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# Mechanisms of Tolerance: Role of the Thymus and Persistence of Antigen in Calcineurin-Induced Tolerance of Renal Allografts in MGH Miniature Swine

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## 1. Introduction

The induction of donor-specific tolerance remains a major goal of clinical transplantation. Partially inbred MGH miniature swine, in which swine leukocyte antigens (SLA) have been defined and fixed, have been utilized extensively as a preclinical model for tolerance induction. This preclinical large-animal model is an invaluable tool for studying the mechanism of transplantation tolerance. Recently, we have investigated the role of the persistence of donor antigen in the maintenance of tolerance and the peripheral regulatory cells' ability to confer tolerance to naïve animals.

Mechanisms of tolerance can be elucidated in part by attempting to interfere with (or "break") the tolerant state. We have previously reported that a short course of calineurin inhibition permits the uniform development of long-term, donor-specific tolerance to renal allografts in juvenile miniature swine. Once tolerant, animals that undergo graft nephrectomy and immediate retransplantation accept donor-MHC matched kidneys while maintaining stable renal function without further immunosuppression.

Utilizing this model we have attempted to break tolerance using several strategies. These have included administration of recombinant IL-2 to provide additional T-cell help, manipulation of the host thymus, and removal of donor grafts. Our data indicate that (1) presence of an intact thymus is essential for the induction, but not for the maintenance of tolerance; (2) the persistence of the donor renal graft is essential for the indefinite continuation of tolerance; and (3) the pathway of donor antigen presentation, in tolerant and previously tolerant animals, is important for preservation or loss of the tolerant state. Although obvious limitations exist with regard to animal studies, our consistently reproducible results using MHC inbred miniature swine provide a unique opportunity to study the mechanisms of transplantation in animals physiologically similar to humans. As we have previously shown, our results are highly suggestive of what will occur in clinical human transplantation protocols.

## 2. Abbreviations

APC: Antigen Presenting Cell
CTL: Cytotoxic T-Lymphocytes
CyA: Cyclosporine A
FoxP3+: Forkhead Box 3
HSC: Hematopoietic Stem Cells
IL-2: Interleukin 2
MGH: Massachusetts General Hospital
MHC: Major Histocompatibility Complex
PBL: Peripheral Blood Lymphocytes
SLA: swine leukocyte antigens
UNOS: United Network for Organ Sharing

## 3. Importance of tolerance

According to UNOS, over 110,000 patients are currently awaiting organ transplantation in the United States. However, in 2010 there were only 14,506 donors. Although the benefits of valued organ transplants are substantial, patients must accept the risks of the required immunosuppression. Immunosuppressive protocols generally include T-cell depletion in the perioperative period, followed by initiation of calcineurin inhibition, mycophenolic acid, and steroids. Many centers have been successful in reducing or eliminating the use of chronic steroids, but patients are still susceptible to the morbidity associated with immunosuppression (1,2).

Transplantation tolerance, or immunologic non-responsiveness to donor antigen, would reduce the morbidity observed with immunosuppression (3). Immunosuppression has been associated with malignancy, infection, end-organ damage, and economic burden. As many as 30% of renal transplant recipients develop skin cancer within 10 years of transplantation (4) similar results have been reported in the cardiac transplant patient population (4,5). Furthermore, viral and bacterial infections are more likely to occur in the immunocompromised patient and although death rates from infection have decreased every decade for the last 30 years, patients at the extremes of age are still considered high-risk for infectious complications (6). End-organ failure is also a potential complication of immunosuppression; renal failure, likely due to calcineurin inhibitors, has been associated with chronic immunosuppression (7,8). Additionally, the lifetime economic cost of immunosuppressive drugs alone following kidney transplantation range from $68,000 to $88,000 depending on the selected regimen (9,10).

Tolerance strategies would potentially alleviate the risks of malignancy, organ-failure, and infectious complications as well as the cost associated with anti-rejection medications. Recent clinical successes in inducing tolerance to kidney (11,12) and liver (13) grafts have proven that tolerance strategies are clinically applicable.

