The Degree of Phylogenetic Disparity of Islet Grafts Dictates the Reliance on Indirect CD4 T-Cell Antigen Recognition for Rejection

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Cellular xenograft rejection involves a pronounced contribution of CD4 T-cells recognizing antigens in association with recipient MHC class II molecules. However, the requirement for such "indirect" antigen recognition for acute islet xenograft is not clear, especially as a function of the phylogenetic disparity between the donor and recipient species. In vitro studies show that C57BL/6 (B6) mouse T-cells respond directly to either allogeneic BALB/c or phylogenetically related xenogeneic WF rat stimulator cells while having undetectable responses to phylogenetically disparate porcine stimulator cells. Although all types of grafts rejected acutely in wild-type mice, this response demonstrated markedly differing dependence on host MHC class II antigen presentation, depending on the donor species. While BALB/c islet allografts were acutely rejected in B6 MHC class II–deficient (C2D) recipients, WF rat xenografts demonstrated marked prolongation in C2D hosts relative to wild-type recipients. Interestingly, neonatal porcine islet (NPI) xenografts uniformly survived long term (>100 days) in untreated C2D hosts despite transfer of wild-type CD4 T-cells, demonstrating that survival in C2D recipients was not secondary to a lack of CD4 T-cells seen in such mice. Taken together, these results show a marked hierarchy in the requirement for host MHC class II–restricted indirect pathway in the rejection of pancreatic islet grafts. Thus, while cellular rejection of porcine xenografts is generally quite vigorous, this pathway is relatively finite, displaying a major reliance on host MHC class II–dependent antigen presentation for acute rejection. *Diabetes* **52:1433–1440, 2003**

Understanding the mechanism of transplant rejection is critical for the development of antirejection strategies and induction of transplantation tolerance. The role of cell-mediated immunity in the rejection of islet allografts has been well studied, and considerable progress has been made in identifying the cellular requirements for the induction of this response (1–5). Most studies indicate that optimal islet allograft immunity requires both CD4 and CD8 T-cells. Monoclonal antibody therapies, including anti-CD4 (1–3), anti-CD8 (3,4), or a combination of these antibodies (3), have been shown to induce long-term islet allograft survival. Likewise, optimal reconstitution of islet allograft immunity in immune-deficient SCID mice requires both CD4 and CD8 T-cell transfer (6). Other studies have demonstrated long-term allograft survival of MHC class I–deficient islets transplanted in fully allogeneic recipients (5,7). Such studies demonstrate a major role for CD8 T-cells and donor MHC class I antigens, in addition to CD4 T-cells, for efficient islet allograft rejection.

In contrast, the role of cellular immunity in the rejection of islet xenografts is less well understood. Unlike islet allograft immunity, xenograft immunity does not appear to require MHC class I–restricted CD8 T-cells (4,8,9) nor host MHC class I expression (9). These results indicate that neither CD8 T-cells nor their corresponding MHC class I target antigens are required for islet xenograft rejection. Moreover, islet xenograft rejection generally appears to be less dependent on donor antigen-presenting cells (APCs) than allograft rejection (4,10). Based on such findings, it has been suggested that a major pathway for islet xenograft immunity is through the indirect antigen presentation whereby xenoantigens are processed by host APCs in association with MHC class II molecules and presented to CD4 T-cells (10,11). CD4 T-cells appear to be the predominant T-cell subset involved in xenograft rejection, and CD4 T-cells can trigger acute xenograft rejection independently of either CD8 T-cells or antibody-producing B-cells (12–14). However, "direct" T-cell reactivity to xenogeneic APCs can occur in some species combinations. Several groups have described robust primary xenogeneic responses in the mouse–anti-rat (15,16) or human–anti-porcine combinations (17–20). In contrast, there have been reports that show diminished or undetectable primary direct responses to xenogeneic APCs for other

species combinations, such as human–anti-mouse (21) and mouse–anti-primate (22). Also, CD8 T-cells have been shown to contribute to xenograft rejection in cases where indirect, host MHC class II–restricted reactivity cannot occur (11,23). Therefore, the relative contribution of direct and indirect antigen recognition as a function of the species combination remains controversial.

The aim of this study was to examine cell-mediated immunity to islet allo- and xenografts by comparing the relative contribution of the indirect (host MHC class II–restricted) pathway in the rejection of pancreatic islet allografts versus phylogenetically related (rat) or unrelated (porcine) xenografts in mice. Results show that the intensity of direct T-cell–dependent responses to xenogeneic APCs in vitro decreases as the phylogenetic disparity between stimulating and responding species increases. Correspondingly, islet xenograft rejection becomes increasingly dependent on the indirect pathway of antigen presentation as the phylogenetic distance between donor and recipient increases. Islet allografts are acutely rejected in the absence of host MHC class II recognition while rat islet xenografts demonstrate greatly prolonged but not indefinite survival. Strikingly, we find that porcine islet xenografts are spontaneously accepted indefinitely in MHC class II–deficient recipients regardless of whether such animals are reconstituted with wild-type CD4 T-cells. Thus, in contrast to some xenograft models, porcine islet xenograft rejection in mice is relatively finite by being markedly dependent on the host MHC class II–restricted indirect pathway of antigen recognition.

