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A potential tolerogeneic role of skin grafts in previously tolerant animals

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A POTENTIAL TOLEROGENEIC ROLE OF SKIN GRAFTS IN PREVIOUSLY TOLERANT ANIMALS

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We have previously shown that long-term tolerance to class I disparate renal allografts in miniature swine is induced by a short course of Cyclosporine A (CyA). In these tolerant animals (TOL), the tolerance has been shown to involve T regulatory cells (Treg) and to persist for 3 to 4 months after the graft is removed. Naïve animals can be sensitized to major histocompatibility complex (MHC) class I mismatched renal allografts by inoculation with either class I peptide or by a donor-type skin graft. Six weeks after graftectomy, peptide immunization similarly sensitized TOL swine, but challenge with donor skin failed to sensitize (n=3). In this study, we further investigated the tolerogenicity of skin grafts under these conditions by challenging simultaneously with peptide and skin graft.

Miniature swine underwent bilateral nephrectomy and MHC class I mismatched renal transplantation with a 12-day course of CyA to induce tolerance. 100 days after transplantation, graftectomy was performed and recipient-matched kidneys transplanted.

Six weeks later, pigs were simultaneously challenged with donor-type class I peptide and donor-type skin grafts. The effect on *in vitro* and *in vivo* immunity was determined.

In contrast to animals treated only with peptide, all of which were sensitized to 2nd renal allografts (rejection in 4-6 days) and developed strong anti-donor cellular and antibody responses (n=3), 2/5 recipients showed only a transient anti-donor cellular response and no or little anti-donor antibody production and maintained their second donor-type class I mismatched renal allografts long-term with normal creatinine. An additional animal experienced prolonged survival (11 days), and the final 2 animals rejected within 5-7 days. Challenging with second donor-type skin grafts and third party skin graft indicated that hyporesponsiveness to the donor was specific.

In animals tolerant of a class I mismatched renal allograft, which would be expected to be sensitized by class I peptide (indirect pathway of sensitization) at 6 weeks after graftectomy, a simultaneous donor-matched skin graft appeared to prevent sensitization in 2 of 5 and prolong survival in a third. These data are consistent with expansion of Treg following a class I mismatched skin graft, presumably by the direct pathway of activation.

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I. Introduction

Background: The Importance of Tolerance

Despite the enormous progress which immunosuppressive drugs have permitted in the field of transplantation, there are also major drawbacks to these drugs. All such agents cause nonspecific suppression of the immune system, which must be balanced to avoid rejection but not completely eliminate immune function. Patients must then stay on chronic immunosuppressive therapy for the remainder of their lives, facing the major complications of too much or too little immunosuppression, including infection and rejection respectively as well as post-transplant lymph proliferative disorders (PTLD), direct toxicity of immunosuppressant medications, diabetes, and other metabolic derangements.

Therefore, the induction of tolerance remains a major goal of transplantation immunology. Tolerance has various definitions. The most basic definition of tolerance is acceptance of a graft without immunosuppression, and "immune tolerance" denotes donor-specific hyporesponsiveness *in vitro*. Tolerance may be local, meaning that only the specific organ is accepted while other donor-type organs would be rejected, or systemic, meaning that all donor-type organs would be accepted (1). In our laboratory, the tolerance we seek encompasses 1) long-term acceptance of the donor graft with stable graft function without continuing immunosuppression, 2) *in vitro* evidence of donor-specific hyporesponsiveness, and 3) an otherwise competent immune system that remains capable of responding to third-party antigens.

There are four major reasons why scientists aim to reach tolerance in the clinical setting. The first, as mentioned above, is that immunosuppressant's have several drawbacks, including potentially severe toxicity as well as a relatively high treatment failure rate, meaning that organs are sometimes rejected despite treatment. Even when acute rejection episodes resolve, repeated episodes may eventually cause chronic allograft failure.

The second reason is the continuing problem of chronic rejection. As improved induction regimens have prolonged graft survival by preventing acute rejection, thereby achieving long-term acceptance, it has become increasingly clear that chronic rejection remains as a challenge to long-term graft survival. Chronic rejection is poorly understood but is thought to result from several factors, which include non-immunologic vascular inflammatory processes, innate immunity, and antibody-mediated damage to fibrointimal layers of graft vasculature (2). It has been shown to be responsible for late rejection of grafts that have avoided acute rejection in both animal models (3, 4) and in the clinic (5-7). Because of the immunologic component, chronic rejection is less likely to occur if tolerance has been induced.

The third reason is the growing demand for organs in comparison to the relative shortage in supply. By avoid rejection, tolerance will decrease the need for replacement organs, which would be especially difficult to find for these highly sensitized patients (8). Tolerance would also overcome the especially large immunologic hurdles that currently prevent alternative sources of organs, such as xenotransplantation.

Fourth, the induction of systemic tolerance would allow genetically identical organs to be interchanged. This has obvious implications for the future if organ cloning, tissue engineering, or xenotransplantation from an inbred herd become clinically applicable. Regarding the latter, as the thymus is a major component of some current experimental xenotransplantation regimens (9) and the juvenile thymus is more effective for tolerance induction (10), this would allow a thymus to be taken from a juvenile donor and an identical organ from a more size-appropriate donor.

For these reasons, achieving tolerance and understanding its underlying mechanisms has long been a goal of transplantation immunology, and it is the driving force behind this project.

The Animal Model

Studies in mice and rats have been responsible for much of the progress which has been made in the past few decades in understanding the biology and immunology of transplantation. Unfortunately, however, despite numerous demonstrations of transplantation tolerance in rodent models, there have been few examples in which comparable protocols for the induction of tolerance have been successful in large animal models or in patients. In fact, most protocols that are successful in rodents, such as those involving mixed chimerism, have yet to achieve reliable success in large animal models (11). There are also notable differences between rodent and large animal immune systems such as the inability to fully deplete T cells in large animals (11) and the lack of constitutive

expression of major histocompatibility complex (MHC) class II on the vascular endothelium of rodents (12). The lack of antigen presentation by donor endothelium obviously affects the immunogenicity of rodent grafts in a way that does not occur in large animal models.

It is therefore clear that large animal models are desirable for determining the potential clinical applicability of tolerance-inducing protocols. Miniature swine currently provide the only large animal model available in which selective matching for class I and/or class II antigens can be reproducibly performed and are therefore of particular significance for examining the role of these antigens in tolerance induction as well as in understanding the mechanism of the tolerance induced. Inbred miniature swine provide a unique opportunity to study transplantation immunity in genetically defined large animals, and our herd of partially inbred Massachusetts General Hospital (MGH) miniature swine, in which swine leukocyte antigens (SLA) have been well defined, has been utilized extensively as a preclinical model for tolerance induction (note: SLA is the swine equivalent of human leukocyte antigen (HLA)) (13, 14). It is hoped that studying the tolerance observed in this system may reveal new approaches for the induction of tolerance in clinical situations.

MGH Miniature Swine

Miniature swine have been developed over the past thirty-five 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 (13, 15). At present, there are three homozygous SLA haplotypes: SLA^{a}, SLA^{c}, and SLA^{d}. There are also five lines bearing intra-SLA recombinant haplotypes as illustrated in Figure 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. 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. In addition, one subline of SLA^{dd} animals has been chosen for further inbreeding in order to produce a fully inbred line of miniature swine (coefficient of inbreeding >94%). These animals demonstrate long-term acceptance of all reciprocal skin and organ grafts without immunosuppression (14).

Another advantage of these swine is that, for several reasons, they may be the most suitable donors of xenografts for humans, largely because of their availability, but also because of their favorable breeding characteristics and the similarity of many of their organ systems to those of humans. These animals have a variety of properties that make them highly suitable as potential donors of xenogeneic tissues and organs: a) Size: These animals achieve adult weights of approximately 120 - 140 kilograms, similar to humans; b) Physiology: Many organ systems of swine have been shown to be highly similar physiologically to their human counterparts, including the skin, the cardiovascular system, renal function, pulmonary function, and the digestive system (16); c) Breeding

Characteristics: Like their domestic counterparts, miniature swine have very favorable breeding characteristics for the production of donor animals. In fact, swine are one of the few large animal species in which it is possible to carry out a genetic breeding program. Swine have large litter sizes (5 - 10 offspring), early sexual maturity (5 months), short gestation time (114 days), and frequent estrus cycles (every three weeks); d) Potential for Genetic Engineering: Because of these breeding characteristics, it is possible to incorporate any number of transgenes into a line of miniature swine designed to be appropriate as a donor species. The most important application of genetic engineering to pig donors has been the knockout of Gal expression through nuclear transfer (17-19), which has produced the "GalT-KO" animals (see below).

Tolerance of Renal Allografts and the "Basic Model"

By performing transplants between animals with known haplotypes using the MGH miniature swine herd, it has been possible to create models of tolerance induction and to gain understanding of the mechanisms behind this tolerance. Initial experiments explored the outcomes of transplantation between animals that were either matched for both MHC class I and II or were MHC-identical except for either class I or class II.

The Role of "Minor" Antigens

When renal transplants were carried out without exogenous immunosuppression between fully MHC-matched animals, recipients developed specific and long-term (>100 days) transplantation tolerance to these grafts in approximately 1/3 of cases (n=112) (20, 21). Further study showed that, as these animals were fixed and matched for both MHC loci,

rejection was due to non-MHC antigens, otherwise known as “minor antigens” (22). By selectively breeding acceptor animals for several generations, resulting generations have become more closely matched at minor antigen loci, with the result that spontaneous acceptance of fully MHC-matched organs has increased from 27.3% to 64.5% (23). This rate of rejection in 1/3 of cases is not only instructive as to the immunogeneic effect of minor antigens, even in otherwise identical animals, but it also affects the project discussed in this thesis since an important step in our model involves transplantation of a recipient-matched kidney without immunosuppression.

The role of minor antigen in the rejection of fully MHC-matched organs is further instructive in comparison to its effect in other circumstances. As will be described in detail below, we have developed a protocol by which tolerance can be reliably induced to renal grafts mismatched for MHC class I. As will also be described in detail below, in animals that have become tolerant to these class I mismatched grafts, the original graft can be removed and replaced by an organ with the same MHC genotype with a 0% rejection rate despite the total lack of immunosuppression. As these replacement organs differ from the original grafts in their minor antigens but are never rejected, an interesting question, therefore, is why minor antigen differences are responsible for rejection of up to 1/3 of fully MHC-matched organs (in which there is natural self-tolerance to the MHC antigens) but never of MHC-mismatched organs in the scenario above (in which there is induced tolerance to the MHC class I antigen). The explanation for this finding illustrates the different mechanisms responsible for self-tolerance versus induced tolerance. In brief, T cells with specificity for self-MHC do not exist due to negative selection in the thymus

(central deletional tolerance), whereas tolerance of MHC-disparate donors involves peripheral mechanisms as well, especially via the development of donor-specific regulatory T cells (Tregs). It has been shown in both rodent and large animal models that in animals made tolerant to a particular antigen, Tregs are able to suppress a response to a third-party antigen coexpressed with the tolerated antigen (24-27), a process known as "linked suppression." Similarly, in the presence of Tregs specific for donor MHC, as occurs in our model of induced tolerance to donor MHC class I, the coexpression of minor antigens would not cause rejection. The mechanisms involved in central and peripheral tolerance and the specific role of Tregs in peripheral tolerance will be further discussed below.

Transplantation Across a Class I Mismatch

When renal grafts differed only for one haplotype at class I (e.g., transplantation of SLA^{ag} organs into SLA^{ad} recipients) and were matched for class II, the rate of long-term acceptance after transplantation without immunosuppression in 128 animals was 30%, the same as for fully-matched grafts (20, 23). Moreover, those that did accept their kidneys did so after experiencing a severe rejection crisis between postoperative weeks 2-4, with the development of cytotoxic immunoglobulin (Ig) M, but not IgG, directed against donor class I. Despite this reaction, the kidneys were accepted with long-term normal renal function, and subsequent donor-matched skin grafts survived for an extended period of time compared to either third-party grafts or donor-matched grafts placed on naïve animals (20). This could indicate that an active immunologic process is

responsible for tolerance in this model, and that this process does not produce sufficient T cell help to cause rejection.

The development of tolerance following an immunologic reaction involving deficient T cell help led to trials in which T cell help was pharmacologically limited via calcineurin inhibitors after transplantation. When kidneys were transplanted without immunosuppression across a two-haplotype class I mismatch barrier, in which class II was identical, they were uniformly rejected within 3 weeks. In contrast, juvenile miniature swine (aged 3 to 8 months) that received a short course (12 days) of Cyclosporine A (CyA) uniformly experienced long-term tolerance (LTT) specific to the donor (n=8) (28, 29), and this result has subsequently been repeated in hundreds of animals without rejection. This is referred to as our "Basic Model." Cyclosporine must be given at a high dose (target range 400-800 ng/dl) which, while high enough to have toxicity in animals and humans, is tolerable with reversible effects if given in a short course (30, 31).

Transplantation Across a Class II Mismatch

As opposed to class I mismatched grafts, all class II mismatched grafts were rejected without immunosuppression (20). However, 5 of 7 animals achieved LTT to these grafts after receiving a short course of high-dose CyA (32).

Transplantation Across a Full MHC Mismatch

As with class II mismatched grafts, all fully MHC-mismatched renal grafts were rejected without immunosuppression. A short course of high-dose CyA prolonged survival but did not lead to LTT (28), although it was subsequently found that all animals could be made LTT by a short course (12 days) of high-dose (35-80 ng/ml) tacrolimus (FK506) (33).

Potential Mechanisms of Transplantation Tolerance

Mechanisms of transplantation tolerance have been broadly categorized as "central" or "peripheral" on the basis of whether donor-specific T cells are rendered unresponsive or deleted during their maturation in the thymus or after they have left the thymus, respectively.

Central Tolerance

As mentioned above, central tolerance is the process via which the bulk of self-tolerance is maintained and involves deletion of self-reactive T cells through negative selection in the thymus (34, 35), although recent studies show that deletional tolerance of self-antigens not encountered in the thymus may be mediated by AIRE-expressing cells in the periphery (36). Induced central tolerance of non-self antigens similarly occurs through deletional mechanisms and has been achieved through mixed hematopoietic chimerism, whether following bone marrow transplantation or peripheral administration of hematopoietic stem cells (HSC) (37-47). Deletion of alloreactive T cells occurs when developing T cells are exposed to alloantigen on HSC, which can occur without precondition in utero or during the neonatal period (48) or after ablation of preexisting mature T cells later in life (49, 50).

Peripheral Tolerance

Peripheral tolerance takes several forms. The common theme linking all of these forms of peripheral tolerance is exposure of alloreactive T cells to antigen in an environment that is not conducive towards stimulating an immune reaction such as occurs when costimulation, survival signals, or inflammatory cytokines are lacking or if T cell activation is actively inhibited by exogenous immunosuppression.

The first form of peripheral tolerance is anergy, which is a state in which T cells encounter and recognize their cognate antigen but do not activate or proliferate in response to it. It is thought that anergy results when antigen is presented in the absence of either costimulation or activating cytokines such as IL-2, and anergy can often be reversed by the addition of IL-2 (51, 52).

A second mechanism is peripheral deletion. T cell clones may be deleted either actively, by restimulation of activated T cells by large amounts of antigen in the presence of Fas and IL-2, or passively, by lack of growth factors or other survival signals, often when costimulation is absent (53). One specific form of peripheral deletion is exhaustion. Unlike anergy, in which T cells become reversibly unresponsive, exhaustion is an irreversible process that can result from a particularly robust immunologic response to an antigen. This is thought to cause multiple rounds of replication and continuous differentiation of T cells into short-lived cytotoxic effector T cells, which depletes that particular clone since it is known that eukaryotic cells only divide a limited number of

times. In terms of function, peripheral deletion, including exhaustion, may play an important role in eliminating autoreactive T cells (54).

A third mechanism is clonal ignorance, in which T cells fail to encounter their antigen, as when antigen resides in immune privileged sites, and fail to activate or provide T cell help to effector B and T cells (55). It may also occur if T cells encounter antigen presented by non-professional APCs, in which case they sometimes fail to activate (52).

The last peripheral mechanism, and the one on which this thesis focuses primarily, is regulation/suppression, in which a population of T cells actively inhibits a specific immune response (56, 57). This function results from the action of Tregs, which have been identified functionally as suppressors *in vitro* (58). They have also been identified phenotypically by cell surface markers such as CD25, which is often present on Tregs (59); by associations with certain cytokines, such as TGF-beta, IL-4, and potentially other TH2 signals (60, 61); and by the presence of foxp3 transcripts (62).