## 4. Antigen presentation of allogeneic antigens and tolerance

Tissue antigens are any proteins which when presented by the major histocompatability complex lead to immunologic responses (14,15). Proof of this MHC restriction earned Zinkernagel and Doherty the Nobel Prize in 1996 for work that was done in the 1970s (15).

In transplantation, these antigens can be presented by either donor or recipient antigen presenting cells (APCs). The presentation of antigen to recipient lymphocytes potentially leads to allosensitization of recipient T-cells. In direct alloantigen presentation, donor tissue antigens are recognized by recipient T-cells in the context of donor MHC molecules. Conversely, indirect antigen presentation occurs when alloantigens are recognized in the setting of recipient MHC. Recently, one group described an additional "semi-direct" mechanism in which the recipient cells acquire the intact MHC from the donor and subsequently present antigen in the context of the newly acquired donor MHC (16).

A comprehensive understanding of antigen presentation is helpful for elucidating both transplantation rejection and tolerance. In allotransplantation, passenger lymphocytes and professional APCs within the transplanted organ present donor antigen to recipient T-cells leading to acute rejection (17,18). Indirect presentation of donor antigen may continue indefinitely and is generally associated with chronic rejection (19). These two pathways, however, are not mutually exclusive as early rejection episodes portend increased risk of future chronic rejection (20). While these alloreactive stimuli are known to play a role in rejection, small and large animal studies of tolerance have also demonstrated the importance of donor antigen presentation in the establishment of a tolerogenic milieu (21-23).

Mechanisms of transplantation tolerance have been broadly categorized as "central" or "peripheral" on the basis of whether T-cells are rendered unresponsive during their maturation in the thymus or after they have left the thymus, respectively (24-26). Deletion is thought to be the mechanism responsible for central tolerance, whereas peripheral tolerance is likely mediated by anergy, suppression, or ignorance (27). Central tolerance can be induced by exposing newly developed T-cells to alloantigens on the progeny of hematopoietic stem cells (HSC) injected either at a very early stage in the development of the immune system, either in utero or in neonates (28,29) or in adult animals following ablation of mature T cells (30-32). This tolerance is deletional and presumably utilizes the same process of negative selection that is responsible for self-tolerance during T-cell maturation in the thymus (29,33). In addition to bone marrow transplantation, another strategy to induce central tolerance is thymic transplantation. We, and others, have demonstrated successful induction of tolerance with donor thymic grafts across allogeneic and xenogeneic barriers in small and large animal models (34-36).

Peripheral tolerance to alloantigens has been induced in many ways, generally by providing alloantigen in a non-stimulatory fashion or at a time when the aggressive alloreactive response has been simultaneously averted. Peripheral tolerance has been ascribed to the same processes as those invoked to explain peripheral tolerance to self – i.e. anergy(37-41), peripheral deletion(42,43), clonal ignorance (32) and regulation (44-49).

Investigation of peripheral cellular suppression began over 40 years ago, though the implications of these findings were not immediately evident. Tada et al. demonstrated that when KLH primed T-lymphocytes were passively transferred to mice prior to immunization with DNP-KLH, antibody formation was inhibited. In contrast, when these cells were transferred after immunization with DNP-KLH, antibody formation was not inhibited. These results demonstrated that a peripheral T-cell response was suppressing immunologic response to immunization (50,51). This phenomenon was studied extensively in the 1970s and 1980s, and Castagnoli et al. found that the supernatant from antigen-specific suppressive thymoma cells was capable of suppressing an alloreactive response to the same antigen. These and other data suggested that the effects of purported T suppressor cells are mediated by one or more soluble factors (52,53). Our understanding of these suppressive

cells was broadened further when Sakaguchi et al. demonstrated that the lack of ostensible suppressor cells in nude/nude mice led to severe autoimmune disease, which could be rectified by inoculation with nude/+ thymocyte suspensions. Sakaguchi and his colleagues then successfully phenotyped these suppressor/regulatory cells as $CD4^{+ve}$, $CD25hi^{+ve}$ (and some years later, FoxP3+) and showed that these cells are likely responsible for tolerance of self, and possibly necessary for the maintenance of peripheral tolerance (47,54).