RESEARCH DESIGN AND METHODS

Animals. Inbred male C57BL/6ByJ (B6, H-2^b), C57BL/6 CD45.1 congenic (C57BL/6J.SJL-Ptprc^{a}Pep3^b/ByJ), C57BL/6-rag1$^{tm1/mom}$ (B6 $rag1^{-/-}$, H2^b), and BALB/cByJ (BALB/c, H-2^d) were purchased from The Jackson Laboratory (Bar Harbor, ME). Male MHC class II–deficient (I-A$_\beta^{-/-}$) (C2D, H-2^b) mice were obtained from Taconic Farms (Germantown, NY). Wistar-Furth (WF, RT1^U) rats were purchased from Charles River Breeding Laboratories (Wilmington, MA) and 1- to 3-day-old Landrace-Yorkshire (1.5–2 kg body wt) neonatal pigs of either sex were from the University of Alberta farm. Recipient mice were rendered diabetic by a single intravenous injection of streptozotocin (160 mg/kg body wt; Calbiochem, La Jolla, CA) 4–5 days before transplantation. All diabetic recipients had two consecutive nonfasting blood glucose levels above 17 mmol/l. Blood samples were obtained from the tail vein to monitor glucose levels using a Precision glucose meter (MediSense, Bedford, MA). All mice and rats were housed under pathogen-free condition and fed standard laboratory food, given acidified drinking water ad libitum, and cared for according to the guidelines established by the National Institutes of Health.
Flow cytometry. Freshly isolated lymphocytes before and after CD4 T-cell enrichment were directly labeled with fluoroscein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (clone RM 4.4; Becton Dickinson [BD] Pharmingen, San Diego, CA), CD8β (clone 53–6.7; BD Pharmingen), TCR (clone H57–597 BD Pharmingen), and CD45R/B220 (clone RA3–6B2, Pharmingen). Frequency determinations were calculated from single-parameter fluorescence histograms on an Elite flow cytometer (Coulter Electronics, Palo Alto, CA) after gating on viable lymphocytes.
Mixed lymphocyte reaction and cytotoxic T-lymphocyte assays. Lymph node and spleen cells were prepared by dissociation into single-cell suspension by mechanical disruption between glass slides. Erythrocytes from spleen were depleted by ammonium chloride solution buffer (Sigma). Where indicated, purified CD4 or CD8 T-cells were obtained from lymph nodes by negative selection using enrichment immunoaffinity columns (Cedarlane Laboratories, Hornby, ON) following the manufacturer's instructions. Isolated CD4 or CD8 T-cells were collected and the purity of eluted cells was assessed by flow cytometry. Such purified populations contained <0.5% of the depleted T-cell subset or B-cells (CD19$^+$ cells). Mixed lymphocyte cultures were established by combining 2×10^5/well responder cells (B6, C2D lymph node cells or purified CD8$^+$ T-cells) with 3×10^5 irradiated (2,500 rad) splenic stimulator cells from BALB/c mice, WF rats, or neonatal pigs. Quadruplicate