Such suppressor cells have been used to explain tolerance in various models. The presence of peripheral donor-specific Tregs explains the concept of linked suppression discussed above and observed in several animal models (25-27). More broadly, Tregs also make possible "dominant tolerance," which is a state of tolerance that persists once attained and includes both linked suppression as well as "infectious tolerance." Infectious tolerance denotes the ability of Tregs from a tolerant animal to render adoptively

transferred lymphocytes tolerant or to make a recipient tolerant when adoptively transferred themselves in high enough number (27, 63, 64).

Similarly, many of the features of tolerance in the MGH miniature swine model can best be explained on the basis of regulatory T cell populations. For example, it has been shown that T cells from animals made tolerant to a class I mismatch kidney with a short course of high-dose CyA are capable of suppressing anti-donor responses of T cells from naïve swine *in vitro* (65, 66), especially when enriched for CD25+ cells (67). As in rodent models, Tregs in the MGH miniature swine model are also capable of linked suppression (24). Moreover, preliminary experiments in our laboratory show that adoptive transfer of long-term tolerated renal grafts is possible in this model, likely because of donor-specific Tregs within the graft (unpublished data). As the presence of a young, active, and normally-functioning thymus is required in the recipient for the induction of tolerance in this model (10, 68), the mechanism for tolerance most likely involves generation of donor-specific Tregs in the thymus. Immediately after transplantation, donor antigen reaches the thymus, either by donor APCs (direct antigen presentation) or by recipient APCs presenting peptides of donor antigen (indirect antigen presentation). At the same time, mature alloreactive T cells are suppressed by the CyA regimen. Therefore, donor antigen does not cause acute rejection but has the chance to be presented to newly developing T cells, including Tregs that are generated constitutively in the thymus (69). These Tregs can then migrate from the thymus and promote peripheral tolerance of the donor (70, 71). Of note, there is probably also a small central deletional component that contributes to tolerance in this mechanism.

Recent Breakthroughs in Tolerance

Two recent findings make tolerance more clinically possible in the near future. In the first, new regimens for pig-to-baboon xenotransplants appear to show evidence for T cell tolerance. These experiments utilize "GalT-KO" swine, which are pigs that lack the α-1,3-Galactose (Gal) moiety found on the cell membrane of all species except humans and Old World primates and which is the antigen responsible for hyperacute rejection of these organs. Baboon recipients of combined kidney/thymus grafts from GalT-KO swine show donor-specific unresponsiveness *in vitro* and early baboon thymopoiesis in the porcine thymus tissue (72). The second finding, which has been successfully and reproducibly performed in humans, is a new combined bone marrow/kidney transplant regimen that has allowed the complete withdrawal of immunosuppression with stable graft function (73).

Breaking Tolerance

Having achieved a method of reliable induction of tolerance across a class I mismatch barrier, my laboratory previously explored methods of breaking tolerance with the goal that understanding how tolerance is broken will better allow us to understand how tolerance can be created and maintained. We have utilized several approaches to breaking tolerance, only the last of which has been successful. The first strategy was to perform thymectomy on tolerant animals. We found that the induction of tolerance could be disrupted by thymectomy during the initial period but that thymectomy at a later time point could not break tolerance that had already been created (74). We next attempted to

expand the anti-donor alloreactive T cell population. Our attempt to do so via exogenous administration of IL2 was unsuccessful (75), and placement of donor-matched skin grafts increased anti-donor responses *in vitro* but did not affect graft function (76). We were also unable to break tolerance by infusing primed anti-donor peripheral blood mononuclear cells (PBMC) from an animal that had rejected a donor-matched organ (unpublished data).

We recently achieved the first successful breaking of tolerance in a large animal model by removing the donor antigen from the recipient (nephrectomy of a tolerated class I mismatched renal graft) for an extended period of time (referred to as the "absence of antigen period"). When a second donor-matched kidney was transplanted without immunosuppression either immediately after graftectomy or one month later, the new grafts were universally accepted, with a transient creatinine increase in the delayed group. However, when three animals were retransplanted three months after removal of the first graft, one animal rejected the graft by day 55, and the others eventually accepted the second grafts suggesting that a 3-month absence of donor antigens represents the border line period for the loss of tolerance (Fig. 2A) (77).

In addition, we found that, unlike our previous attempts to expand the anti-donor alloreactive T cell population while the graft was present, doing so during the absence of antigen period further accelerated the breaking of tolerance. When donor MHC class I peptide was injected midway through the absence of antigen period, anti-donor cellular responses increased dramatically, anti-donor IgG was formed, and subsequently-

transplanted donor-matched kidneys were rejected in 3-5 days with evidence of accelerated acute humoral and cellular rejection. Interestingly, however, attempting to expand the alloreactive population by placing donor-type skin midway through the absence of antigen period had no effect on tolerance. Large anti-donor cellular responses were formed in these previously unresponsive animals after skin graft rejection, but no anti-donor IgG developed (although a single animal developed IgM). Despite this *in vitro* evidence of T cell sensitization, however, none of these recipients showed accelerated rejection of a second donor-matched kidney graft. One of three animals had stable renal function until day 30 and then experienced delayed rejection, and the other two accepted their kidneys long-term with stable renal function after a transient rise in creatinine during the initial period (likely representing acute rejection crisis) (Fig. 2B). Of note, these clinical courses are similar to those seen in recipients of 3-month delayed second kidney grafts not preceded by skin grafts (77).

The findings described above raise interesting questions regarding the contrasting effects of donor-type MHC peptide and skin grafts during the absence of antigen period. Having reviewed our model of tolerance induction as well as the mechanisms of tolerance involved therein, one can understand how answering these questions might further clarify the process of tolerance induction and maintenance. Such was the aim of the work described in this thesis.

II. Statement of Purpose, Hypothesis, and Specific Aims

As our previous data demonstrate that the breaking of tolerance was accelerated by inoculation of previously tolerant animals with donor MHC class I peptide during the absence of antigen period but not by placement of donor-type skin grafts (even when these grafts were rejected), we hypothesized that, while both donor-type peptide and skin grafts are immunogeneic, skin grafts play an additional tolerogeneic role. To evaluate this hypothesis, we placed donor-type skin and peptide at the same time during the absence of antigen period in the present study. Our specific aims were as follows:

1) Confirm the ability of donor MHC class I peptide to sensitize during the absence of antigen period when placed alone;
2) Observe whether donor-type skin grafts fail to sensitize when placed at the same time as donor MHC class I peptide during the absence of antigen period as they do when placed without peptide (i.e., to assess whether skin grafts merely fail to sensitize or whether there is a tolerogeneic effect of skin grafts on previously tolerant animals that is strong enough to counteract the powerful immunogeneic effect of peptide); and
3) Use *in vitro* analysis to evaluate the mechanism behind this novel function of skin grafts.

III. Methods

Animals

Transplant donors and recipients were selected from our herd of partially inbred miniature swine at 4–7 months of age. These swine have been inbred to homozygosity at the class I and class II MHC (termed swine leukocyte antigen [SLA] in pigs) loci, as described previously (15). At present, three homozygous haplotypes are available for study. In addition, a number of intra-MHC recombinant haplotypes (derived from spontaneous recombination events) are also available (78). In this study, the recipient haplotype was SLA^{dd} (homozygous class I^{d} and homozygous class II^{d}), and the donor haplotype was SLA^{gg} (homozygous class I^{c} and homozygous class II^{d}). Genotyping has been controlled by strict pedigree breeding and confirmed by microcytotoxicity testing using allospecific antisera. All transplants in this study were performed on swine that were MHC class II matched (i.e., MHC class I and minor antigen–disparate). All donor-recipient pairs were confirmed to be mutually reactive on a preintervention assay of cell-mediated lympholysis (CML). All animal care and procedures were in compliance with the "Principles of Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (revised 1996).

Kidney Transplantation

The surgical procedures for kidney transplantation and retransplantation have been previously described in detail (28, 68). Briefly, a paramedian incision was made in the abdomen, the various tissue layers incised, and the bowels retracted to expose the kidney. Gerota's capsule was dissected away. The ureter, renal artery, and renal vein were then sequentially ligated and divided, and the kidney removed. The graft kidney was placed orthotopically. The renal vein and then artery were then anastomosed to the inferior vena cava and aorta respectively using 6-0 Prolene (Ethicon, Somerville, NJ) running sutures. The ureter was anastomosed to the bladder using 6-0 Prolene running sutures. Hemostasis was achieved and the abdomen closed.

Skin Grafts

Split-thickness skin grafts (4×3 cm) were harvested from donors with a Zimmer dermatome and placed on graft beds on the dorsum of recipients. Donor-matched skin was SLA^{gg}. Third-party class I mismatched skin was SLA^{hh} (homozygous class I^{a} and homozygous class II^{d}). Skin grafts were assessed for viability by color, warmth, and softness to touch. They were assessed daily by an observer blinded to the source of the grafts and were regarded as rejected when they became dark in color, cool, and rough.

Immunosuppression and Rejection Monitoring

CyA (Sandimmune) was provided by Novartis Pharmaceutical Corp. (Hanover, NJ) and administered as an intravenous suspension. CyA was administered daily at a dose of 10 to 13 mg/kg (adjusted to maintain a blood level of 400–800 ng/ml) for 12 days, starting on

the day of the primary renal transplantation. Whole blood trough levels were determined by a monoclonal radioimmunoassay. Rejection was monitored primarily clinically by serum creatinine levels and was confirmed by histological analysis of biopsy specimens.

Histopathology and Immunohistochemistry

Renal open-wedge biopsies were performed during periods of renal failure and at postmortem. Allograft rejection was scored by standard pathologic criteria according to the Cooperative Clinical Trials in Transplantation criteria (79) (see Table 1). Immunohistochemical staining for anti-donor immunoglobulin (Ig) M and IgG deposition in renal allografts was examined by fluorescence microscopy using frozen sections stained with saturating concentrations of fluorescent isothiocyanate-labeled goat anti-swine IgM or IgG (68).

Table 1: National Institutes of Health- Cooperative Clinical Trials in Transplantation Classification of Acute Renal Allograft Rejection

Type I	Mononuclear infiltrate in >5% of cortex, at least 3 tubules with tubulitis in 10 consecutive high-power fields from the most severely affected areas, and at least 2 of the 3 following features: edema, activated lymphocytes, or tubular injury.
Type II	Arterial or arteriolar endothelialitis, with or without type-I features.
Type III	Arterial fibrinoid necrosis or transmural inflammation, with or without thrombosis, parenchymal necrosis, or hemorrhage.

Source: Colvin, Kidney Int. 1996 Sep;50(3):1069-82.

Allopeptide Immunization

As previously mentioned, allopeptide immunization was performed to evaluate the effect of sensitizing recipients with donor-type class I MHC peptide. Most of the polymorphic sites of the two known class I MHC loci in the pig (designated P1 and P14) are contained within the hypervariable regions of the [alpha]$_1$ and [alpha]$_2$ domains, as determined by comparison of the MHC class I^c (donor type) and MHC class I^d (recipient type) genetic sequences (80). Four MHC class Ic peptides spanning the full length of the hypervariable regions of the P1 [alpha]1 helix were synthesized (81) and labeled as PC1-1 (amino acids [aa] 3–27), PC1-2 (aa 35–52), PC1-3 (aa 53–73), and PC1-4 (aa 71–90). Three MHC class I^c peptides spanning the full length of the hypervariable regions of the P14 [alpha]1 helix were synthesized and labeled as PC14-1 (amino acids [AA] 3–27), PC14-2 (AA 45–59), and PC14-3 (AA 60–85). Peptide purity was >90%, as verified by high-performance liquid chromatography and mass spectrometry. Peptides were provided by the Biological Chemistry and Molecular Pharmacology (BCMP) Biopolymers Lab at Harvard Medical School. The length of the peptides was chosen to optimize binding to class II molecules. Previous studies have shown that recipients rejecting lung grafts (either acutely or chronically) spontaneously develop T cell reactivity to these same peptides and that preoperative immunization with these synthetic donor-derived peptides causes accelerated rejection in comparison to non-immunized controls (82).

In this study, 500 µg of each peptide in 750 µL of complete Freund's adjuvant (CFA) were injected subcutaneously 6 weeks after graftectomy of the original donor kidney. Peripheral blood mononuclear cells (PBMC) from the prospective recipients were tested

for in vitro proliferative responses against each individual allogeneic peptide three weeks later (see below), and immunized pigs were rechallenged with individual peptides to evaluate in vivo delayed-type hypersensitivity (DTH) responses two weeks after immunization (see below).

Delayed-Type Hypersensitivity (DTH) Responses

In the immunized group, DTH responses were evaluated 2 weeks after allopeptide immunization by injecting 100 µg of each individual PC1 and PC14 peptide in 0.1 mL phosphate-buffered saline (PBS) intradermally into separate sites on the neck of the pig. PBS (0.1 mL) was used as a negative control, and 100 µg of Mycobacterium tuberculosis H37 RA (MTB) was used as a positive control. Induration was measured 48 hr after injection by blinded observers using calipers. Positive responses were defined as having a diameter of induration greater than 10 mm. Induration between 5 and 10 mm was considered to be an intermediate response, and negative responses had less than 5 mm of induration.

Media

Tissue culture media used for CML assays consisted of Roswell Park Memorial Institute (RPMI) 1640 (GIBCO Invitrogen) supplemented with 6% fetal calf serum (Sigma, St. Louis, MO), 100 U/mL penicillin (GIBCO Invitrogen), 135 µg/mL streptomycin (GIBCO Invitrogen), 50 µg/mL gentamicin (GIBCO Invitrogen), 10 mM HEPES (Fisher Scientific, Pittsburgh, PA), 2 mM l-glutamine (GIBCO Invitrogen), 1 mM sodium pyruvate (BioWhittaker, Walkersville, MD), 0.1mM nonessential amino acids

(BioWhittaker), and 5×10-5M [beta]-2 mercaptoethanol (Sigma). The effector phase of the CML assay was performed using Basal Medium Eagle (GIBCO Invitrogen) supplemented with 6% CPSR-3 (Sigma) and 10 mM HEPES (Fisher Scientific).

Medium for flow cytometry consisted of Hanks' balanced salt solution (HBSS; GIBCO Invitrogen, Carlsbad, CA) supplemented by 1g/L Bovine Serum Albumin and 1g/L sodium azide.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC were prepared from freshly collected, heparinized whole blood diluted approximately 1:2 with Hanks' balanced salt solution (HBSS; GIBCO Invitrogen, Carlsbad, CA) as previously described (68). Mononuclear cells were obtained by gradient centrifugation using Lymphocyte Separation Medium (Organon, Teknika, Durham, NC), washed once with HBSS, and contaminating red cells were lysed with ACK Buffer (B&B Research Laboratory, Fiskeville, RI). Cells were then washed with HBSS and resuspended in tissue culture medium. All cell suspensions were stored at 4°C until utilized in cellular assays. Antigen presenting cell (APC) and T cell preparations were isolated from PBMC by nylon wool passage.

Primary Cell-Mediated Lympholysis Assay

The procedure for primary CML assays has been described elsewhere (28, 65, 68). Lymphocyte cultures containing 4×10^6 responder and 4×10^6 stimulator PBMC (irradiated with 2500 cGy) were incubated for 6 days at 37°C in 7.5% CO_2 and 100% humidity.

Bulk cultures were harvested, and effectors were tested for cytotoxic activity on ^{51}chromium (Amersham, Arlington Heights, IL)- labeled lymphoblast targets. Effector cells were incubated for 5.5 hr with target cells at E/T ratios of 100:1, 50:1, 25:1, and 12.5:1. Three target cells were tested in each assay: MHC-matched PBMC to the effectors, donor-matched PBMC, and third-party PBMC. Supernatants were then harvested using the Skatron collection system (Skatron, Sterling, VA) and ^{51}chromium release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as percent specific lysis (PSL), calculated as: PSL=([experimental release (cpm)–spontaneous release (cpm)]/[maximum release (cpm)–spontaneous release (cpm)])×100%. For assays in this project, self-type cells were SLA^{dd}, donor-type cells were SLA^{gg}, and third party cells were SLA^{hh}, which share class IId with the donor and recipient but differ with both by having class Ia.