## 5. Importance of large animal models

While small animal data are valuable for defining mechanisms, large animal studies are imperative for the development of preclinical models (55). Numerous strategies for tolerance induction in rodents have proven fruitful, though few have been successfully translated to humans, non-human primates, or other large animals (55,56). Likely owing to the increased complexity of the immune system in large animals and longer-term exposure to environmental antigens, the results from tolerance induction protocols in large animals have been less successful than rodent studies (55-57). Another possible cause for this difference is that MHC class II expression on endothelial cells that is the first target in the alloreactive response to in solid organs. Murine "resting" endotherial cells do not express MHC class II while swine, whereas primate endothelial cells do (58).

## 6. MHC inbred MGH miniature swine: A unique large animal model to study mechanisms of acceptance/rejection

Miniature swine have been developed in our laboratory over the past thirty years as a model system for studies of transplantation biology. Swine were chosen for this purpose because they represent one of the few large animal species in which breeding characteristics make genetic experiments possible. Swine have a relatively large litter size (3-10 offspring) and a short gestational cycle (3 months). They reach sexual maturity at approximately 6 months of age, and sows have an estrous cycle every 3 weeks. These breeding characteristics have made it possible to develop MHC homozygous lines of miniature swine in a relatively short time and have also made it possible to isolate new MHC recombinants, to breed them to homozygosity, and to carry out short-term backcross experiments in order to identify and study the segregation of genetic characteristics (59). Our miniature swine thus represent the only large animal model in which MHC genetics can be reproducibly controlled. As such, these animals have been particularly useful in assessing the effects of MHC matching on rejection and/or tolerance induction (60,61).

At present, we maintain swine of three homozygous SLA haplotypes, $SLA^a$, $SLA^c$, $SLA^d$ and five lines bearing intra-SLA recombinant haplotypes as illustrated in Fig. 1. All of these lines differ by minor histocompatibility loci, thus providing a model in which most of the transplantation combinations relevant to human transplantation can be mimicked. Thus, for example, transplants within an MHC homozygous herd simulate transplants between HLA identical siblings, while transplants between herds resemble cadaveric or non-matched sibling transplants. Likewise, transplants between pairs of heterozygotes can be chosen to resemble parent into offspring or one-haplotype mismatched sibling transplants (22). In addition, we have chosen one subline of our $SLA^{dd}$ animals for further inbreeding, in order to produce a fully inbred line of miniature swine. This subline reached a coefficient of inbreeding of >96%, leading for the first time to long-term acceptance of reciprocal skin grafts (62). These animals

have also made it possible to carry out adoptive transfer experiments for the first time in a large animal model, as reported here (Scalea et al, manuscript in preparation).