cultures in a total volume of 0.2 ml of Eagle's modified essential medium supplemented with 10% FCS, 1×10^{-5} mol/l 2-mercaptoethanol, and 1% antibiotics were established in 96-well flat-bottom plates at 37°C in 10% CO$_2$ in air. T-cell proliferation was detected by pulsing cells of primary culture with 1 μCi [^{3}H]thymidine for 6 h on the indicted day of culture. Cells were harvested onto glass microfiber filters (Wallac, Turku, Finland), and counting of samples was done using the Wallac beta emission counter (Gaithersburg, MD). Parallel bulk cultures were established in 24-well cultures using 2×10^6 responding cells with 2×10^6 irradiated stimulator cells in 2.0 ml of media as described above. Cultures were assessed for cytotoxic activity using a standard 4-h ^{51}Cr release assay (24) on spleen (BALB/c) or lymph node (WF rat) blast target cells.
Islet isolation and transplantation. BALB/c and WF rat pancreatic islets were isolated by collagenase (Sigma) digestion of the pancreas, purified by Histopaque (Sigma) density gradient, and then handpicked for transplantation as described (24). Neonatal porcine islets were isolated as previously described (25). Briefly, neonatal pigs were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreas was removed, placed in Hanks' balanced salt solution (HBSS), cut into small pieces, and digested with 2.5 mg/ml collagenase (Sigma). Digested tissue was filtered through a 500-μm nylon screen and was then cultured for 5–7 days in HAM's F10 medium (Gibco Laboratories, Grand Island, NY) containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, Canada), 0.5% BSA (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 3 mmol/l CaCl$_2$, 10 mmol/l nicotinamide (BDH Biochemical, Poole, U.K.), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C (5% CO$_2$, 95% air). A total of 500 freshly isolated rodent islets or 2,000 cultured NPIs were transplanted under the left kidney capsule of diabetic B6 or C2D mice as previously described (24). Engraftment was considered successful when blood glucose level was ≤10 mmol/l. Graft rejection was defined as the first of consecutive days of hyperglycemia (≥12 mmol/l glucose), and rejection was confirmed by histological analysis of the graft. Nephrectomy of the graft-bearing kidney was performed on recipients with long-term graft function (>100 days posttransplant) to confirm the graft dependence of euglycemia.
Adoptive transfer of purified CD4 T-cells. Two thousand NPI were transplanted under the left kidney capsule of diabetic B6 rag1$^{-/-}$ or C2D recipients. One week after normalization of blood glucose, B6 rag1$^{-/-}$ and C2D mice bearing established porcine islet xenografts were injected intraperitoneally with 1×10^7 CD4 T-cells purified from lymph nodes of congenic B6.CD45.1 mice to distinguish the donor from recipient T-cells. Alternatively, some C2D mice transplanted with NPIs were injected with purified CD4 T-cells 1 day before transplantation to mimic the lymphocyte composition of wild-type B6 mice before transplantation. Islet xenograft function was monitored as described above, and animals were killed at the time of hyperglycemia (graft rejection) or at >100 days if blood glucose levels remain normal.
Immunohistochemical staining. Five micrometer–thick sections of formalin-fixed tissues were stained to determine the presence of insulin-containing cells. Insulin-containing cells were detected by applying guinea-pig anti–porcine insulin primary antibody (1:200; Dako, Carprinteria, CA) for 30 min, followed by the addition of biotinylated goat anti–guinea pig IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA). All such paraffin sections were counterstained with Harris' hematoxylin and eosin. The avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories) and 3,3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA) were used to produce a brown color.

CD4 and CD8 T-cells were determined with tissues snap frozen in –70° sections immersed in OCT compound. Five-micrometer sections of tissue were air dried for 10 min then fixed with acetone at 4°C for 10 min. Sections were then washed in PBS, and endogenous peroxidase activity was quenched by incubating the tissue in 3% hydrogen peroxide in PBS for 6 min. After quenching, sections were washed and then blocked with 20% normal goat serum (Sigma) in PBS. Rat anti-mouse CD4 antibody (1:500; BD Pharmingen) or rat anti-mouse CD8 antibody (1:200; BD Pharmingen) was applied to each section for 30 min at room temperature. Biotinylated rabbit anti-rat IgG secondary antibody (1:200; Vector Laboratories) was added and incubated for 20 min. ABC/HP reagent (Vector Laboratories) was applied and sections were counterstained with Harris' hematoxylin.
Statistical analysis. Statistical differences between samples in vitro were sought using paired t test. Nonparametric one-way ANOVA (Kruskal-Wallis rank sum test) was used to determine difference in the transplant experiments. A P value <0.05 was considered to be statistically significant.