Secondary Co-culture Cell-Mediated Lympholysis Assay

As previously described (65), lymphocyte cultures from experimental animals containing 4×10^6 cells were primed with donor-type 4×10^6 PBMC (irradiated with 2500 cGy) and incubated as a regulator for 6 days at 37°C in 7.5% CO_2 and 100% humidity. Then regulator cultures were harvested, and 2×10^6 regulator cells and naïve matched type 2×10^6 PBMC (responder) and 4×10^6 donor-matched stimulator PBMC (irradiated with 2500 cGy) were incubated for 6 days at 37°C in 7.5% CO_2 and 100% humidity. Bulk cultures were incubated with and effectors were tested for cytotoxic activity on ^{51}chromium (Amersham, Arlington Heights, IL)-labeled lymphoblast targets. Effector cells were incubated for 5.5 hr with target cells at E/T ratios of 100:1, 50:1, 25:1, and

12.5:1. Three target cells were tested in each assay: PBMC SLA-matched to the effectors, donor-matched PBMC, and third-party PBMC. Supernatants were then harvested using the Skatron collection system (Skatron, Sterling, VA) and ^{51}chromium release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as PSL, calculated as above. Once again, for assays in this project, self-type cells were SLA^{dd}, donor-type cells were SLA^{gg}, and third party cells were SLA^{hh}, which share class IId with the donor and recipient but differ with both by having class Ia.

Mixed Lymphocyte Reaction (MLR) Peptide Proliferation Assay (PPA)

To evaluate the ability of a recipient to mount a proliferative T-cell response to an indirectly presented peptide antigen, a peptide proliferation assay (PPA) using thymidine incorporation was performed as previously described (83). In brief, 4×10^5 recipient-matched PBMC were cultured with 50 µg/mL of individual allopeptides for 5 days in triplicate plates. The culture was then pulsed with [^{3}H]thymidine (1 µCi/well) for 5 hr, and [^{3}H]thymidine incorporation was measured by [beta]-scintillation counting. A stimulation index (SI) for each peptide was expressed as experimental counts per minute divided by media control counts per minute. Based on historical data from 30 naïve pigs tested against each of three allogeneic class I PC14 peptides (81), the average SI of all 30 naïve responses was 1.2. Adding three standard errors resulted in an SI of 2.2. Therefore, SIs greater than 2.3 were deemed to be significant.

Antigen Presenting Cell (APC) Depletion

APCs are known to be adherent and can be negative sorted by adhering to plastic or nylon wool as previously described (84, 85). PBMC were incubated for 4 h at 37°C in 75-cm^2 plastic flask (Falcon #3023, Becton Dickinson Labware, Lincoln Park, NJ) to deplete the adherent cells. To further deplete APCs by negatively sorting adherent cells, the overlying media was then gently removed to avoid disturbing the cells attached to the flask and was run by gravity through a column of sterile nylon wool (Fenwal Laboratories, Deerfield, IL). The flasks were gently washed once with 5 ml of media, which was also run through the nylon wool and collected.

The adherent cells (APC-rich cells) in both the flask and nylon wool were also collected to use for control plates to compare with the APC-depleted plates. Cells were recovered from the flasks by twice adding 5 ml of media, scraping the walls, and aspirating. Cells were recovered from the wool by rinsing with 4ml media and aspirating remaining fluid. Both adherent and nonadherent cell collections were spun at 1800 rotations per minute (rpm) for 10 minutes, placed in 1 ml media, and stored at 4°C for up to 24 hours until usage.

Bulk and APC-Depleted Mixed Lymphocytes Reaction (MLR)

MLR cultures, to test for proliferative response to alloantigen, have been described previously (86). Briefly, 4 x 10^5 responders and an equal number of irradiated (25 Gy) stimulators were incubated in triplicate in 200 µl of standard MLR medium using flat-bottom 96-well plates (Costar, Cambridge, MA). Medium consisted of RPMI 1640 supplemented with 6% fetal pig serum,10 mM HEPES, 1 mM glutamine, 1 mM sodium

pyruvate, 0.1 mM nonessential amino acids, 100 U/mL penicillin, 135 µg/mL streptomycin, 50 µg/mL gentamicin, and 2×10^{-5} M [beta]-2mercaptoethanol. Cultures were incubated for 5 days at 37°C in 4% CO_2 and 100% humidity, after which 1 µCi of [^{3}H]thymidine was added to each well, followed by an additional 5 hour incubation. Cells were harvested onto Mash II glass fibers using a TomTek harvester (Perkin Elmer Wallac, Waltham, MA). ^{3}H incorporation was determined in triplicate samples by liquid scintillation. [^{3}H]thymidine incorporation was measured as counts per minute (cpm) using the Microbeta liquid-scintillation system (Perkin Elmer Wallac, Waltham, MA). A stimulation index (SI) for each reaction was expressed as experimental counts per minute divided by media control counts per minute.

For MLR cultures measuring only response to indirectly presented antigen, stimulator APCs were first depleted as described above, and this cell solution was used in place of the bulk stimulator cell solution. As a control, another set of APC-depleted plates were set up identically with the addition of a smaller number of APC-enriched (2.5×10^4) cells.

For assays in this project, self-type cells were SLA^{dd}, donor-type cells were SLA^{gg}, and third party cells were SLA^{hh} (which share class IId with the donor and recipient but differ with both by having class Ia), SLA^{cc} (which differs from the recipient in both class I and II and shares class Ic with the SLA^{gg} donor), and Yukatan (Yuk, which are completely outbred pigs differing at all MHC loci).

Enzyme-linked immunosorbent assay (ELISA)

As previously described (87), plates were coated with 50 μL of peptides (2 μg/mL) or PBS and incubated overnight at 4°C. The plates were washed twice with 200 μL of PBS + 0.1% Tween20 and then blocked by dispensing 200 μL of PBS + 0.05% Tween20 and 1% BSA with a 1-hour incubation at room temperature. The plates were then washed twice and swine serum at 1:80 dilution in PBS + 0.1% Tween20 was added in triplicate. Following a 1-hour incubation at room temperature, plates were washed twice more. Rabbit anti-pig IgG (1:250) and IgM (1:250) in PBS + 0.05% Tween20 1% BSA was added to each well and incubated for 1 hour at room temperature. Following two more washes, 50 μL SAv-HRP developing solution (1:1000) was added to each well and allowed to incubate for 1 hour at room temperature and in the dark. Another two washes were performed, and hydrolysis was measured adding ABTS peroxidase solutions into each well. Product absorbances were measured in optical density (OD) using a BioRad ELISA plate reader at 405 nm (BioRad, Hercules, CA).

Flow Cytometry

As previously described (83), the presence of anti-donor class-I IgM and IgG in the serum of experimental swine was detected by indirect flow cytometry. Briefly, serum was decomplemented by incubation at 56 degrees Celsius for 30 minutes, and 10 microliters of each serum was added to 5 x 10^6 target cells suspended in 100 microliters. After a 30-60 minute incubation at 4 degrees Celsius and washing twice with media, 15 microliters of appropriate secondary antibody mix containing either a 1:50 dilution of FITC-conjugated goat F_{ab} anti-swine IgM F_c antibodies (Jackson ImmunoResearch, West Grove, PA) or 1:50 dilution of FITC-conjugated goat F_{ab} anti-swine IgG F_c antibodies

(Jackson ImmunoResearch). After another 30-60 minute incubation followed by 2 washes, 10 microliters of propidium iodine at 1:23 dilution was added. Fluorescence-activated cell sorting (FACS) was performed using a Becton Dickinson FACScan microfluorometer (Sunnyvale, CA) and analyzed with WinList analysis software (Verity Software House, Topsham, ME).

Complement-mediated Cytotoxicity

Cytotoxic antibodies binding to target cells were detected by complement-mediated cytotoxic assays, as previously described (8). Target cell suspensions were diluted to 5×10^6 cells/mL in Medium 199 (Cellgro, Herndon, VA) supplemented with 2% fetal calf serum and serially diluted from 1:10 to 1:160. In 96-well U-bottom plates (Costar, Cambridge, MA), 45 µL of the appropriate target cell suspension was incubated with 5 µL of diluted serum or controls for 15 min at 37^{o}C, followed by a second incubation with 25 µL of appropriately diluted rabbit complement. Dead cells were identified by staining for 30 minutes with 10 µL of 7-AAD. Data were acquired, and the percentage of dead cells was assessed using a Becton Dickinson FACScan (San Jose, CA) and analyzed with WinList analysis software (Verity Software House, Topsham, ME).

Contribution of Student Researcher

Joshua Weiner performed all procedures and analysis detailed in the Methods and Results sections. David Sachs and Kazuhiko Yamada advised me in analysis. Yoshinori Ishikawa, Kazuhiko Yamada, Justin Etter, and Shannon Moran assisted in surgeries. Justin Etter, Shannon Moran, and Hanzhou Hong provided occasional technical support with assays.

IV. Results

Immunogenicity of Peptide

It has been demonstrated previously that inoculation with donor MHC peptide sensitizes recipients to reject grafts bearing the same donor MHC in an accelerated fashion (81, 82, 87, 88). Before conducting our experiment, we first demonstrated that our batch of donor-type class Ic peptide was also immunogeneic. This was demonstrated in two ways. First we inoculated a naïve SLA^{dd} pig (pig #17050) with class Ic peptide and observed *in vitro* and *in* vivo responses. We found that the animal responded to the peptide *in vitro* with increased MLR responses in a peptide proliferation assay (Fig. 3A) and with production of anti-peptide IgG in an ELISA (Fig. 3B), but it did not produce antibody against SLA^{gg} cells, which display MHC class Ic (Fig. 3C). MLR response to SLA^{gg} cells increased dramatically after peptide inoculation, especially when antigen was indirectly presented (Fig. 4A). This is consistent with the idea that the pig was sensitized on an indirect level since peptide can only be presented indirectly. Although anti-donor MLR responses were increased after peptide, CML responses to donor cells did not increase after peptide inoculation (Fig. 4B). When this sensitized pig was then injected with SLA^{gg} PBMC 59 days after peptide inoculation, both direct and indirect anti-donor responses increased even further (Fig. 5A), and both large anti-donor CML responses and SLA^{gg} IgG now formed as well as shown in Figures 5B and 6.

Second, we demonstrated that our batch of peptide was capable of sensitizing recipients in a manner capable of causing accelerated rejection of a subsequently placed SLA^{gg}

kidney (in this case 21 days after peptide inoculation). When this animal (pig #18524) was injected with peptide, anti-donor MLR responses formed as before, and the kidney was rejected in an accelerated acute cellular and humoral fashion as evidenced by increase in anti-donor CML response (data not shown), acute increase in creatinine and fall in platelets (Fig. 7A), and the large, hemorrhagic-appearing graft at necropsy (Fig. 7B). Of note, this result occurred despite our normal CyA induction regiment with which 100% of class I mismatched kidney grafts are accepted in non-sensitized animals.

Experimental plan

Having shown that our batch of peptide was immunogeneic, we evaluated the combination of peptide-plus-skin during the absence of antigen period. Juvenile SLA^{dd} swine were made tolerant to two-haplotype class I mismatched/class II matched renal grafts from SLA^{gg} swine with a 12-day course of cyclosporine A (CyA) as previously described (28). After these animals displayed long term tolerance (approximately 100 days with stable renal function and *in vitro* evidence of donor-specific hyporesponsiveness), a 3 month "absence of donor antigen" period was created by removing the renal allograft for 12 weeks. In order to maintain renal function in the recipients while the donor renal grafts were absent, recipients were given a self-type (SLA^{dd}) kidney on the day of graftectomy. Grafts that are fully MHC-matched but differ in minor antigens are typically accepted without immunosuppression in approximately 2/3 of recipients in our experience, sometimes with a transient and minor increase in creatinine approximately 1 week postoperatively. Six weeks after the nephrectomy of the primary kidney (i.e., 6 weeks prior to the second donor kidney transplant), recipients

were challenged simultaneously with donor-type (SLA^{gg}) skin grafts and subcutaneous injection of donor MHC class I (class Ic) peptides. After an additional 6 weeks, a second donor-type (SLA^{gg}) kidney was transplanted without immunosuppression. The experimental plan is detailed in Figure 8.

Anti-donor responses in the initial period

Induction of tolerance of class I disparate renal allografts

We have previously reported that a 12-day course of CyA facilitated the induction of tolerance of class I disparate renal allografts in juvenile (3-8 months of age) MGH miniature swine (28). This was the case in all 5 of our experimental animals as well. The clinical courses of 3 of these animals were unremarkable with stable creatinine throughout. Two animals (18349 and 18354) had early peaks in creatinine that were found to be caused by ureteral stenosis during exploratory surgery, and the problem resolved with reanastomosis. By the time of graftectomy, all 5 pigs had creatinine stably in the normal range and showed donor-specific unresponsiveness or hyporesponsiveness on in vitro assays. In the 3 animals for whom these data were available, secondary co-culture CML showed suppression of naïve recipient-matched cells against donor-type cells.

Small increase in anti-donor responses in some swine during absence of antigen period

We have previously documented that anti-donor cellular responses increase slightly after the removal of the donor-kidney and replacement with recipient-matched kidney (the so-called “absence of antigen period”) (77). We have also shown that roughly 1/3 of the

recipient-matched kidneys are rejected due to disparities in minor antigens (20). This pattern was seen in our experimental animals. One animal rejected its matched kidney at day 35 and was saved by a rescue transplant of another recipient-matched kidney. Biopsy at the time of graftectomy showed cellular but not humor rejection, consistent with a process mediated by minor antigen differences. The other animals experienced only very slight creatinine increases between days 10-12. In 4/5 animals, there was a small increase in anti-donor CML responses in the absence of antigen, although no increase in MLR response.

Effects on tolerance of combined donor-type skin plus class I peptide during the absence of antigen period

Recipients reacted to peptide in vivo and in vitro

All 5 experimental animals reacted to the donor-type Class I peptide *in vivo* and *in vitro*. Delayed type hypersensitivity (DTH) responses to class Ic peptides were analyzed in all recipients immunized with the class Ic PC14 peptides 14 days after inoculation. PC14-3 elicited the strongest positive DTH response in the immunized animals. The immunized pigs showed brisk DTH responses to the M. tuberculosis H37 RA positive control and negative responses to the phosphate-buffered saline control. These results confirmed the presence of indirect alloantigen presentation *in vivo* and validated the immunogenicity of specific class I MHC peptides. To assess the *in vitro* reactivity of recipient lymphocytes to individual class Ic peptides, MLR peptide assays were performed with lymphocytes from these animals three weeks after immunization in the absence of donor kidney antigens. There was no T cell proliferative response to any of the PC14 (class Ic) peptides

before immunization. After immunization, T cell responses to PC14-3 developed, which was consistent with the positive DTH responses observed 14 days after immunization. 3/5 swine also had increases in IgG directed against the peptide on ELISA.

Skin survived same length of time as when placed without peptide

Donor-matched (SLA^{gg}) skin grafts all engrafted initially and were then rejected between 15-18 days later. Of note, this is the same length of survival as when skin was placed without the concurrent inoculation with peptide in tolerant animals during the absence of antigen period (77). We also found that skin grafts briefly became darker and more mottled between 6-9 days after placement. This could represent vascularization, but it could also be a rejection crisis during which the alloreactive and tolerogeneic reactions compete and thereby, presumably, provide the immunologic stimulus for the development of Tregs. Therefore, to evaluate this hypothesis, we set up a primary co-culture in 2 animals 7 days after skin grafting. If this was a time of Treg stimulation expansion in the blood, it might be possible to see suppression of naïve anti-donor response by these cells. However, we instead saw an increase in the anti-donor response (data not shown). Possible reasons for this finding are outlined in the Discussion.

Anti-donor cellular responses increased after skin-plus-peptide

All animals had increased specific CML responses to donor cells after skin-plus-peptide. These responses were large in 3 animals and moderate in 2, and the differences did not correlate with whether these animals eventually rejected their kidneys (Fig. 9). All animals also had increased specific MLR responses to donor cells. In two animals, this

response was seen only in the bulk wells but not in the wells in which indirect antigen presentation was isolated. In 2 animals, the responses were higher in the indirect wells than in the bulk wells. The absence of indirect response did not correlate with whether these animals eventually rejected their kidneys, however the predominance of the indirect response was seen in the 2 animals who survived longest after their second donor-matched kidney transplant (Fig. 10).

Possible increase in suppressive cells

Flow cytometry showed no consistent changes in Foxp3 cell number or percentage in peripheral blood after skin-plus-peptide; however, 3 of 4 animals tested had increased CD25+ and CD25/CD4++ cell percentages during this period (data not shown). This might potentially represent an expansion of cells capable of performing a regulatory function during the period after skin-plus-peptide.

Clinical course after transplantation of 2nd donor-matched kidney (SLA^{gg}) without immunosuppression

In our previous experience, differences in survival were stark after 2nd donor-matched kidney transplantation without immunosuppression when either donor-type skin or peptide were placed solitarily during the absence of antigen period. Swine that received peptide alone rejected their kidneys in an accelerated acute humoral and cellular fashion within 3-5 days; those that received skin alone either survived long-term or rejected in a delayed fashion (77). Our purpose in this current experiment was to evaluate which of

these effects would predominate when animals were challenged simultaneously with skin and peptide.