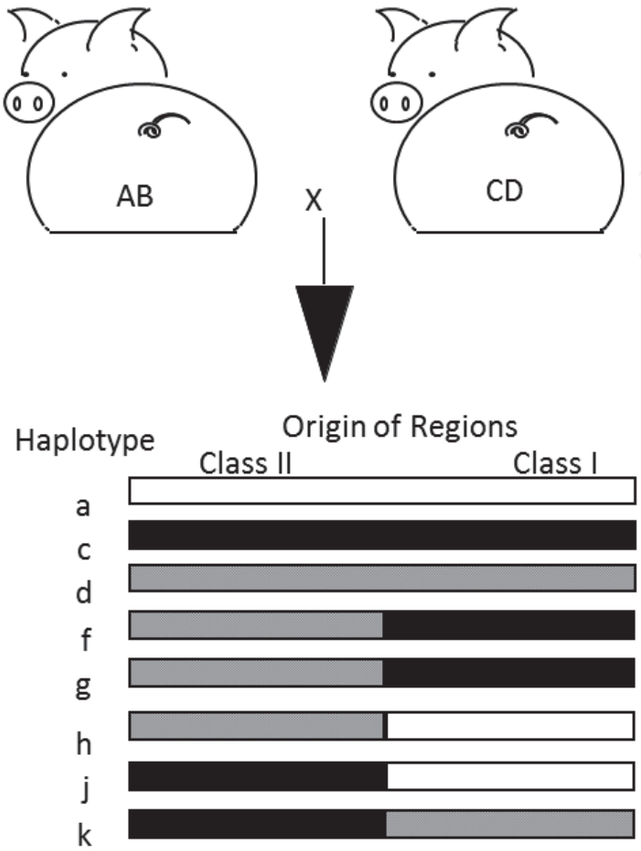


Fig. 1. The serial breeding of MGH swine has led to fixed, defined MHC classes. Transplantation between lines of these swine allows researchers to mimic living-related and cadaveric organ transplantation.

## 7. Thymic-dependent and antigen-dependent tolerance in MHC miniature swine renal transplant models

### 7.1 Induction of tolerance with a short course and high dose calcineurin inhibitor in MGH miniature swine

i. **Effects of CyA on renal allograft survival across a selective MHC barrier:** We have attempted to induce tolerance using pharmacological limitation of T cell help. For this purpose, class I mismatched renal transplants were performed using a short course of treatment with Cyclosporin A (CyA) (63,64). We chose to study the effect of CyA across a selective *two-haplotype class I mismatched, class II matched* barrier, since without immunosuppression such recipients uniformly reject renal allografts within three weeks without immunosuppression (Fig. 2). As reported in our initial study of this treatment regimen, a twelve-day course of CyA (10-13 mg/kg/day) induced long-term, specific tolerance in eight of eight *two-haplotype class II matched, class I mismatched* recipients(64). This result has been reproduced subsequently in more than fifty additional CyA-treated

animals, 100% of which develop long-term tolerance across a class I disparity. It is important to note that although this dose and the resulting blood levels (400-800 ng/dl) are high with respect to clinically acceptable values, the toxicity caused by such levels clinically is generally reversible if discontinued after a two- week course (65).