RESULTS

In vitro reactivity of mouse lymph node T-cells to allogeneic and xenogeneic APCs. Initial studies set out
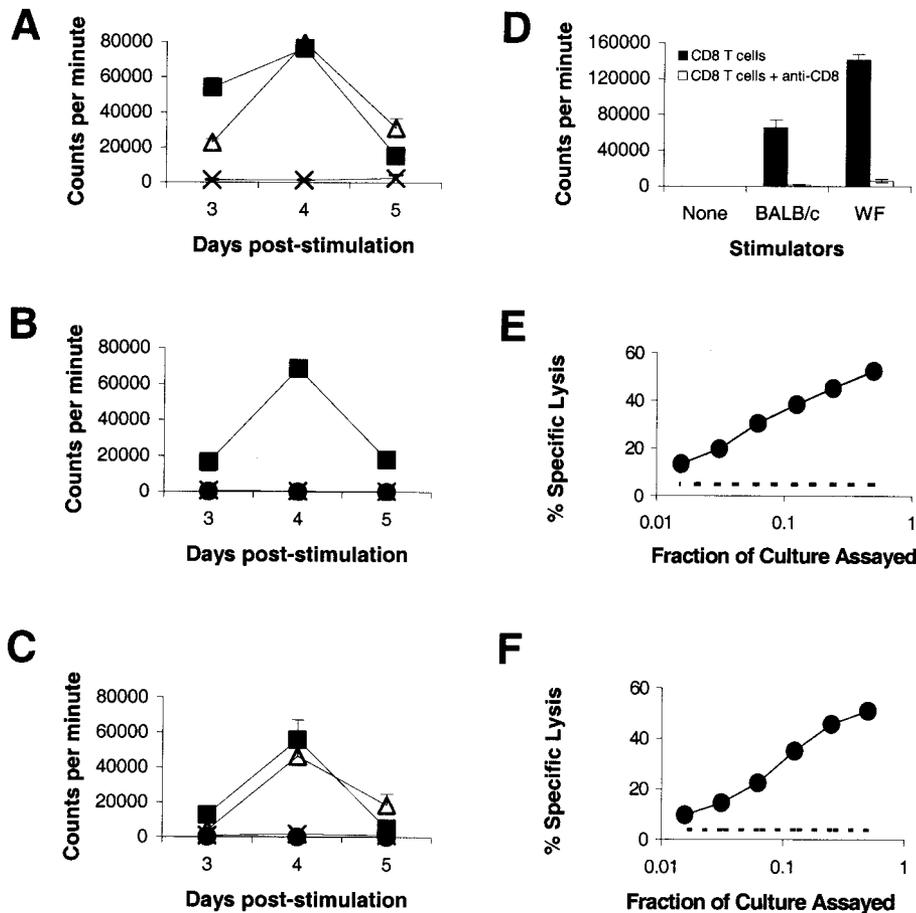
FIG. 1. In vitro proliferative and cytotoxic response of B6 lymph node cells against allogeneic or xenogeneic WF rat or porcine stimulator cells. *A*: Wild-type B6 lymph node cell proliferative response to BALB/c (■) and WF rat (△) splenocytes. *B*: Wild-type B6 lymph node cell response to BALB/c and porcine splenocytes (●). Although B6 T-cells failed to respond to porcine stimulators, they responded vigorously to control allogeneic stimulator cells. *C*: MHC class II–deficient B6 lymph node cell proliferative response to BALB/c, WF rat, and porcine splenocytes. *D*: Peak (day 4) response of purified B6 CD8 T-cells to both allogeneic BALB/c and rat splenocytes with or without anti-CD8 antibody. *E*: Cytotoxic response against allogeneic BALB/c stimulator cells. *F*: Cytotoxic response against xenogeneic WF rat stimulator cells. Lower limit indicates the lower limit of detection as assessed by mean + 3 SD of spontaneous release of ^{51}Cr from target cells alone. Responding cells cultured in media alone did not lead to cytotoxic activity above the lower limit of detection (not shown). All data shown are representative of at least three experiments using different responder/stimulator combinations. Nonstimulated responder cells (x) serve as control.

to determine the degree of direct reactivity of mouse T-cells to allogeneic BALB/c, phylogenetically related rat, or phylogenetically disparate porcine stimulator cells in vitro. Both wild-type B6 and B6 MHC class II–deficient ($I-A_\beta^{-/-}$) lymph node cells responded vigorously to both allogeneic BALB/c and xenogeneic WF rat spleen cells (Fig. 1*A* and *C*). Although there was mild variability between experiments, the vigor of reactivity to allogeneic versus rat xenogeneic APCs was comparable in most cases. In stark contrast, there was consistently undetectable reactivity against porcine stimulatory cells (Fig. 1*B*). In parallel experiments, several culture parameters were varied to determine that the undetectable reactivity to porcine cells was not due to in vitro assay conditions. Variations in conditions included the additional stimulation with anti-mouse CD28 antibody (final concentration of 5 μg/ml) or the use of different sources of porcine stimulator cells such as peripheral blood, lymph node, or spleen from neonatal and adult pig. Also, the length of culture assayed (2–7 days) and the number of responder-stimulator cell ratios were varied in the event that the cell numbers or kinetics required for detecting direct anti-porcine reactivity in vitro significantly differed from corresponding allogeneic responses. Despite these multiple in vitro conditions, the magnitude of reactivity to porcine stimulator cells remained comparable to unstimulated lymph node cells (not shown), suggesting that mouse T-cells were inherently unresponsive to porcine APCs. Furthermore, porcine lymph node cells proliferated in response to allogeneic porcine stimulator cells or to