Upon reperfusion of the 2nd donor-matched kidneys, we observed spot hemorrhages in all grafts within 1 hour. This is consistent with the deposition of preformed anti-donor antibody which developed during the absence of antigen period, most likely after skin-plus-peptide. Two of these animals developed slightly larger areas of darkness in their grafts but continued to make urine soon after reperfusion. Another 2 animals, despite patent anastomoses and adequate renal artery pulse, developed diffusely dark kidneys which felt soft to the touch and produced very little urine during surgery.

We found that 2 of 5 animals survived long-term after receiving a second donor-matched kidney without immunosuppression. An additional animal experienced slightly prolonged survival (11 days). The final 2 animals rejected within 5-7 days with acute increases in creatinine and eventually uremia and internal bleeding. Figure 11 shows survival of all five animals after second donor-matched kidney transplantation compared to pigs who received either peptide or skin alone. Figure 12 shows creatinine levels for the five skin-plus-peptide animals after their second donor-matched kidneys. Of the long-term survivors, one (17944) experienced a mild and transient rise in creatinine between postoperative days 8-10. Creatinine then returned to baseline and remained at that level throughout. Note that the slight increase in creatinine at the end of this animal's course was due to severe pneumonia diagnosed clinically and confirmed by necropsy and histology. The other long-term survivor (18354) experienced a more pronounced early

rise in creatinine, which eventually returned to baseline. The large increase in creatinine in the later part of this animal's course was due to a prolonged period of sepsis, which required treatment with intravenous fluids and multiple rounds of antibiotics.

The correlation between appearance of the kidney during surgery and survival was not perfect. However, the two longest surviving kidneys showed either mild spot hemorrhages or slightly larger darker areas with urine production. The other kidney with larger areas of darkness and urine production was in the animal that experienced slightly prolonged survival. The kidneys that were diffusely dark and soft without urine production were in the two animals with the shortest survival.

In all second donor-matched kidneys, histology showed IgM and IgG deposition as early as 1 hour after reperfusion, which is consistent with our intraoperative observations. Additional findings are as follows. In the first long-term surviving animal, histology after sacrifice showed grade II cellular rejection. The second long-term survivor showed no evidence of rejection of necropsy but had slight glomerulitis of uncertain etiology. The animal who rejected on day 7 showed acute humoral rejection at necropsy. At time of submission, final analysis of histology data from necropsy of the other two early rejectors was still being completed, but we believe that the results will also show acute humoral rejection based on the gross appearance of these organs at necropsy**.** Figure 13 contrasts the gross necropsy findings in long-term acceptors versus rejecters.

In vitro responses after 2nd donor-matched kidney (SLA^{gg}) without immunosuppression

In vitro responses correlated with clinical course after second donor-matched kidney transplantation without immunosuppression. In the two animals that survived long-term, CML responses against donor cells, which had increased after skin-plus-peptide, became negligible within the first month after transplantation. The animals maintained normal CML responses to 3rd party cells (Fig. 9).

Bulk and indirect MLR responses could not be assessed in animals who rejected in the early period after transplantation. However, in the two animals who survived long-term with stable renal function, both bulk and indirect MLR responses, which had increased after skin-plus-peptide, became negligible after transplantation. Third-party responses remained appropriate (Fig. 10).

Neither clinical course nor in vitro responses were affected by additional donor-antigen challenge after transplantation

The two animals who maintained their second donor-matched grafts long-term were further challenged with additional donor antigen. In one animal, challenge was in the form of a second donor-matched skin graft, which was placed 2 months after the renal graft. Self and third-party grafts were placed at the same time. The donor skin was rejected after 17 days, which is the same kinetics as when donor skin is placed either in an animal bearing a tolerated kidney graft or during the absence of antigen period after this graft has been removed. In contrast, a self skin graft was accepted indefinitely, and a third-party skin graft was rejected within 5 days. The other long-term surviving animal

was challenged by an additional inoculation with donor-type Class Ic peptide 2 months after the renal graft. In both pigs, renal function was entirely unaffected, and there was either a small or negligible increase in anti-donor CML and MLR responses. In the animal who was challenged with skin, both MLR and CML responses to the third party donor increased, and anti-third-party IgG became detectable in the serum within 1 week. No anti-donor antibodies were detectable.

The role of humoral responses

The contribution of humoral responses in this experiment was complex. It is now the focus of our ongoing research and will be addressed in detail in the future. Briefly, however, levels of anti-donor IgG peaked roughly 2 weeks after skin-plus-peptide and thereafter decreased in the animals who had long-term or prolonged survival . These antibodies were found to be only minimally cytotoxic against donor cells in our complement-dependent antibody-mediated cytotoxicity assays. In contrast, levels of anti-donor antibodies were consistently significantly elevated and highly cytotoxic in the two animals who rejected most quickly (data not shown).

V. Discussion

In the absence of exogenous immunosuppression, skin grafts are usually quite immunogenic, and the sensitization caused by these grafts causes accelerated rejection of a donor-matched graft placed later. However, we have previously shown that, when placed on tolerant swine in whom the original donor organ has been removed for several weeks, donor-matched skin grafts do not appear to have this effect. When a new donor-matched kidney is placed without immunosuppression 6 weeks after skin, rejection is not accelerated, and the new organs may even survive indefinitely. This is similar to the pattern seen when no skin graft is placed during the absence of antigen period but stands in stark contrast to the accelerated rejection that occurs when donor peptide is placed during this time (77).

By challenging recipients with both donor-type peptide and skin at the same time during the absence of antigen period, we now demonstrate not only that donor skin does not hasten its own rejection but that the concurrent inoculation with donor skin and peptide also does not hasten rejection of donor skin and at least partially negates the immunogeneic effect of peptide alone. When recipients are inoculated with peptide alone during the absence of antigen period, tolerance is always broken, and future grafts are rejected by severe acute cellular and humoral rejection within 3-5 days (77). When skin and peptide were placed together, however, tolerance was preserved in 2 of 5 animals, and survival was prolonged in a third. In the 2 long-term survivors, kidney function remained normal after the initial period. Moreover, *in vitro* parameters of tolerance were

restored. Both anti-donor MLR and CML responses, which had increased substantially after skin-plus-peptide, once again became hyporesponsive or unresponsive. Finally, this tolerance was found to be stable and could not be broken even by additional inoculation with donor-type skin or peptide, neither of which affected function of the donor kidney or increased anti-donor responses *in vitro*.

Although the number of animals in this series was not large, the difference between the accelerated acute rejection seen in all animals receiving peptide alone versus the possibility of long-term survival in the skin-plus-peptide group was dramatic, and we saw a difference in *in vitro* parameters between the groups as well.

The most likely mechanism for this phenomenon involves the balance between alloreactive and suppressive T cells. The evidence for this lies in several observations from our well-established model, in which we reliably induce tolerance of Class I mismatched renal allografts, and in other models of tolerance induction. We have shown that tolerant animals have stable graft function and *in vitro* evidence of donor-specific hyporesponsiveness (28). Moreover, T cells from these tolerant animals are capable of suppressing the anti-donor responses of naïve T cells when cultured together, which is compatible with the idea that Tregs develop after transplantation and play an integral role in maintaining tolerance peripherally (65, 67, 89).

Because of these Tregs, tolerance cannot be broken as long as the graft remains in place, even if the recipient is challenged with either donor skin or peptide. Graft function

remains normal even if CML responses are increased (76, 90-92). Even though tolerogeneic or suppressive host cells infiltrating the graft may help maintain tolerance at the local level (93), the maintenance of tolerance in our model is more a function of the host than of the graft as we and others have shown that tolerance is maintained if the graft is removed and immediately replaced with a donor-matched organ without immunosuppression. In fact, we have shown that tolerance is maintained for up to 4 months in the absence of antigen (77), and this time period is even longer in the rodent model (94). Beyond this time period, however, rejection of donor-matched organs begins to be observed (63).

The most likely mechanism for these observations is the changing balance between Tregs and alloreactive T cells over time. Tregs progressively decrease in number, potency, and/or affinity after donor tissue has been removed (91, 94, 95). On the other hand, unlike in central (e.g., deletional) mechanisms of tolerance, alloreactive cells remain present in our tolerant animals, as indicated by the increased anti-donor CML responses when donor-type class I/third-party class II skin grafts are placed during this time (data not shown). Therefore, the relative balance between the residual Tregs and alloreactive memory cells at any given time determines whether grafts are rejected or whether tolerance is preserved, and the progressive decrease in Tregs is the most likely reason why tolerance is lost with time (77, 92, 94, 96). Evidence for this can be seen in our demonstration that anti-donor CML responses increase during the absence of antigen period.

As outcomes differ dramatically when recipients are challenged with peptide versus skin during the absence of antigen period, we propose that the particular balance of Tregs and alloreactive cells differs between these two processes. There are several possible mechanisms that could explain this difference. One involves the type of antigen presented. As the injected peptide is simply a polypeptide portion of the MHC class Ic antigen, only class I antigen can be presented after peptide inoculation. However, a wide range of donor antigens can be presented after skin grafting, including both MHC antigen as well as minor antigens. Presentation of these other donor antigens may expand the Treg population in a way not possible with presentation of Class I peptide alone. It is known that presentation of MHC peptides is much more immunogeneic than presentation of non-MHC peptides (97), and it is therefore possible for presentation of non-MHC peptides to lead to a tolerogeneic state (98-101).

A second possible mechanism involves the manner in which host APCs present donor tissue antigens versus peptide. That is, tissue antigen may be taken up by APCs and presented in draining lymph nodes in a way that favors lack of alloreactivity relative to suppression or anergy. The predominance of dendritic cells may somehow play a role in this mechanism. In contrast, presentation of peptide antigen may occur more diffusely by various APCs throughout the body and in a way that favors expansion of alloreactive cells relative to Tregs.

A third reason is that placing donor-matched skin during the absence of antigen period

simply affects the kinetics of Treg loss since it literally restores the presence of antigen. We and others have demonstrated that, once tolerance has been created, it is not possible to break as long as the graft remains in place (63, 76, 102, 103).

A fourth and less likely hypothesis is that any remaining DCs in the graft may be in an immature form that does not express much MHC Class II, CD80, or CD86. It has recently been shown that immature DCs, such as those found in cornea grafts after transplantation, may cause T cells to become Tregs rather than alloreactive cells (104). However, it might be that these immature DCs exist only in immunoprivileged sites and are not found in our model. Nonetheless, the idea of tolerance facilitated by antigen presentation by immature or semi-mature DCs has been proposed elsewhere as well (105-107).

While any of these hypotheses could explain our findings, we propose that the most likely mechanism involves the particular mode of allopresentation (i.e., direct versus indirect presentation). As peptide immunization lacks actual donor cells, including APCs, the donor antigen can be presented only indirectly; in contrast, antigen can be presented both directly and indirectly after skin grafting as the grafts include donor APCs.

There are two ways in which direct allopresentation may contribute to a tolerogeneic state. The first is that only in direct allopresentation is cell-to-cell contact possible between donor APCs and recipients CD4+ T cells. We have previously shown that this cell-to-cell contact is necessary for the expansion of the Treg population (65). Therefore, direct presentation in previously tolerant animals would allow for the expansion of the

Tregs in a way not afforded by indirect presentation (108). Evidence for this hypothesis can be seen in our flow cytometry data showing that, while foxp3+ cells do not increase after placement of skin and peptide, CD25+ and CD4+/CD25+ T cells do. It is not clear if the lack of increase in foxp3+ cells is significant. Perhaps our assay is not sensitive enough for detecting subtle changes in peripheral blood, which has very low numbers of foxp3+ cells. It is also possible that we observed an increase in induced Tregs as opposed to natural Tregs.

This model supposes that, while Tregs are expanded by direct allopresentation after skin grafting, they are not activated by peptide since cell-to-cell contact between donor and recipient cells is not possible through the indirect pathway alone. On the other hand, alloreactive cells, which remain present at low levels, are stimulated by peptide through the indirect pathway in a way that Tregs cannot be. The activated alloreactive cells, in the setting of relatively decreased Treg number or potency in the absence of antigen, would then be able to provide T cell help to both cytotoxic T cells and B cells.

The second mechanism by which direct allopresentation may contribute to a tolerogeneic state is that direct allopresentation by non-APCs may play a progressively tolerogeneic role after transplantation. Direct presentation certainly contributes to rejection in the early period when the presence of donor APCs is highest (97). However, direct presentation plays a decreasing role in rejection with time, mostly because the number of donor APCs decreases with time (81, 109-112). This is also seen in human allograft recipients (113).

Not only does direct presentation plays a decreasing role in rejection with time, but it also appears to play an increasingly tolerogeneic role as donor APCs decrease, thus leaving the remaining direct presentation by non-APCs alone (81, 113). In this hypothesis, donor APCs initially migrate to host lymph nodes, where they activate alloreactive T cells. These T cells migrate to the graft, where they secrete cytokines that stimulate graft endothelial cells to express Class II. In a long-term surviving graft, these Class II-expressing donor cells are all that remain of direct presentation once donor APCs are depleted over time. As they neither express costimulation nor migrate to lymph nodes, they foster either anergy or suppression (114-116). Tolerance created through this mechanism has been shown to persist even if costimulation is eventually added (117). In fact, after the acute period, the presence of donor antigen expressed by Class II on donor cells seems to inhibit cell-mediated rejection as grafts lacking Class II expression are rejected more rapidly (97). It has been shown that direct presentation by graft endothelial cells, which express class II but are not professional APCs, downregulates anti-donor activity through interactions with donor-specific primed/memory T cells (CD45RO+). These alloreactive memory cells decrease after encountering donor antigen presented directly by non-APC donor cells (such as class II-expressing vascular endothelium) while CD45RA+ (naïve) cells do not. This is substantiated by fact that CD45RO+ cells circulate to the graft, while CD45RA+ cells do not, and that CD45RO+ cells decrease in number after contact with alloantigen presented by parenchymal graft cells (117, 118). More specifically, these potentially tolerogeneic graft parenchymal cells seem to have a tolerogeneic effect on both memory and naïve cells. For memory cells (CD45RO+), the mechanism seems to be anergy, since responses are restored by IL-2 in culture. For naïve

cells (CD45RA+), the mechanism seems to be deletion subsequent to activation without costimulation, as the process can be prevented by blocking the CD95 receptor (117).

Since it seems that direct allopresentation without costimulation is at least partially responsible for fostering a tolerogeneic state, the question becomes why? The most likely answer is that it modulates the cytokine profile. It has been demonstrated that such "sustained suboptimal antigenic stimulation" fosters peripheral generation of both natural and induced Tregs through a pathway requiring the presence of TGF-beta, which both raises the threshold for T cell activation and directly promotes differentiation into Tregs (119). This is consistent with recent studies showing that TGF-beta plays a large role in the formation of Tregs (120, 121). This pathway may also engender tolerance through resulting release of IL-4 or IL-10 and activation of the Th2 pathway (119, 122). Although controversial, there is some evidence that Th2 pathway, and its cytokines, can help facilitate a tolerogeneic state (123). In euthymic hosts, the mechanism appears to be a mixture of anergy and suppression, with the suppression seemingly depending on cytokines such as IL-10, IL-4, and IL-2, which possibly function to foster an environment favoring Th2 T cell activity (63). This could explain one mechanism by which direct allopresentation fosters tolerance and could also explain the decrease in suppression as these cytokines decrease in the absence of antigen.

As our hypothesis involves a balance between alloreactive and tolerogeneic cells, a logical extension is that the effect of skin is not all-or-nothing but rather a spectrum in which competition between the alloreactive effects of both peptide and skin and the

tolerogeneic effect of skin determines where on the continuum between rejection and tolerance the animal will fall. This would explain why anti-donor cellular and humoral responses differed between animals. As of yet, we do have not identified a marker that predicts which competing impulse will win.

In previous experiments, we have been able to observe the competition between the immunogeneic and tolerogeneic impulses in the form of rejection crises within the first 2 weeks after transplantation followed by long-term tolerance. We believe that this struggle represents the initial alloresponse, during which activation and expansion of both alloreactive and tolerogeneic cell populations occur. Evidence for this is that Treg upregulation requires exposure to antigen and T cell activation (56, 66, 124-127), and we have shown that, in the early period after transplantation, the population of recipient T cells and macrophages infiltrating both accepting and rejecting grafts were relatively similar (128). Moreover, when long-term tolerance occurs after transplantation of one-haplotype class I mismatched renal grafts without immunosuppression, it is preceded by a severe rejection crisis with formation of IgM but not IgG directed against donor class I (20, 23). This finding serves as additional evidence that an active immunologic process contributes to Treg development. Conversely, it has been shown that suppressing T cell activation and IL-2 formation via calcineurin inhibitors during the early period after transplantation interferes with expansion and maintenance of Tregs and inhibits long term tolerance (129).