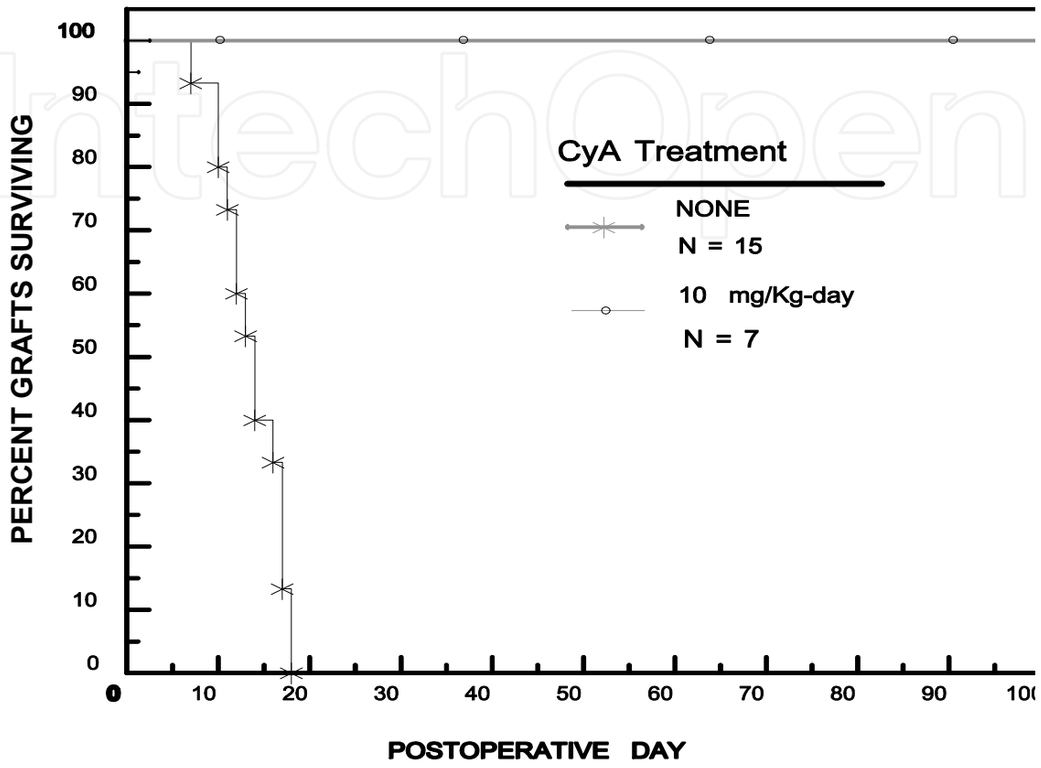


Fig. 2. With a short-course of high-dose Cyclosporine A, tolerance is uniformly established across an MHC class-I mismatch in MGH miniature swine (*n*>50).

ii. **Effects of CyA on renal allograft survival across other selective MHC barriers:** We have also studied whether tolerance is induced with a 12-day course of CyA therapy across further immunologic disparities. Although the CyA regimen was capable of prolonging renal allograft survival across a full MHC barrier, it did not induce long-term tolerance in any of the animals tested (64). However, since pharmacologic help limitation by CyA should be possible regardless of the MHC disparity, we also tested the effect of the CyA regimen on renal transplants selectively mismatched for class II or for one full haplotype (66,67). Of seven *CyA-treated class I matched, class II mismatched* kidney transplants, five (71%) were accepted long-term, whereas two rejected on days 20 and 39, respectively (68). Similarly, of six CyA-treated recipients of single haplotype mismatched kidney allografts, two rejected (on days 31 and 37), while four (67%) accepted long-term. Thus, tolerance was possible across both class II and single haplotype full mismatches, but with a lower success rate and with a less stable clinical course than across a selective class I mismatch.

iii. **Effects of Tacrolimus on renal allograft survival across other selective MHC barriers** According to the latter hypothesis, increasingly potent immunosuppression of T-cell help would be required for increasing the extent of the MHC disparity (i.e. class I < class II < one full haplotype < two full haplotypes). Our subsequent results using

Tacrolimus corroborate this finding (69). We have demonstrated that Tacrolimus at blood levels between 35 and 80ng/ml facilitates tolerance induction not only across a class I barrier, but also across a two-haplotype full MHC barrier in-vivo (69). However, like CyA-induced tolerance across class I mismatched barrier, thymic-dependent mechanisms are involved in this Tacrolimus-induced tolerance (70). Tolerance was only induced in juvenile hosts (see more details blow).

### 7.2 Mechanisms of tolerance using high-dose calcineurin inhibitors

To understand better the mechanisms of tolerance induction, both the role of thymus (i.e. aging) and persistence of donor antigens in tolerance induction/maintenance have been studied.

i. *Thymic dependent tolerance:* A series of experiments were performed using aged and thymectomized animals. The data from these studies demonstrated that when aged animals underwent class-I mismatched kidney transplantation followed by 12-days of CyA, tolerance could not be induced. Similarly, thymectomy prior to kidney transplantation interfered with the development of tolerance across the same class-I barrier (71). Interestingly, thymectomy in a maintenance period (beyond 6 weeks after transplantation) did not abrogate tolerance (70). Recent work from this laboratory has also demonstrated thymic dependent mechanisms play important role in tacrolimus induced tolerance in both miniature swine and nonhuman primates (Yamada et al manuscript in preparation). These studies indicated that that 1) tolerance induction is thymus-dependent, whereas tolerance-maintenance is not and 2) an interaction between central and peripheral mechanisms of tolerance is likely occurring in the stably tolerant animal.