phytohemagglutinin demonstrating that porcine APC populations could actively stimulate under the culture conditions examined (not shown). Finally, we tested the possibility that porcine APCs may be inherently inhibitory to mouse T-cells. Addition of various concentration of the nitric oxide synthase inhibitor aminoguanidine (26) in a concentration range of 2.0–0.125 mmol/l was performed and subsequently failed to restore activity, suggesting that nitric oxide was not inhibiting cultures. Also, B6-responding cells were stimulated with a mixture of allogeneic BALB/c and porcine APCs in vitro. Such coculture of allogeneic with porcine APCs did not inhibit the response to allogeneic cells (not shown), which again suggested that porcine APCs were not intrinsically inhibitory to mouse cells in vitro. Taken together, these results suggest that mouse T-cells have a markedly reduced capacity to respond directly to porcine stimulator cells relative to either allogeneic or concordant rat xenogeneic stimulator cells in vitro. It should be noted that the culture conditions used were intentionally biased to detect direct reactivity to stimulator cells, rather than detect responder APC-dependent "indirect" reactivity in vitro. Previous studies indicate that using screened FCS lots with very low background activity, flat-bottom plates, and lymph node–responding cells that are relatively low in responder myeloid APC content combine to give low primary indirect responses to allogeneic or xenogeneic cells (R.G.G., unpublished observations). Thus, primary responder APC-dependent reactivity would be unlikely to be detected in these in vitro studies.
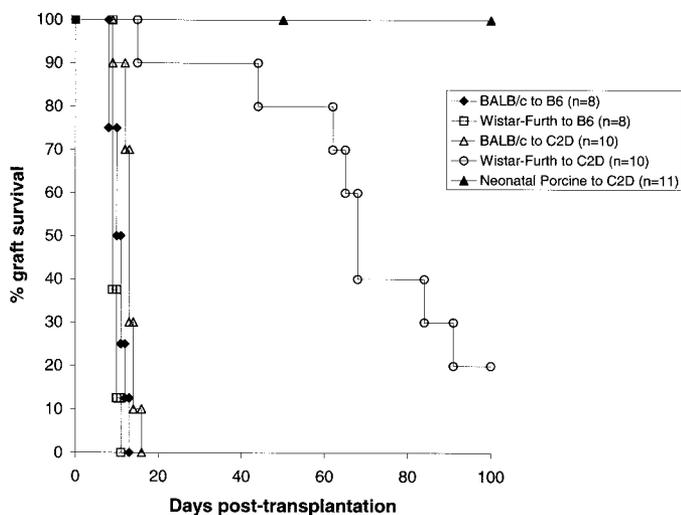
FIG. 2. Islet graft survival in streptozotocin-induced diabetic wild-type B6 or B6 MHC class II–deficient (C2D) mice.

The relative requirement for host MHC class II for islet allograft versus xenograft rejection in vivo. To determine the requirement for host MHC class II in allograft and xenograft rejection, islets were transplanted into either wild-type B6 or C2D mice. Rejection of allogeneic BALB/c islet allografts and WF rat islet xenografts occurred at a comparable rate in wild-type B6 recipients with mean graft survival times of 10.4 ± 0.6 and 9.5 ± 0.3 days, respectively (Fig. 2). All C2D recipients rejected allogeneic BALB/c islet grafts in similar tempo to that seen in wild-type B6 mice (12.9 ± 0.5 days), indicating that host MHC class II was not required for primary acute islet allograft rejection. In contrast, WF rat islet xenografts enjoyed markedly prolonged survival in C2D mice ($>69.7 \pm 7.9$ days), though most rat xenografts were eventually rejected in C2D recipients. Two mice in this group retained their graft until the end of the study (>100 days posttransplantation) and became diabetic after nephrectomy of the graft-bearing kidney, indicating that euglycemia was indeed graft dependent. Because either BALB/c or WF rat stimulator cells triggered comparable in vitro proliferative responses from either B6 or C2D lymph node cells, we determined whether purified CD8 T-cells from C2D mice also responded to allogeneic versus WF rat xenogeneic stimulator cells. Results show that purified CD8 T-cells responded vigorously to either BALB/c or WF stimulator cells as assessed by proliferative (Fig. 1*D*) or cytotoxic responses (Fig. 1*E* and *F*). Thus, despite robust CD8 T-cell reactivity in vitro, the rejection of WF rat xenografts was nevertheless much more dependent on host MHC class II expression than was the corresponding rejection of BALB/c allografts.

