone. Future studies need to study the effects of DST *in vivo*. The effect of DST could potentially be the result of Treg expansion. The *in vivo* expansion of Tregs would represent an enormous breakthrough, as these cells could be used as part of a cell therapy treatment for the induction of tolerance.

Kidney grafts that were transplanted from tolerant animals seem to play a role in inducing infectious tolerance, where Tregs create a regulatory environment where they promote expansion of new Tregs that are donor-antigen specific (Burrell et al., 2012). In the 73 day Group C survivor and 46 day Group D survivor there was less cell infiltrate or vasculitis in the transplanted graft as compared to the naïve graft. The apparent protection of the tolerant kidney may be due to passenger graft infiltrating cells, immunoregulatory renal tubular cells (RTEC's), and less immunogenic donor APCs. Also, the passenger T regulatory cells that have been found in LTT graft may regulate a systemic tolerant response after adoptive transfer (Cobbold et al., 2004; Graca, Cobbold,

& Waldmann, 2002). In addition, the chemokine/cytokine milieu of LTT kidneys allows for the expansion of Tregs. Consistent with this, Foxp3 has been seen to be up-regulated in tolerated allografts (Lee et al., 2005). In addition, IL-10 has been documented in the milieu and may also play a role in creating a protective environment (Blanco et al., 1995).

Naïve grafts have a large number of donor MHC-type dendritic cells, while LTT animals have few or no donor-type dendritic cells. Understanding the role these APCs play could help distinguish if the protective aspect in LTT kidneys was due to the absence of the direct pathway of antigen presentation. At the same time, tolerogenic dendritic cells have been shown to be capable of inducing donor-specific T-regs. In addition, a positive feedback loop has been seen where these donor-specific T-regs are able to go back and induce the tolerogenic dendritic cells. This may play a role in maintaining tolerance as the graft consistently provides antigen to the periphery in order to amplify and keep T-reg suppressive function (Burrell et al., 2012). Future studies are needed to examine the role of these dendritic cells in transplantation and determine how they in turn interact with T-regs.

While donor-specific transfusions and PBMC infusion from LTT animals did not have a great effect on inducing tolerance, transfer of DST primed PBMC and kidneys from LTT animals successfully induced tolerance of naïve class I mismatched kidneys. Thus, we believe that the cells from the periphery and in the kidney infiltrate played an important role in maintaining tolerance. As with most processes in the human body, the peripheral and central components work together. Maintaining tolerance needs

interactions between the kidney graft and the periphery. We believe that the transferred Tregs travel to the LTT kidney where they undergo an increase in potency and/or expansion in number caused by antigens from the donor renal graft. To translate our findings to the clinic, we must understand the characteristics of the infiltrate in tolerated kidney grafts and investigate the complex interactions that are occurring between the tolerated kidney and the periphery. Understanding the mechanisms and timing of the graft infiltrating Tregs and peripheral Tregs may allow for tolerance induction without a long-term tolerant kidney.

In tolerance it appears that nTregs can go directly to the graft as they are already present, while iTregs must first be produced in the periphery graft (Burrell et al., 2012). Thus, nTregs may play a greater role during the induction phase of tolerance, while iTregs are important in the maintenance phase of tolerance. As more is understood about these subtypes, therapies to facilitate the expansion of one subtype may be the answer to inducing or maintaining tolerance.

Some new studies in transplantation include the use of a new FDA approved immunosuppressive drug belatacept and study of potential biomarkers that can enhance transplantation tolerance (Burrell et al., 2012). Some of the latest approaches that have used T-regs include transfection of activated CD4 cells with Foxp3 to give Treg status, using regulatory cytokines to induce Tregs, treating patients with immunosuppressive drugs that do not suppress Tregs, and treating patients with drugs that will stimulate nTregs (Burrell et al., 2012). We believe that by understanding the roles of Tregs and being able to manipulate them further, therapies that use Tregs may enable us to utilize

the suppressive nature of Tregs to induce and maintain tolerance. Manipulating Tregs in therapeutic manners to suppress rejection will require a fine balance to exploit the beneficial effects of Tregs suppressive effects to induce and maintain tolerance, while not affecting the rest of the normal immunological responses. In conclusion, the proof that Tregs can be harnessed to induce and maintain tolerance through adoptive transfer will require further understanding of the mechanisms and functions of these cells.

## LIST OF JOURNAL ABBREVIATIONS

Am J Surg	The American Journal of Surgery
Am J Transplant	American Journal of Transplantation
Arch Surg	Archives of Surgery
Clin Pharmacol Ther	Clinical Pharmacology and Therapeutics
Curr Opin Organ Transplant	Current Opinion in Organ Transplantation
Expert Opin Biol Ther	Expert Opinion on Biological Therapy
J Am Soc Nephrol	Journal of the American Society of Nephrology
J Clin Invest	The Journal of Clinical Investigation
J Exp Med	The Journal of Experimental Medicine
J Immunol	The Journal of Immunology
J Intern Med	Journal of Internal Medicine
Kidney Int	Kidney International
N Engl J Med	The New England Journal of Medicine
Pharmgenomics Pers Med	Journal of Pharmacogenomics and Personalized Medicine
Proc Natl Acad Sci U S A	Proceedings of the National Academy of the Sciences of the United States of America
Semin Immunol	Seminars in Immunology

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