*ii. Regulatory mechanisms and stability of tolerance – Class I mismatched kidney transplantation with a 12-day course of CyA:*

a. In vitro assays suggesting involvement of regulatory mechanisms

Because thymectomy post-transplantation did not lead to the abrogation of tolerance, investigators postulated that a suppressive peripheral regulatory mechanism had developed in class I mismatched tolerant model. When peripheral blood lymphocytes (PBL) from long-term tolerant animals were stimulated with donor PBLs, and re-cultured with naïve SLA matched PBLs plus either donor-PBLs or 3rd party PBLs, we observed suppression of the naïve-SLA anti-donor CTL response and maintenance of naïve-SLA anti-3rd party CTL responses (45). This co-culture assay demonstrated in-vitro that a peripheral cellular mechanism of suppression was present. To study activation and effector function of these purported regulatory cells, we performed co-culture assays in which PBLs taken from a tolerant animal were placed in a transwell culture system such that they were separated from donor PBLs by a permeable membrane (69). In previous co-culture CTL assays performed without the membrane barrier, we observed that naïve-recipient SLA matched PBLs were inhibited by the presence of PBLs taken from a tolerant animal. However, when the cells were separated by a permeable membrane, the cells were no longer suppressive (69,72). This demonstrated that 1) direct cell-to-cell contact is required for activation of the peripheral regulatory mechanism and 2) soluble factors produced by the peripheral regulatory cells are themselves incapable of regulatory effector activation in this class I mismatched kidney model.

b. Exogenous T-cell help interfered with the induction of tolerance but not maintenance of tolerance

Based on these in-vivo and in-vitro data, we attempted to further define the role of regulatory cells in the MGH miniature swine model, by attempting to abrogate tolerance in

animals. Because a lack or T cell help likely plays an essential role in calcineurin-induced tolerance, we administered exogenous IL-2 either 1) during the induction period (Days 8. 9 and 10) or 2) once animals were long-term tolerant (73). Much like our thymectomy experiments, we found that whereas IL-2 administration can prohibit the induction of tolerance if administered perioperatively, treatment with exogenous IL-2 failed to abrogate tolerance in long-term tolerant (LTT) animals (73). To further distill the role of T-cell help required for the anti-donor cellular response in tolerant animals, we challenged LTT animals with skin grafts from class-I donor/class-II third party donors instead of IL-2 (74). We reasoned that the class-II disparate graft may be capable of providing the necessary T-cell help by stimulating the alloreactive CD4+ population. Although recipient animals experienced a brief rejection crisis following skin grafting, they remained tolerant in the long-term (74). Thus, once established, the peripheral mechanism of tolerance is steadily stable, and capable of suppressing further stimulation with donor antigen.

c. Role of graft in maintenance of tolerance

Because 1) removal of the thymus in a tolerant animal did not lead to tolerance abrogation and 2) based on the in-vitro data suggesting that tolerance was mediated by an active cellular process, we next questioned if the graft itself was providing the tolerogenic stimulus for maintenance of tolerance. To test this hypothesis, we designed several experiments in which the tolerated graft was removed and replaced by a donor-MHC matched graft (21) (75). In the first experiment, long-term tolerant animals underwent graft nephrectomy and immediate retransplantation with a donor-MHC matched graft. As previously published, each animal uniformly accepted the retransplanted graft and never experienced rejection. In the next experiments, we introduced a period of "absence-of-donor-antigen" by removing the tolerated kidney from LTT animals and replacing it with a self-MHC matched graft to support the life of the animal. Then, at 1 and 3 months, animals were retransplanted with a donor-MHC matched graft. When animals bearing self-MHC matched grafts underwent retransplantation from actual donors immediately after primary graft nephrectomy, all animal accepted kidney with stable renal function(21). However, we observed a brief rejection crisis followed by uniform acceptance when second kidneys were transplanted at one month after the primary graft nephrectomy. Moreover, as the period of absence-of-donor-antigen was increased to 3 months, retransplantation was followed by significant rejection crisis in two of three animals. One animal completely rejected the retransplanted graft within 2 months and the other had severe rejection episodes (21). Furthermore, when skin grafts from class-I donor/class-II third party donors were transplanted onto animals during absence of donor kidneys, second kidneys transplanted 3 months after primary kidney nephrectomy were uniformly rejected in an accelerated manner (<7 days), indicating a "broken tolerance" (Yamada et al., manuscript in preparation). This series of experiments confirmed that the kidney plays an essential role in the maintenance of tolerance. Because we observed rejection of the retransplanted donor-MHC matched graft following periods without donor antigen present, it is likely that the kidney provides a constant tolerogenic stimulus via a peripheral mechanism which is required for maitenence of T-regulatory cells.