We then examined the requirement for host MHC class II expression in the rejection of discordant porcine islet (NPI) xenografts. In vitro experiments described above resulted in undetectable direct reactivity of mouse T-cells against porcine APCs. Such results implied that the rejection of porcine xenografts should be greatly dependent on the indirect, host MHC class II–restricted pathway of antigen presentation. To test this proposition, NPI xenografts were transplanted into wild-type B6 or in B6 C2D host as mentioned above. These neonatal porcine islet-like

tissues require several weeks to restore euglycemia in mice (25), so acute rejection required histological definition. NPI xenografts engrafted and eventually restored euglycemia in immune-deficient B6 rag1$^{-/-}$ mice, indicating the viability and capacity for function of the NPI xenografts. However, acute cellular rejection of NPI xenografts occurred within 10–12 days posttransplantation in five of five wild-type B6 recipients, as determined by histological examination of the grafts (Fig. 3*E*). Such grafts demonstrated complete destruction of donor tissue architecture and florid mononuclear cell infiltration. However, in contrast to the rejection of allografts and most WF rat xenografts, NPI transplants uniformly functioned and survived long term in C2D hosts ($>100 \pm 0.0$ days; $P < 0.01$ vs. BALB/c allograft in C2D). Diabetic C2D recipients of NPI xenografts restored their normal blood glucose levels within 8–10 weeks posttransplantation in a similar time course to that found in parallel control diabetic B6 rag1$^{-/-}$ mice (not shown). All C2D mice bearing long-term functioning NPI xenografts returned to hyperglycemia following nephrectomy of the graft-bearing kidney, confirming that the euglycemic state was graft dependent.

Examination of islet allografts and xenografts from wild-type B6 mice at the time of rejection revealed no intact islets left within the graft and mononuclear cell infiltration (Fig. 3*A*, *C*, and *E*). Similarly, no intact allogeneic islets remained in grafts harvested from C2D mice on day 11 posttransplantation (Fig. 3*B*), and few intact islets were observed at the transplant site of late-rejecting (>60 days) WF rat islet xenografts in C2D recipients (Fig. 3*D*). In contrast, examination of NPI xenografts in C2D mice harvested >100 days posttransplantation showed intact and fully differentiated islets containing numerous insulin-producing cells (Fig. 3*F*). Examination of cellular infiltrates in wild-type B6 recipients consisted predominantly of CD4 and CD8 T-cells (not shown). In C2D recipients, minor CD4 infiltration and greater CD8 infiltration was found in both islet allografts and rat islet xenografts (not shown). In most cases, a mild peri-islet mononuclear cell accumulation consisting of both CD4 and CD8 T-cells was also present at the site of the long-term functioning (>100 days) porcine xenografts (not shown).

