**MATERIALS AND METHODS**

**Animals:** Animals were cared for according to the guidelines of the Massachusetts General Hospital Institutional Animal Care and Use Committee. The immunogenetic characteristics of this herd and of the intra-MHC recombinant haplotypes available have been described previously (1) (2). Recipient animals were all of the SLA\(^{dd}\) haplotype.

**Kidney Transplantation and Re-transplantation:** The surgical procedures for primary transplantation and re-transplantation of kidneys have been previously described in detail (3) (4).

**Skin Grafts:** Split-thickness (0.22 mm) skin grafts (4×3 cm) were harvested from donors with a Zimmer dermatome and placed on graft beds on the dorsum of recipients. Donor and donor-matched skin were SLA\(^{gg}\) (class I\(^c\) class II\(^d\)). Third-party class I-mismatched skin was SLA\(^{hh}\) (class I\(^a\) class II\(^d\)). Skin grafts were assessed for viability by color, warmth, and softness to touch. They were assessed daily and were regarded as rejected when less than 10% of the graft appeared to be viable.

**Immunosuppression and Rejection Monitoring:** CyA (Sandimmune) was generously provided by Novartis Pharmaceutical Corp. (Hanover, NJ) and administered as an intravenous suspension at a dose of 10 to 13 mg/kg/day (adjusted to maintain a blood level of 400–800 ng/ml) for 12 days, starting on day 0 (the day of the primary renal transplantation). Whole blood trough levels were determined using an ARCHITECT immunochemistry analyzer (Abbott Labs, Abbott Park, Illinois).
Histopathology and Immunohistochemistry: Renal open-wedge biopsies were performed on days 30 and 60 or for episodes of renal dysfunction. Allograft rejection was scored by standard pathologic criteria (5). Immunohistochemical staining for anti-donor immunoglobulin IgM and IgG deposition in renal allografts was examined by fluorescence microscopy on frozen sections (6).

Antibodies and Flow Cytometry: The presence of anti-donor class-I IgM and IgG in the serum was detected by indirect flow cytometry. Fluorescence-activated cell sorting (FACS) was performed using a Becton Dickinson FACScan microfluorometer (Sunnyvale, CA).

Synthetic MHC Class-Ic Peptides: 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 Ic (donor type) and MHC class Id (recipient type) genetic sequences (7). Four MHC class-Ic peptides spanning the full length of the hypervariable regions of the P1 alpha-1 helix were synthesized (8) 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-Ic peptides spanning the full length of the hypervariable regions of the P14 alpha-1 helix were synthesized and labeled as PC14-1 (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.
Allopeptide Immunization: 500 µg of each peptide in 750 µL of complete Freund's adjuvant were injected subcutaneously 6 weeks after graftectomy of the original donor kidney. Peripheral blood mononuclear cells (PBMCs) from the prospective recipients were tested for in vitro proliferative responses against individual allogeneic peptides 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.

Delayed-Type Hypersensitivity (DTH) Responses: DTH responses were evaluated 2 weeks after allopeptide immunization by injecting 100 µg of each 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 and 72 hours 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.

Preparation of Peripheral Blood Mononuclear Cells (PBMCs): For separation of PBLs, freshly heparinized whole blood was diluted 1:2 with Hank’s balanced salt solution (HBSS; GIBCO BRL, Gaithersburg, MD) and the mononuclear cells were
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obtained by gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC) as previously described (4).

**Cell-Mediated Lympholysis (CML) Assay:** The procedure for CML assays has been described elsewhere (3,9-11). 51Cr release was determined on a gamma counter and the results were expressed as percent specific lysis (3,12).

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\text{% specific lysis} = \frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{maximum release (cpm)} - \text{spontaneous release (cpm)}} \times 100\%
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**Lymphocyte - 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 (13). Briefly, recipient PBMCs were cultured for 5 days with purified class-I and 3’rd party peptides. On the fifth day, recipient PBMCs were pulsed with radioactive thymidine, and a beta-counter was used to determine thymidine uptake. Stimulation indices greater than three standard errors from the mean of naïve cultures were deemed to be significant.

**Antigen Presenting Cell (APC) Depletion:** APCs were removed by negative sorting, using adherence to plastic and nylon wool, as previously described (14) (15). Briefly, PBMC were incubated for 4 h at 37°C in 75-cm2 plastic flask (Falcon#3023, Becton Dickinson Labware, Lincoln Park, NJ) and then passed over a column of sterile nylon wool (Fenwal Laboratories, Deerfield, IL).
APC-rich cells were also collected to use for control plates to compare with the APC-depleted plates. Cells were recovered from the flasks and aspirated into a sterile pipet. 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 h until usage.

**Bulk and APC-Depleted Mixed Lymphocytes Reaction (MLR):** MLR cultures, to test for proliferative response to alloantigen, have been described previously (16). Briefly, 4 x 10^5 responders and an equal number of irradiated (25 Gy) stimulators were incubated in 200 µl of standard MLR medium using flat-bottom 96-well plates (Costar, Cambridge, MA). Cultures were incubated for 5 days at 37°C in 4% CO₂ 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-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.

**ELISA:** As previously described (13,17), plates were coated with 50 µL of peptides (2 µg/mL) or PBS and incubated overnight at 4°C. The plates were washed and blocked by dispensing 200 µL of PBS + 0.05% Tween20 and 1% BSA with a 1-h incubation at room temperature. After washing, 1 hour incubation at room temperature, and rewashing, 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. Fifty µL SAv-HRP of developing solution (1:1000) and ABTS peroxidase were added, separated by a washing step. Absorbance was measured using a BioRad ELISA plate reader at 405 nm (BioRad, Hercules, CA).

**Flow Cytometry:** As previously described, the presence of antidonor class-I IgM and IgG in the serum of experimental swine was detected by indirect flow cytometry using monoclonal goat-anti-swine IgM and goat-anti-swine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Fluorescence-activated cell sorting (FACS) was performed using a Becton Dickinson FACScan microfluorometer (Sunnyvale, CA).

**Complement-mediated Cytotoxicity:** Cytotoxic antibodies binding to target cells were detected by complement-mediated cytotoxicity assays (18). Briefly, target cell suspensions of 5×10^6 cells/mL were serially diluted from 1:10 to 1:160. Targets were incubated with 5 µL of diluted serum, or controls, for 15 min at 37°C, followed by a second incubation with 25 µL of appropriately diluted rabbit complement in 96-well U-bottom plates (Costar, Cambridge, MA). Dead cells were identified by staining with 7-AAD. Data were acquired, and using a Becton Dickinson FACScan (San Jose, CA) and analyzed with WinList analysis software (Verity Software House, Topsham, ME).

**Statistical Analysis:** The statistical significance of outcome differences for experimental versus historic groups was calculated using the Z-test for two proportions, at a confidence level of 95%.
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