We similarly observed the competition between immunogeneic and tolerogeneic impulses in our current model. For example, it could be seen after the second donor-matched kidney transplantation in the two animals that survived long-term. In both, creatinine increased transiently during the first 2 weeks before returning to baseline. It could also possibly be seen in the brief period of darkening and mottling of the donor skin grafts roughly 1 week after transplantation, after which the skin returned to a lighter shade until ultimately rejected due to skin-specific antigens. Both observations are consistent with the hypothesis that the Treg population was expanded by direct allopresentation at these timepoints, although at least some component of the change in skin color could have resulted from new vascularization. Evidence for the struggle between immunogeneic and tolerogeneic impulses could also be seen in our *in vitro* data as both anti-donor CML and MLR responses increased after skin-plus-peptide. While this finding certainly represents expansion of the alloreactive T cell population, it could also represent the alloresponse necessary for the expansion of the Treg population.

The timing of this immunologic struggle is likely important. It is highly probable that, if donor skin were placed in the days or weeks after peptide inoculation rather than simultaneously, the unopposed action of the peptide would lead to expansion of the alloreactive population without the concurrent tolerogeneic effect. Therefore, we suspect that both the donor skin and subsequently the second donor-matched kidney would be rejected in an accelerated fashion.

To date, we have been unable to prove this hypothesis via *in vitro* assays. Primary co-culture assays using peripheral blood drawn when the skin grafts grew darker show increased rather than suppressed anti-donor responses. Therefore, we cannot say definitively that there is expansion of a Treg population that is capable of suppressing naïve or memory alloreactive T cells during this time. It is possible, however, that there is expansion of a Treg population during this time that either does not have a suppressive effect *in vitro* or that does not show such an effect until later. Regarding the former, we and others have shown that demonstration of neither donor-specific hyporesponsiveness nor *in vitro* suppression is necessary for long-term survival of a graft (90). Moreover, our assays may not be sensitive enough to show a suppressive effect from a number of Tregs that may be clinically significant but whose effect is so closely balanced with that of an alloreactive population as to be undetectable *in vitro*. Regarding the idea that an *in vitro* suppressive effect might not be apparent until days to weeks later, a secondary CML co-culture assay performed just prior to second donor-matched kidney transplantation in an animal that later accepted its second kidney long-term did not show a suppressed anti-donor response (data not shown). This could further indicate that a Treg expansion that is clinically significant might not be detectable *in vitro*. It could also mean that our Treg expansion hypothesis is incorrect.

Finally, we draw a few limited conclusions regarding humoral immunity. First, we see that, while class Ic peptide immunization in naïve swine causes formation of antibody directed against peptide but not against SLA^{gg} cells. Anti- SLA^{gg} antibody forms only when recipients are exposed to actual donor tissue, whether in the form of PBMC, skin,

or kidney. This illustrates an important concept about the conformation of the class Ic peptide we inject as compared to actual MHC class Ic. Proteins have a specific conformation, which is determined by the chemical properties and sequence of their amino acids. Antibodies directed against a portion of the protein recognize both the sequence and the conformation. However, if the same portion of the protein is isolated as a polypeptide, it may fold in the native conformation (the conformation in which it exists in the protein) or in any number of other random conformations (130-132). This is depicted artistically in Figure 14.

It remains unclear why class Ic peptide immunization sensitizes recipients against SLA^{gg} cells on a T cell level without stimulating the production of antibody against SLA^{gg} cells as exposure to actual donor tissue does. The reason likely involves differences in conformations between the injectable class Ic peptide and MHC class Ic as discussed above. Given the lack of anti-donor antibody production after inoculation with class Ic peptide in naïve animals, it also remains unclear as to how antibody against SLA^{gg} is produced after peptide inoculation during the absence of antigen period. As we have shown that anti-donor antibody (as opposed to anti-peptide antibody) forms only after exposure to actual donor tissue, and these animals were previously exposed to a donor kidney, the most likely scenario is that B cells were primed against donor antigen earlier when the kidney graft was present but did not produce antibody until exposed to peptide during the absence of antigen period. One possible explanation for this is that the continued presence of donor antigen in a tolerant animal has some sort of suppressive effect on B cells, much as we propose that it has on T cells (perhaps through the same

direct antigen presentation mechanisms hypothesized above). This would be the first time that such an effect is demonstrated. These questions are now the focus of ongoing research in our laboratory.

The second limited conclusion we draw regarding humoral immunity is that there appears to have been some form of anti-donor humoral response following skin-plus-peptide challenge, and differences in this response seem to correspond with different outcomes. We previously described a strong anti-donor humoral response after inoculation with peptide alone during the absence of antigen period, including anti-donor IgG seen on flow cytometry and dark kidneys after reperfusion of donor-matched kidney grafts followed by low urine output that never increased, death within 3-5 days, and evidence of accelerated acute humoral rejection on histology. In our current experiment, we similarly demonstrated an antibody response *in vivo* after skin-plus-peptide as evidenced by the presence of spot hemorrhages after reperfusion of the second donor-matched kidney graft, the early damage to some of the grafts on physical inspection (dark and soft with little urine production), the antibody deposition seen on immunohistochemistry as soon as 1 hour after reperfusion, and the large and hemorrhagic appearance of grafts that rejected acutely. This response was also demonstrated *in vitro* by the increased levels of anti-donor antibody detectable in serum after skin-plus-peptide. The increase was larger and more sustained in animals that rejected acutely than in animals who survived for longer periods after their second donor-matched kidney transplant, and the antibody in these animals was found to be more cytotoxic. It is therefore clear that anti-donor antibody produced after skin-plus-peptide was sometimes lower in amount, in affinity, or in

potency than after peptide alone, and it was in these cases that long-term survival was possible. Animals that maintained higher levels of antibody or of cytotoxicity rejected almost as quickly as those receiving peptide alone. Moreover, the longest surviving kidneys were those with the best clinical appearance after reperfusion. Several questions persist regarding the effect of skin-plus-peptide on antibody. Therefore, the specific modulations in antibody level, specificity, and cytotoxicity after skin-plus-peptide are the subject of our current research and will be discussed in depth in the future.

In conclusion, we describe a possible tolerogeneic role of skin grafts when placed on animals previously made tolerant to that donor. When placed several weeks after removal of the original renal graft, they do not accelerate the breaking of tolerance when a second donor-matched kidney is subsequently transplanted without immunosuppression, and they may in fact at least partially negate the immunogeneic effect of inoculation with donor-type class I peptide. The most likely mechanism is by expanding the previously existing Treg population preferentially to the alloreactive cell population through direct allopresentation. To our knowledge, this is the first report of skin acting in this manner. Future work will aim to repeat these findings with additional animals, explore the nature of the antibody produced, and further characterize the mechanism responsible for prolonged survival after skin grafting during the absence of antigen period.

VI. Figures

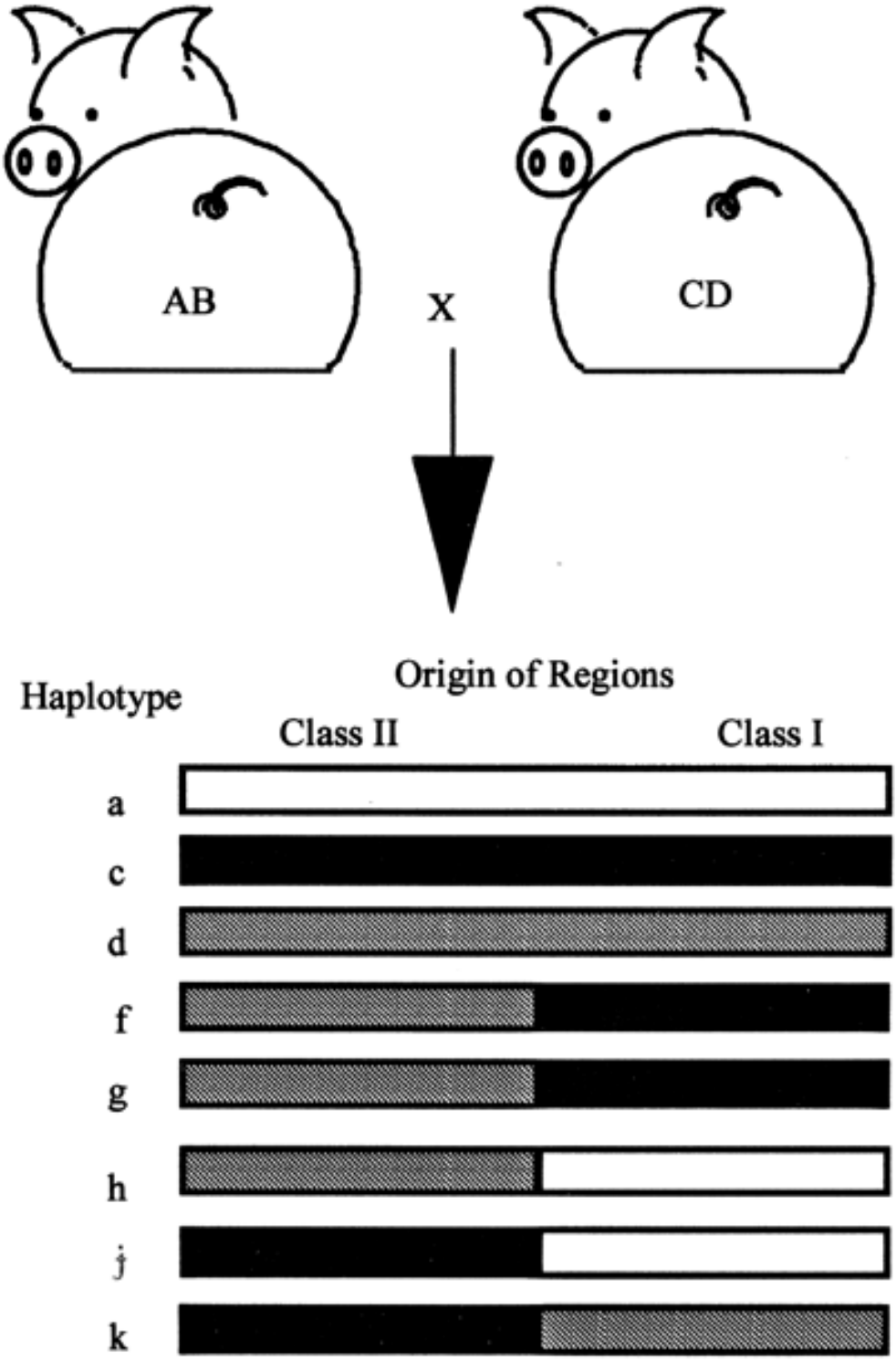
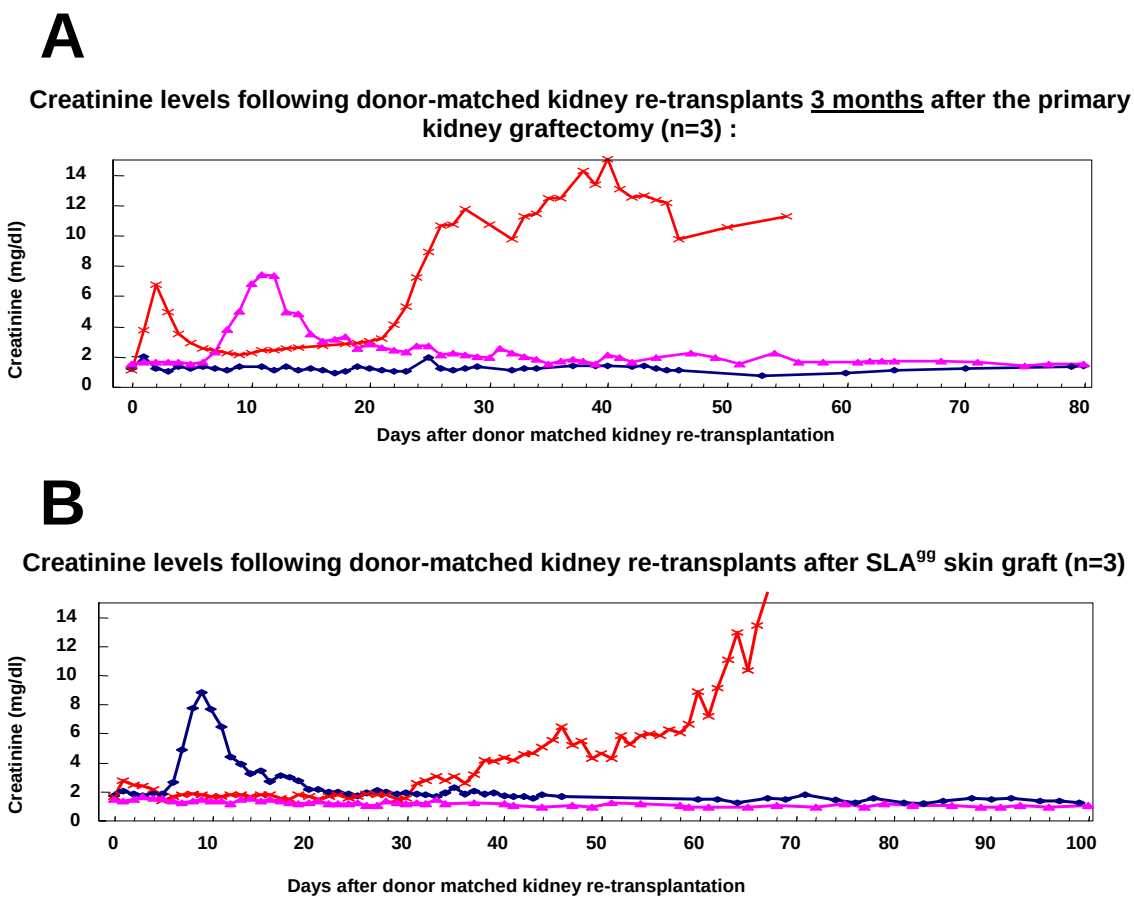


Figure 1: MHC class I and class II haplotype combinations in MGH miniature swine.



Source: Okumi et al. *Transplantation* 85:270-280.

Figure 2: Clinical course following second donor-matched kidney transplants without immunosuppression after absence of antigen period with and without skin grafts.

Following second donor-matched kidney transplants without immunosuppression after a 12 week absence of antigen period, 2 recipients survived long-term with stable renal function (after an early transient increase in one) (A). The same was found when donor-type skin grafts were placed midway through the absence of antigen period (B).