Porcine xenograft acceptance in C2D recipients is not due to CD4 deficiency. Although results clearly indicated that porcine islet xenografts are spontaneously accepted in C2D recipients, this finding could be due to the lack of host MHC class II expression and/or to the corresponding reduction in CD4 T-cells found in C2D animals (27). To determine whether the acceptance of NPI xenografts in C2D hosts was secondary to the CD4 deficiency, C2D recipients were reconstituted with purified CD4 T-cells from B6.CD45.1 congenic T-cell donors. Due to the delayed (8–10 week) maturation of NPI xenografts in mice, it was conceivable that the inherent composition or immunogenicity of NPI grafts could change with time. Therefore, C2D hosts or B6 rag1$^{-/-}$ mice (CD45.2) grafted with NPI xenografts were reconstituted with 10^7 CD45.1$^+$ CD4 T-cells either day -1 before transplantation or 1 week after achieving euglycemia (8–10 weeks posttransplant). We previously found that CD4 T-cells transferred to C2D recipients triggered acute rejection of cardiac allografts, indicating that CD4 T-cells can mediate graft rejection in a
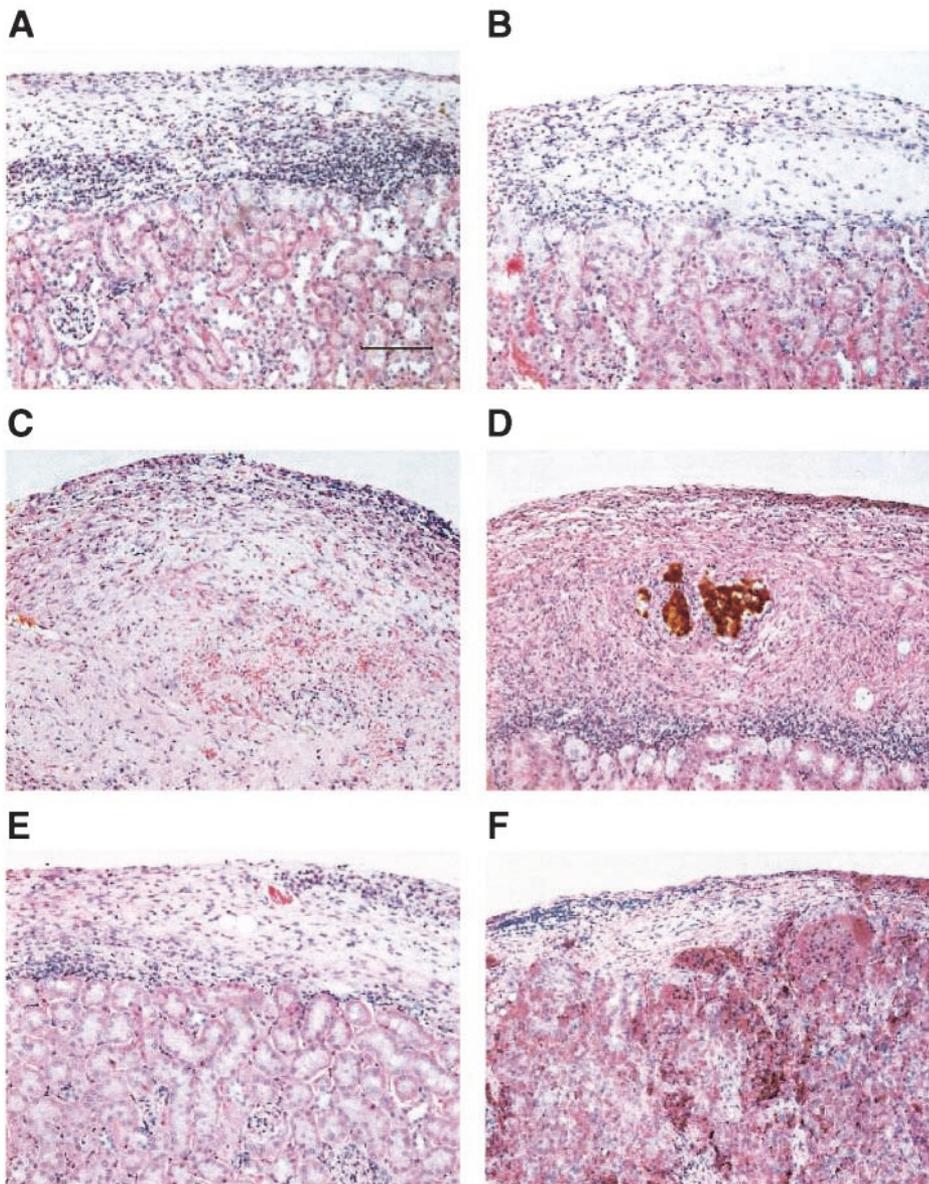
FIG. 3. Islet grafts in wild-type B6 or B6 MHC class II–deficient mice. Allogeneic BALB/c (*A* and *B*) or xenogeneic rat (*C* and *D*) or porcine (*E* and *F*) islet graft bearing kidneys were removed at the time of rejection or at the conclusion of the study (>100 days). No intact islets were observed in wild-type B6 recipients (*A*, *C*, and *E*). In contrast, numerous intact islets positive for insulin were detected in C2D mice transplanted with NPIs but not with BALB/c or WF rat islets (*B*, *D*, and *F*). Scale bar represents 100 μm.

MHC class II–deficient environment (28). The persistence of the transferred CD45.1$^+$ CD4 T-cells in C2D hosts was confirmed by longitudinal assessment of peripheral blood leukocytes and confirmed that the transferred CD4 T-cells comprised 0.2–0.5% of the recipient peripheral blood lymphocytes for at least 60 days post T-cell transfer (not

TABLE 1
Survival of neonatal porcine islet grafts in B6 MHC class II–deficient mice

Transfer of CD45.1$^+$ CD4 T cells	n	Graft survival post–CD4 T-cell transfer (days)
1) None	11	>100 × 11
2) 10^7 CD4 T-cells day −1	5	>100 × 5
3) 10^7 CD4 T-cells postfunction	3	>30 × 3

Streptozotocin-induced diabetic C2D mice were transplanted with 2,000 NPIs. Control C2D recipients were otherwise untreated (group 1). In one group, C2D recipient mice received 10^7 B6.CD45.1$^+$ CD4 T-cells either on day −1 relative to transplantation (group 2) or 1–2 weeks after normal blood glucose has been achieved (group 3).

shown). Despite reconstitution of C2D mice with purified CD4 T-cells, NPI xenografts failed to reject (Table 1). Parallel studies were performed in which purified CD45.1$^+$ CD4 T-cells were adoptively transferred into MHC class II–expressing immune-deficient B6 rag1$^{-/-}$ mice bearing NPI xenografts. Unlike results in C2D mice, reconstitution of MHC class II$^+$ B6 rag1$^{-/-}$ mice with CD4 T-cells led to rapid rejection of NPI grafts within 10 days post T-cell transfer. Histological examination of porcine xenografts in either unreconstituted B6 rag1$^{-/-}$ mice or C2D recipients showed intact islets with extensive insulin staining of islet β-cells (Fig. 4*A*, *C*, and *D*). However, reconstitution of B6 rag1$^{-/-}$ recipients with CD4 T-cells resulted in complete destruction of porcine islets and presence of infiltrating CD4 T-cells in the transplant site (Fig. 4*B*).