d. Current evidence for the role of T-regulatory cells in peripheral tolerance

The previous experiment clarified our understanding of the role that persistence of donor antigen plays in the maintenance of tolerance and provided indirect evidence that this process was mediated by T-regulatory cells (21,45). We then postulated that, if T-regulatory cells were responsible for maintaining tolerance then removal of T-regulatory cells from a LTT animal, may lead to abrogation. Thus, we next attempted to hasten the onset of

rejection by performing an extensive leukapheresis immediately prior to retransplantation of a donor-matched graft following a three month period of absence-of-donor-antigen. In eight of ten animals in this protocol, we observed rejection of a subsequently retransplanted donor-MHC matched graft, and complete or chronic rejection in four of ten animals (Scalea et al, manuscript being submitted). When the leukapheresed cells were evaluated in vitro, we observed that they were capable of suppressing a naïve anti-donor response in a cell mediated lympholysis (CTL) co-culture assay in a donor-specific manner (Scalea et al, manuscript in preparation).

Having determined that these cells were capable of suppressing a naïve response in-vitro (45), we then questioned whether they would be suppressive in-vivo. However, based on our absence-of-donor-antigen and leukapheresis with retransplantation experiments, it appeared that both the kidney and peripheral regulatory cells were important for the continuation of tolerance (21). To resolve the contribution of the circulating cellular compartment versus the kidney itself, we adoptively transferred leukapheresed cells from tolerant donors that were from our most highly-inbred line of miniature swine. We found that when either the LTT kidney was transplanted into a naïve animal, or leukapheresed cells were transferred along with a naïve donor graft, there was prolonged graft survival, but not long-term acceptance (Scalea et al, manuscript in preparation). Conversely, when the leukapheresed cells and kidney were transferred from the same LTT animal, the naïve recipient experienced long-term graft survival. Additionally, these recipients were unresponsive to donor in CML assay as early as day 28, and in one case, as late as 150 days following transplantation (Scalea et al, manuscript being submitted).

## 8. Summary

In summary, our understanding of the mechanisms of tolerance has blossomed over the last several decades. It is clear that while small animal data has helped elucidate the molecular basis for tolerance, large animal studies are required for the development of preclinical models. While animal models have some limitations, our unique model allows us to model accurately the clinical scenarios of clinical transplantation. Using this model, we have demonstrated the importance of the thymus for tolerance induction and the persistence of donor antigen for the maintenance of tolerance. Recent work in our laboratory has demonstrated that stable peripheral tolerance is mediated by a cellular mechanism that is best explained by the presence of T-regulatory cells.

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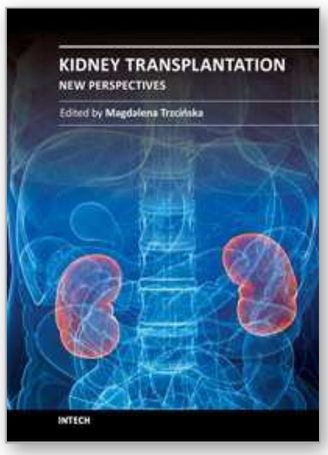


**Kidney Transplantation - New Perspectives**

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Although many years have passed since the first successful kidney transplantation, the method, although no longer considered a medical experiment, is still perceived as controversial and, as such, it triggers many emotions and thatâ€™s why conscious educational efforts are still needed for kidney transplantation, for many people being the only chance for an active lifestyle and improved quality of life, to win common social acceptance and stop triggering negative connotations. Apart from transplantation controversies piling up over years transplantologists also have to face many other medical difficulties. The chapters selected for this book are of high level of content, and the fact that their authors come from many different countries, and sometimes even cultures, has facilitated a comprehensive and interesting approach to the problem of kidney transplantation. The authors cover a wide spectrum of transplant-related topics.

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