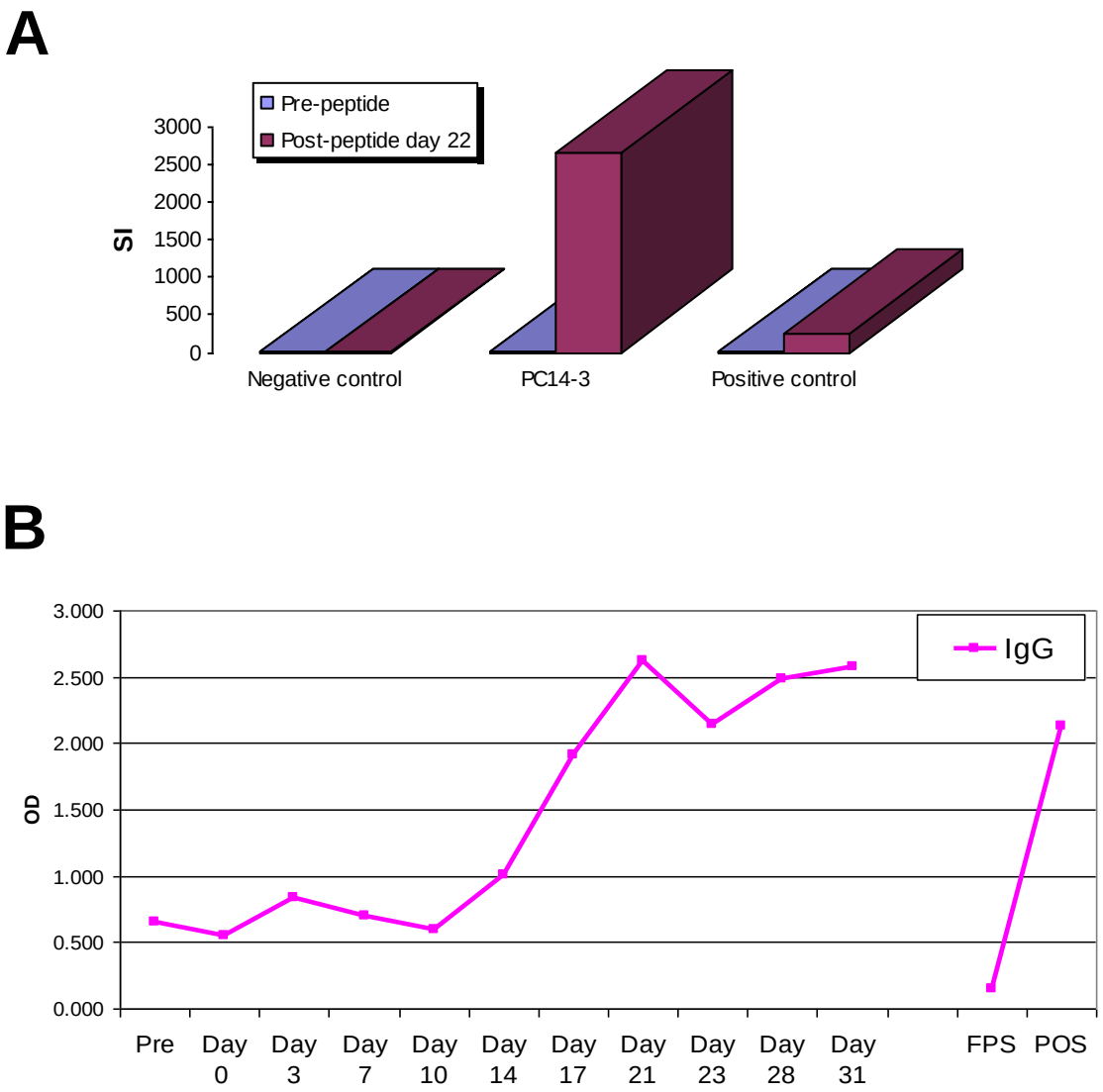
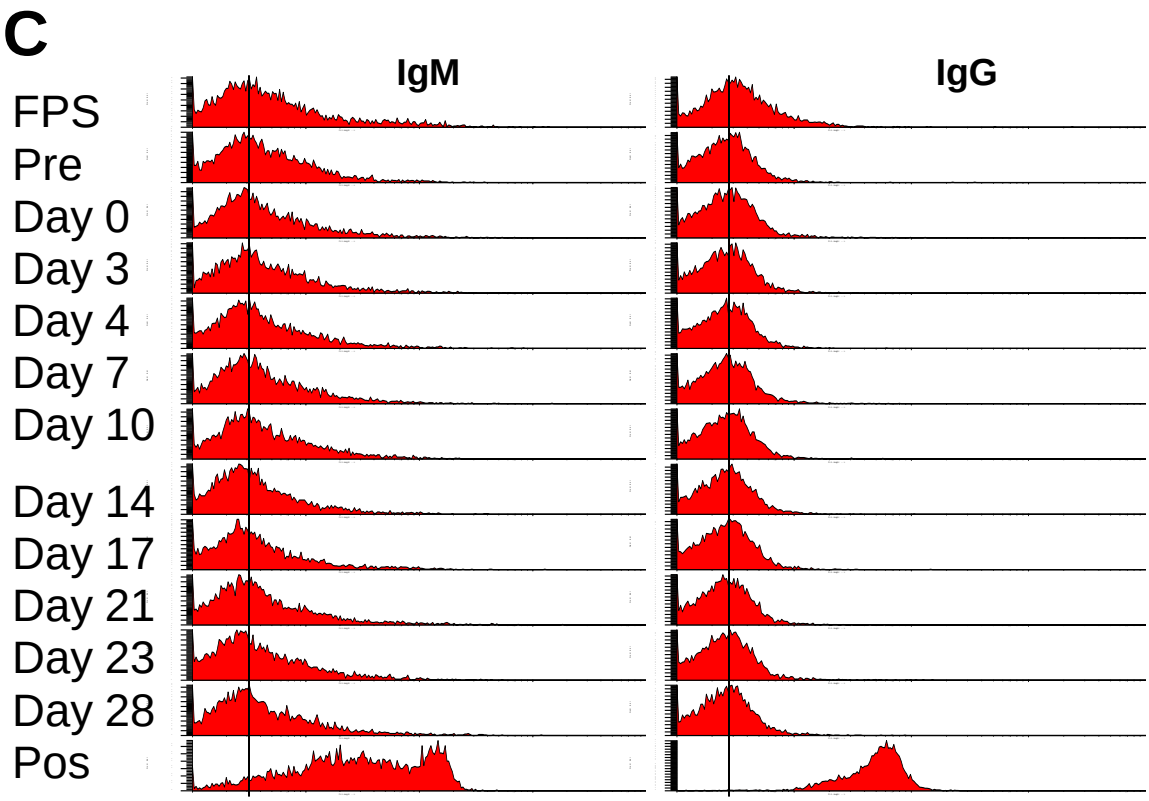


Figure 3: In vitro responses of naïve pig to class Ic peptide immunization.
After inoculation with class Ic peptide, the control pig had a robust MLR response to the PC14-3 peptide in a peptide proliferation assay (SI = stimulation index as described in Methods section) (A) and produced IgG against the peptide by around day 14 as measured by ELISA (OD = optical density) (B), but it did not produce antibody against SLA^{gg} cells (C). In part C, note that FPS refers to our negative control "fetal porcine serum," and our positive control is serum from an animal sensitized against SLA^{gg} cells. The vertical black line in the IgM and IgG rows is drawn through the peak levels in the histogram of the negative control samples and represents the baseline level of antibody.



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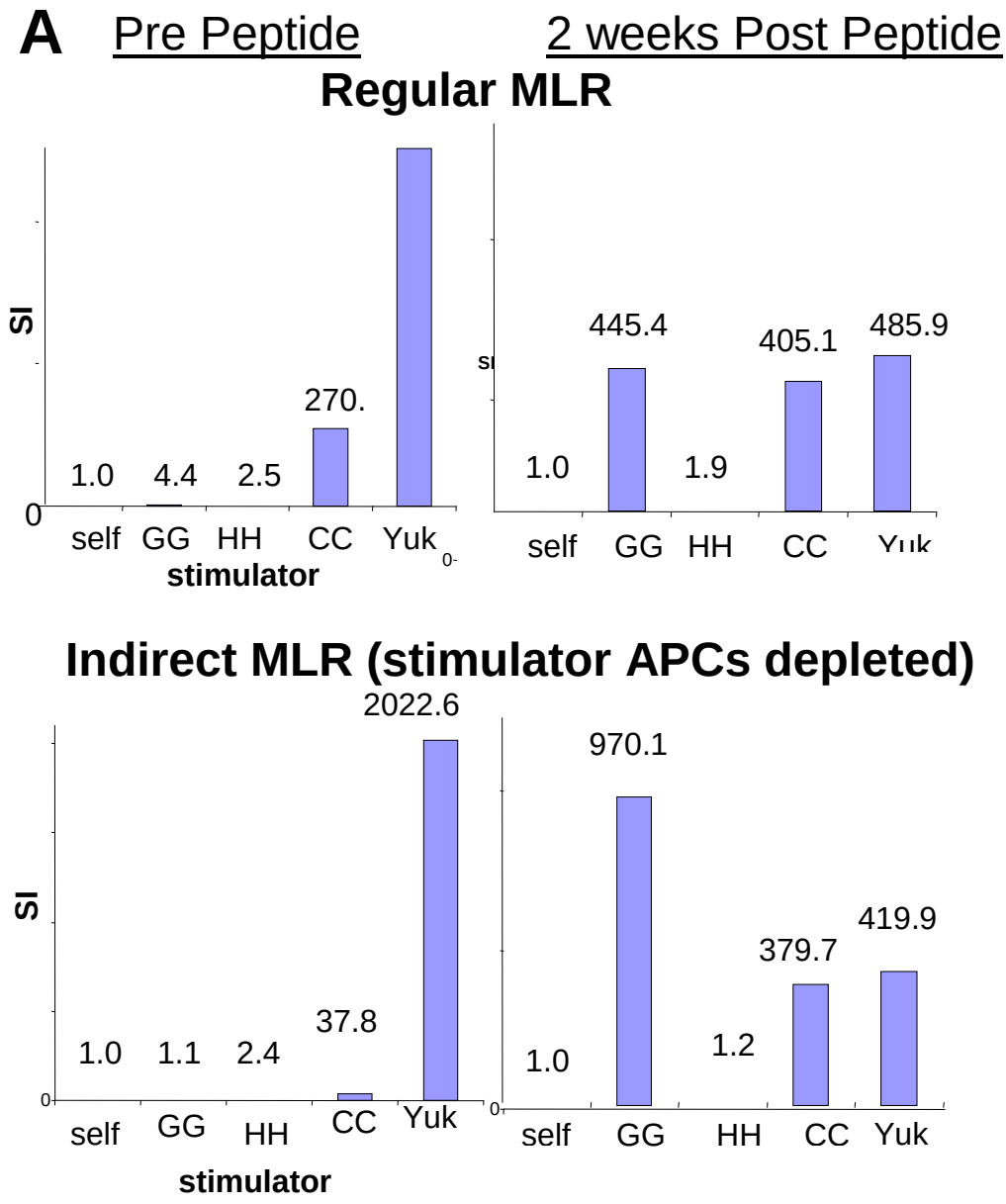
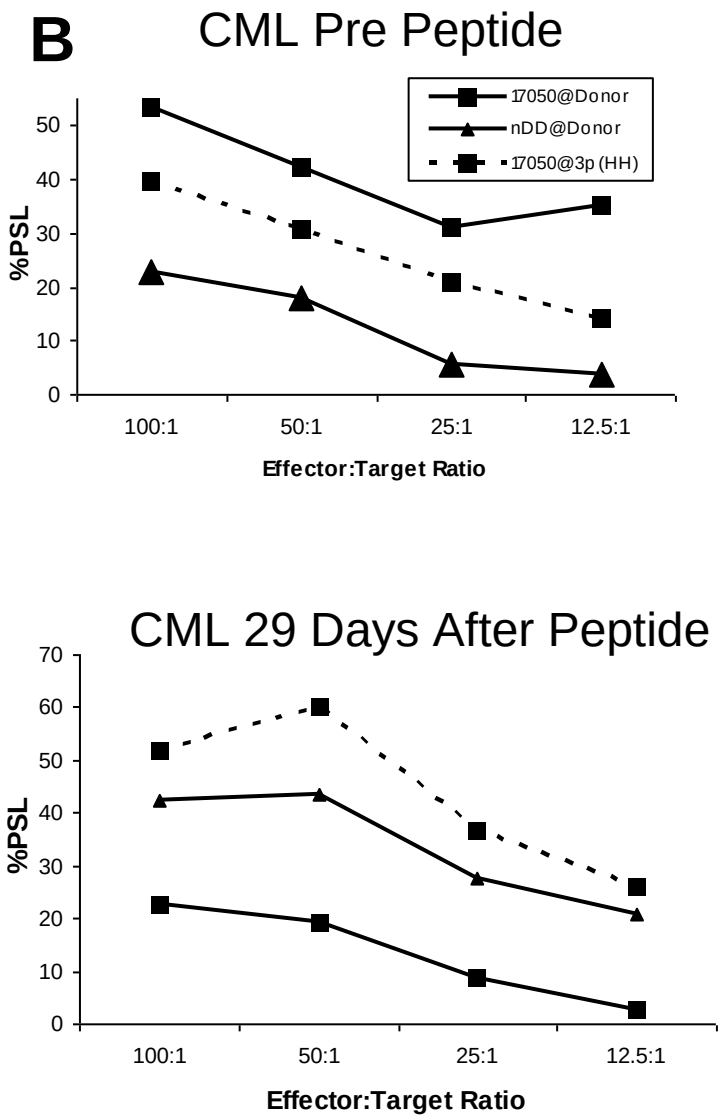


Figure 4: CML and MLR results in naïve pig after class Ic peptide immunization. (A) After this naïve SLA^{dd} pig (17050) was inoculated with class Ic peptide alone, the MLR response to SLA^{gg} cells increased dramatically, especially when antigen was indirectly presented (bottom portion of figure). This is consistent with the idea that the pig was sensitized on an indirect level since peptide can only be presented indirectly. (B) Although anti-donor MLR responses were increased after peptide, CML responses to donor cells did not increase after peptide inoculation. SI = stimulation index. %PSL = percent specific lysis. nDD = naïve SLA^{dd} pig. 3p = third party stimulator (SLA^{hh} in this case). For MLR data, self-type cells were SLA^{dd}, donor-type cells were SLA^{gg}, and third party cells were SLA^{hh} (which shares class IId with the donor and recipient but differs with both by having class Ia), SLA^{cc} (which differs from the recipient in both class I and II and shares class Ic with the SLA^{gg} donor), and Yukatan (Yuk, which are completely outbred pigs differing at all MHC loci).



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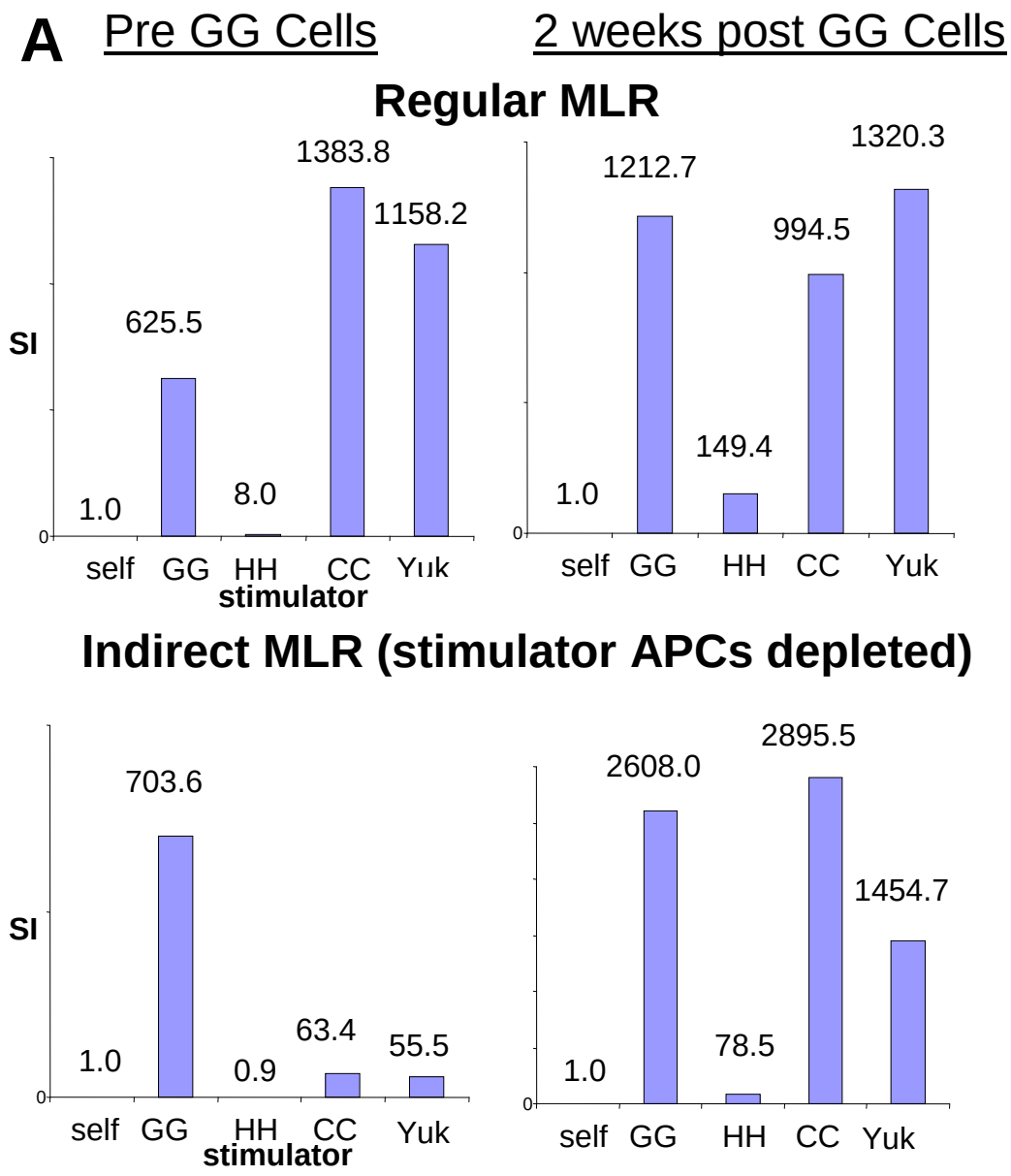
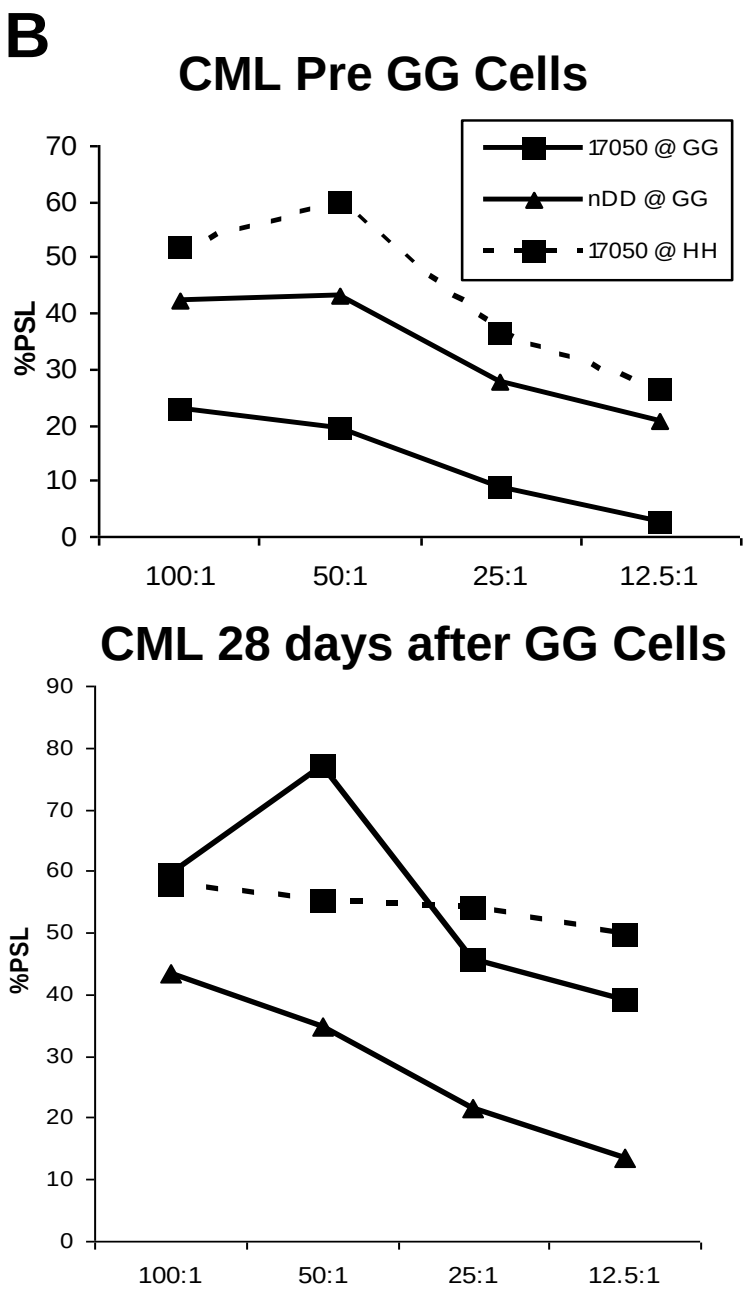


Figure 5: MLR and CML results after injection of donor cells in a pig previously sensitized by class Ic peptide.
After the sensitized control pig was injected with SLA^{gg} cells 64 days after peptide inoculation, anti-donor MLR responses increased even further (A), and now anti-donor CML responses also increased dramatically (B). SI = stimulation index. %PSL = percent specific lysis. nDD = naïve SLA^{dd} pig. 3p = third party stimulator (SLA^{hh} in this case). For MLR data, self-type cells were SLA^{dd}, donor-type cells were SLA^{gg}, and third party cells were SLA^{hh} (which shares class IId with the donor and recipient but differs with both by having class Ia), SLA^{cc} (which differs from the recipient in both class I and II and shares class Ic with the SLA^{gg} donor), and Yukatan (Yuk, which are completely outbred pigs differing at all MHC loci).



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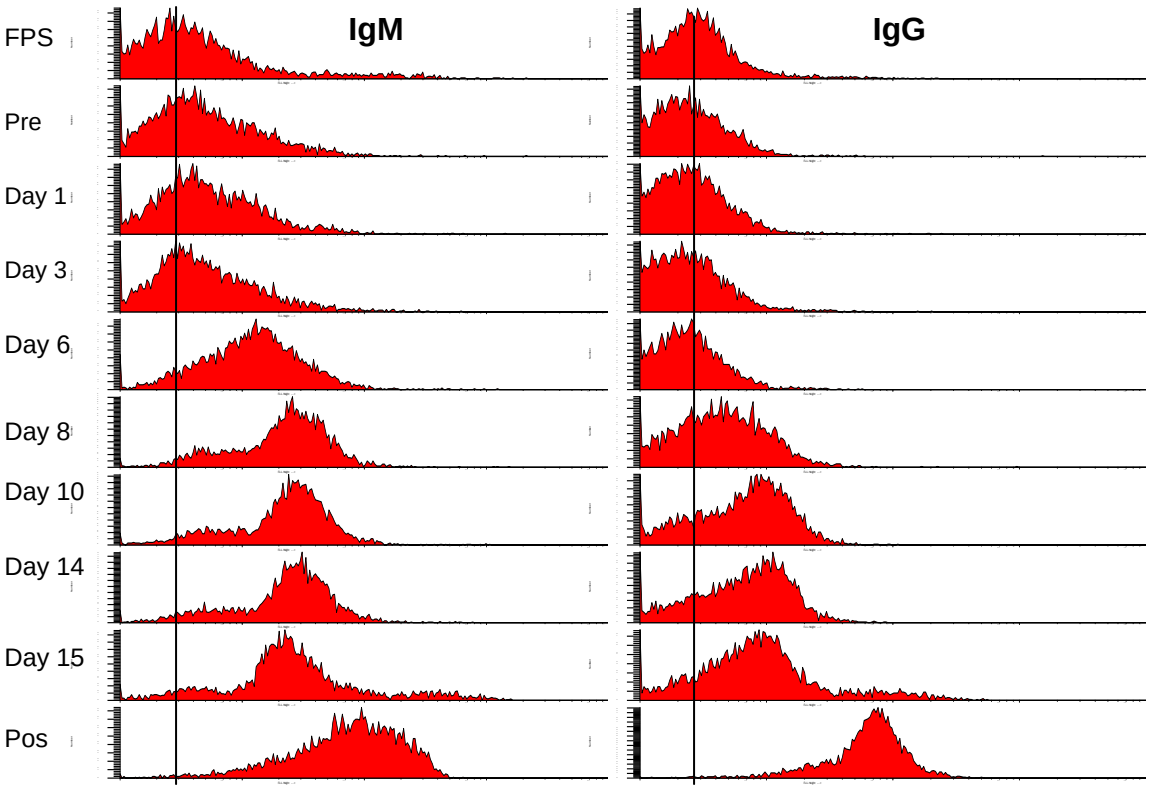


Figure 6: Antibody FACS results after injection of donor cells in a pig previously sensitized by class Ic peptide.
This figure represents the level of antibody in the serum of the recipient that binds to donor (SLA^{gg} cells) as determined by flow cytometry. After injection of SLA^{gg} cells, both anti-donor IgM and IgG were produced. As IgG was produced as early as day 8, this represents a sensitized response due to the prior inoculation with class Ic peptide. FPS refers to our negative control "fetal porcine serum," and our positive control is serum from an animal sensitized against SLA^{gg} cells. The vertical black line in the IgM and IgG rows is drawn through the peak levels in the histogram of the negative control samples and represents the baseline level of antibody.

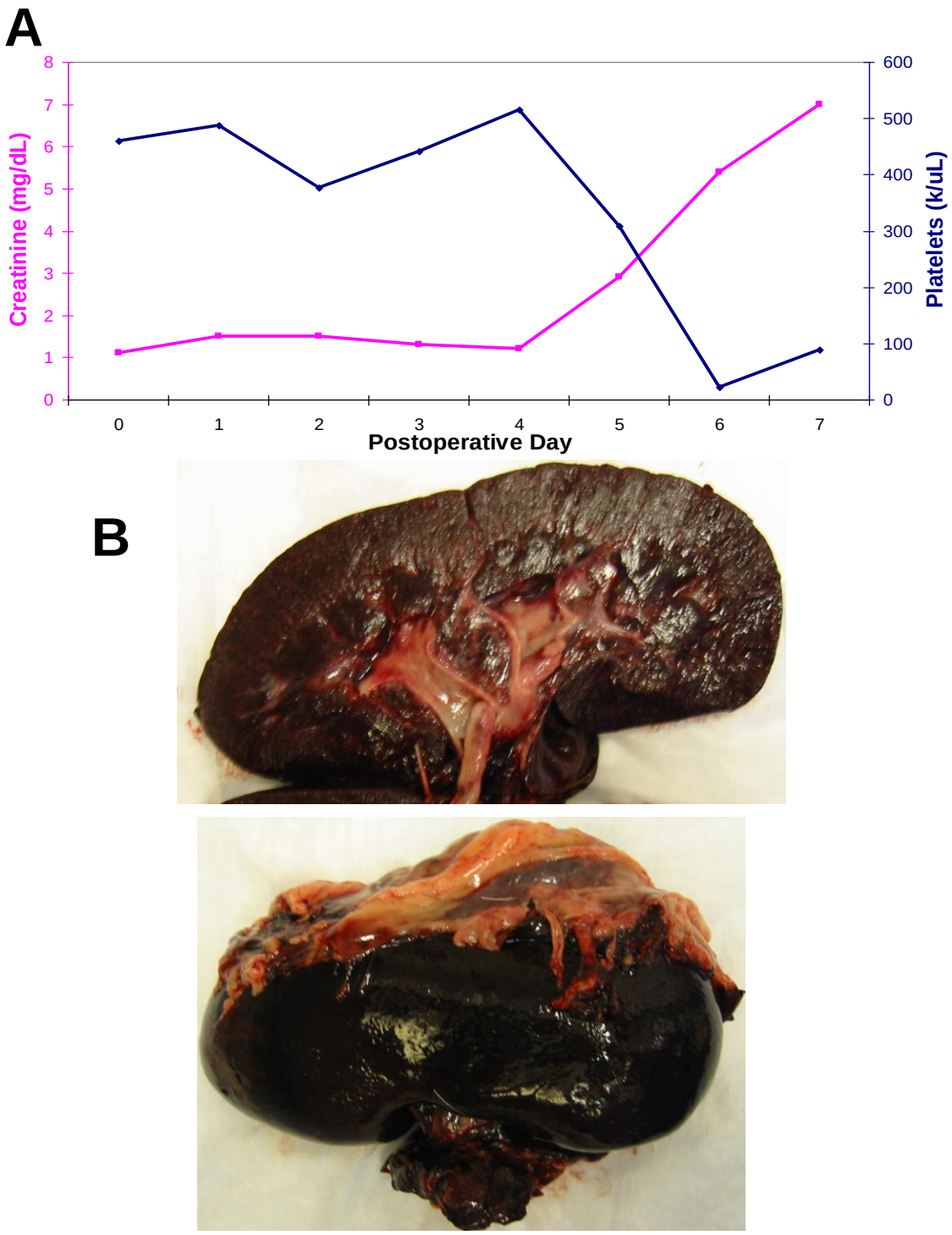


Figure 7: Clinical course and pathology after SLA^{gg} kidney transplant into pig previously sensitized with class Ic peptide immunization.
Acute increase in creatinine and decrease in platelets following transplantation of SLA^{gg} kidney with CyA treatment 21 days after immunization with class Ic peptide (A). This is consistent with accelerated acute humoral and cellular rejection as is the enlarged, hemorrhagic appearance of the graft upon necropsy (shown in hemisection on top and in whole form on bottom) (B).

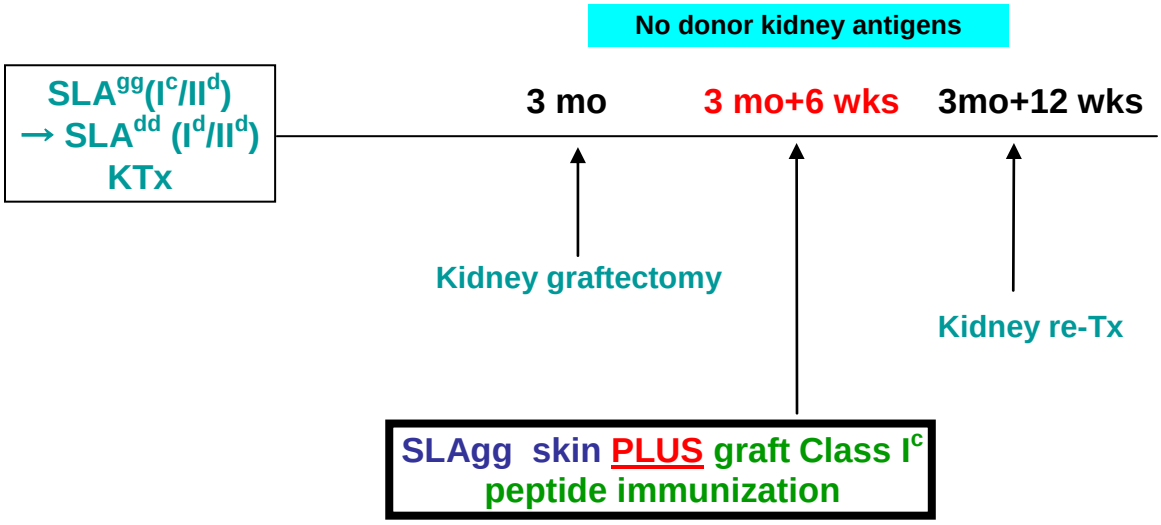


Figure 8: Experimental Plan

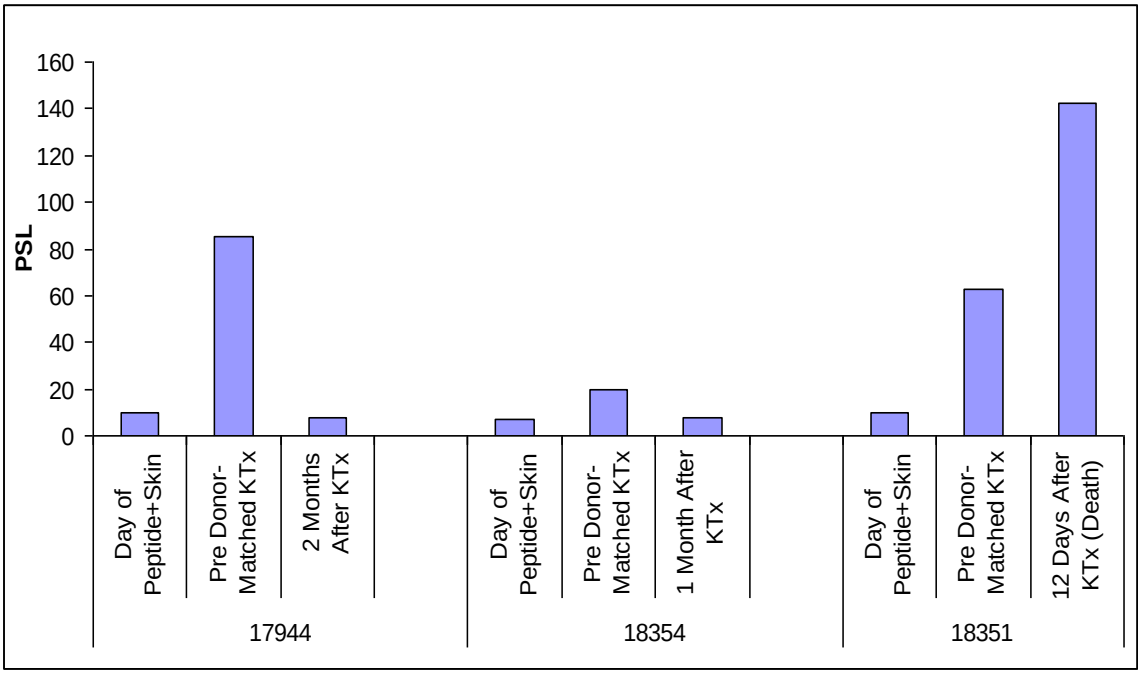


Figure 9: Anti-donor CML responses at various timepoints (50:1 effector:target ratio)

CML data showing anti-donor responses in 2 animals who accepted a 2nd donor-matched kidney long-term after skin-plus-peptide (17944 and 18354) and 1 animal who rejected at day 12 (18351). Timepoints from left to right are 1) day of skin-plus-peptide, 2) 6 weeks after skin-plus-peptide (day of donor-matched kidney transplant without immunosuppression, and 3) after donor-matched kidney transplant. Anti-donor CML responses increased in all animals but returned to baseline after donor-matched transplantation only in animals that accepted their kidneys, demonstrating that skin-plus-peptide did not break tolerance in these animals. Anti-third party responses remained normal (data not shown). PSL = percent specific lysis. KTx = kidney transplant.

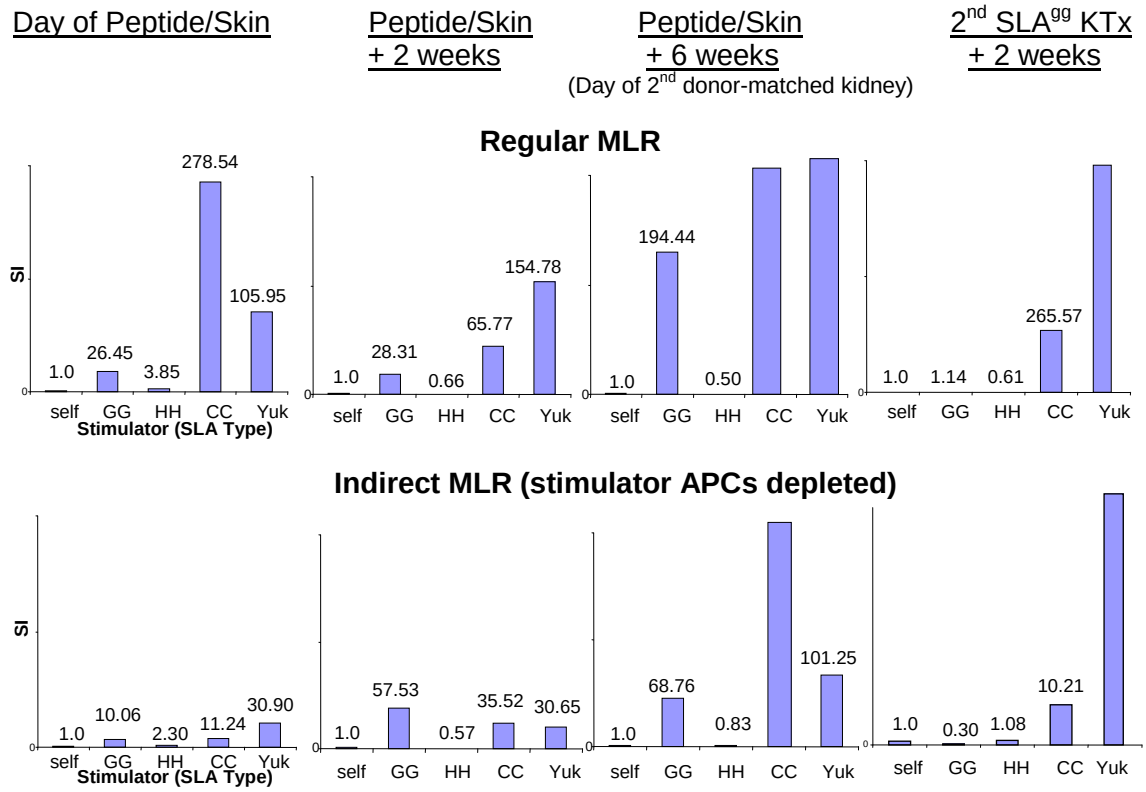


Figure 10: Anti-donor MLR responses of long-term survivor at various timepoints. This figure shows representative bulk and indirect (stimulator APC-depleted) MLR responses of a long-term survivor against various SLA types after skin-plus-peptide and 2nd donor-matched kidney transplant without immunosuppression. In all animals, specific anti-donor bulk and indirect MLR responses increased after skin-plus-peptide. In both long-term survivors, indirect responses predominated initially (significance of this is unclear). Anti-donor responses became negligible after 2nd donor-matched kidney transplantation in both long-term survivors. SI = stimulation index. KTx = kidney transplant. For MLR data, self-type cells were SLA^{dd}, donor-type cells were SLA^{gg}, and third party cells were SLA^{hh} (which shares class IId with the donor and recipient but differs with both by having class Ia), SLA^{cc} (which differs from the recipient in both class I and II and shares class Ic with the SLA^{gg} donor), and Yukatan (Yuk, which are completely outbred pigs differing at all MHC loci).

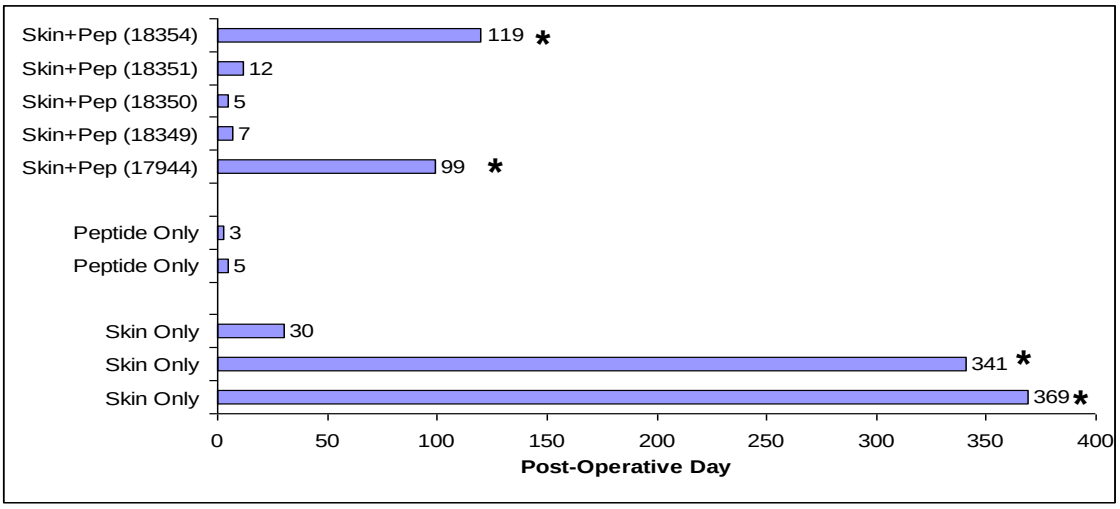


Figure 11: Postoperative survival in days after second donor-matched kidney transplant without immunosuppression in the five animals who received the skin-plus-peptide regimen compared to the two animals who received peptide alone and the three animals who received skin alone.
This illustrates the accelerated rejection in the two animals that received peptide alone contrasted with long-term survival in 2/3 of the skin-alone group and 2/5 of the skin-plus-peptide group. One animal in the skin-plus-peptide group had slightly prolonged survival (12 days). Stars indicate animals that maintained long-term tolerance and were sacrificed due to the end of their experimental course. It is assumed that these four animals would have continued to survive indefinitely.

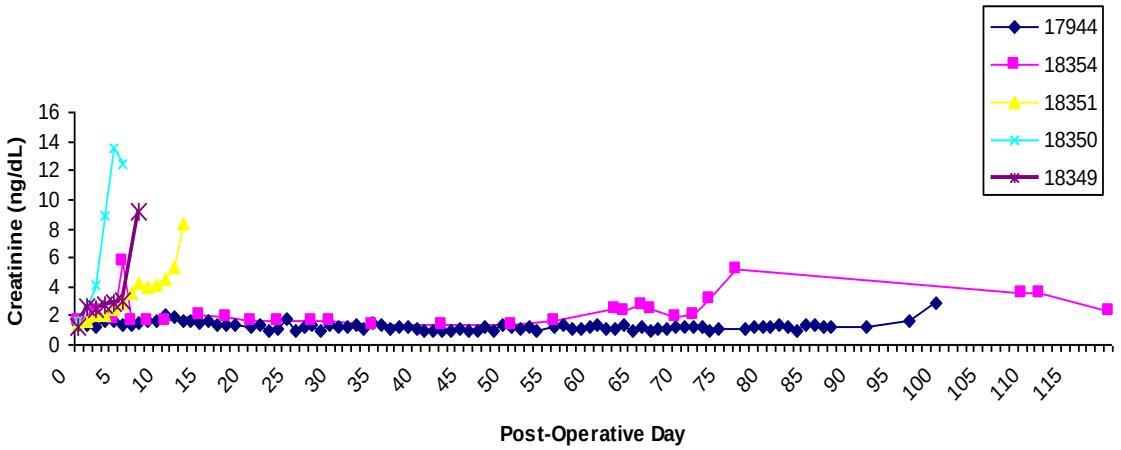
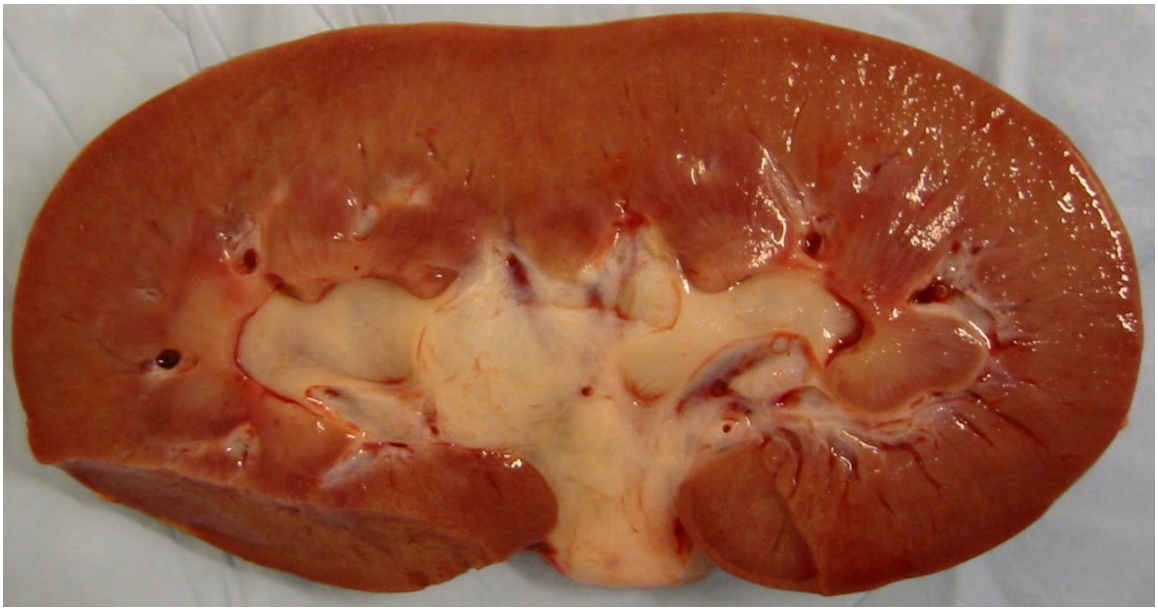


Figure 12: Creatinine levels after 2nd donor-matched kidney transplants without immunosuppression.
Creatinine levels in all 5 skin-plus-peptide animals after 2nd donor-matched kidney transplantation without immunosuppression showing long-term stable kidney function in 2 animals and slightly prolonged survival in another. In contrast, animals that received peptide alone rejected their 2nd donor-matched kidneys between days 3 and 5. The two animals who survived longest (17944 and 18354) were sacrificed due to the end of their experimental course. It is assumed that they animals would have continued to survive indefinitely.

A



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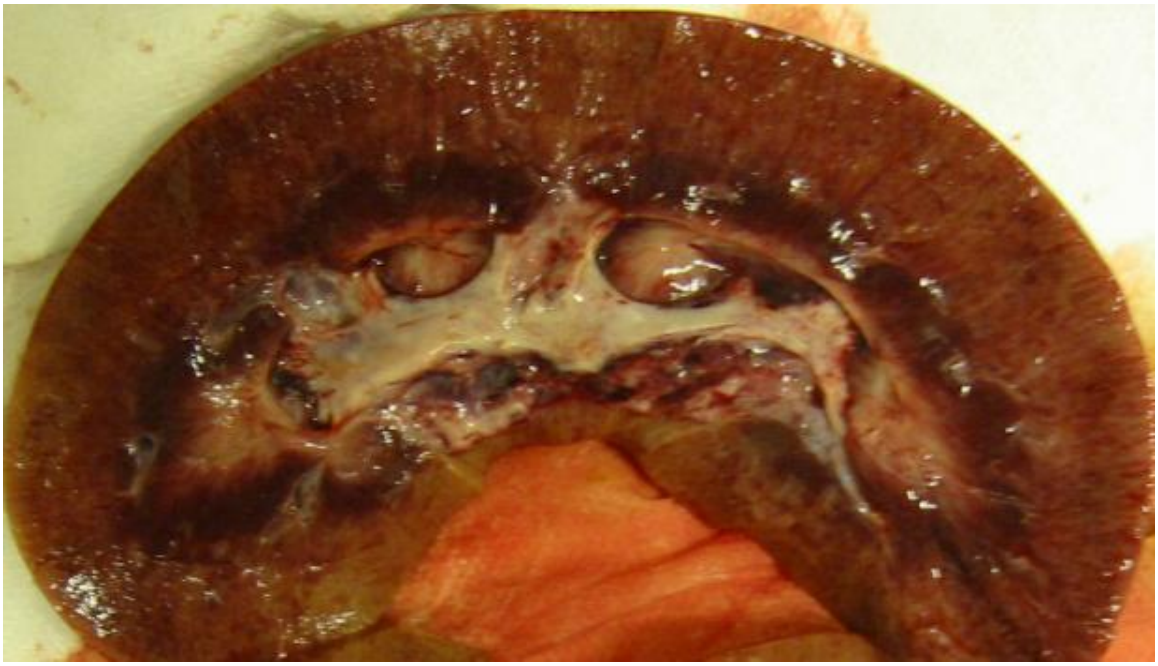

Figure 13: Gross appearance of kidney grafts at necropsy in long-term acceptor versus rejector.

A) Picture of the second donor-matched kidney from a long-term acceptor (17944) at necropsy showing normal appearing kidney.
B) Picture of the second donor-matched kidney from an animal that rejected within 11 days (18351) at necropsy showing dark, hemorrhagic appearing kidney.

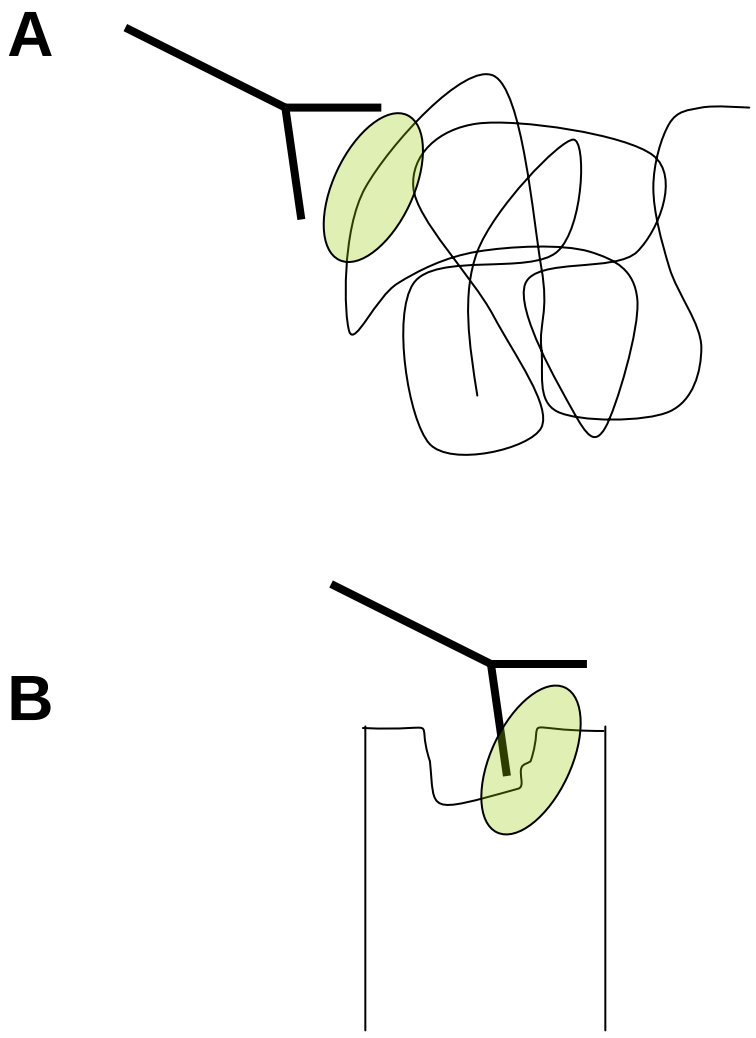

Figure 14: Artistic representation of random polypeptide conformations versus native protein structure.
Polypeptide sequences may exist in random conformations (A) or folded into a specific protein form (B). Antibodies recognize specific regions based on both amino acid sequence and conformation. Therefore, an antibody that binds the specific region (marked in green) in the protein (B) may not recognize the same sequence in a random polypeptide form (A).

VI. References

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