DISCUSSION

The contribution of host MHC class II–restricted antigen recognition to transplantation immunity and tolerance has remained a controversial issue. The present results indicate that there is a marked hierarchy in the requirement
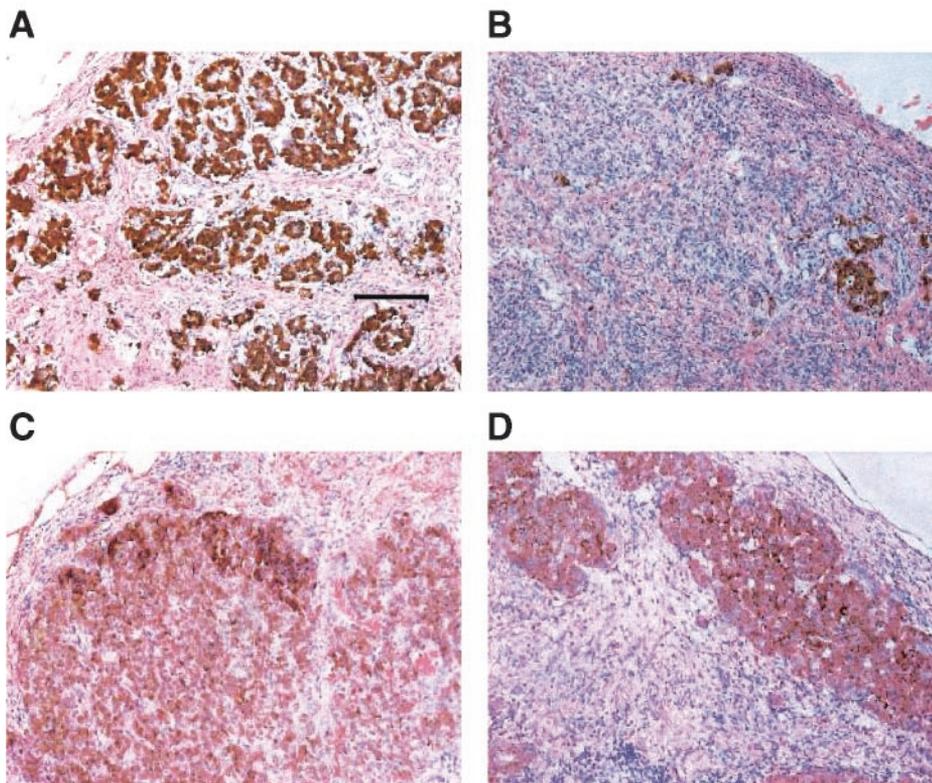
FIG. 4. Histology of islet xenografts in CD4 T-cell–reconstituted B6 rag$^{-/-}$ and C2D mice. Intact islets positive for insulin granules (brown stain) are present in grafts obtained from unreconstituted B6 rag$^{-/-}$ (*A*), unreconstituted C2D (*C*), and reconstituted C2D mice (*D*). While very few intact islets were observed in reconstituted B6 rag1$^{-/-}$ (*B*) recipient. Scale bar represents 100 μm.

for a host MHC class II indirect pathway of antigen recognition for the destruction of pancreatic islet allografts relative to xenografts. This spectrum of reactivity ranged from islet allograft rejection that showed no discernable reliance on host MHC class II recognition, to the rejection of phylogenetically related rat xenografts that showed partial dependence on this pathway, and finally to the rejection of phylogenetically unrelated porcine xenografts that was greatly dependent on an intact host MHC class II presentation pathway. Thus, while cellular xenograft rejection is generally considered to be quite vigorous in vivo (10), there appear to be striking qualitative differences between allograft and xenograft rejection with regard to host MHC class II–restricted reactivity.

In vitro proliferation experiments showed a vigorous direct reactivity of lymph node cells (from wild-type B6 or C2D mice) after stimulation with allogeneic BALB/c or xenogeneic WF rat spleen cells. This contrasts with the lack of detectable reactivity of mouse T-cells to porcine APCs under a wide variety of experimental conditions. Thus, mouse T-cells generated strong responses when stimulated with allogeneic BALB/c or closely related xenogeneic WF rat spleen cells but had a limited capacity for generating primary in vitro proliferative responses to more phylogenetically disparate porcine APCs. Other studies have also shown strong proliferative response to allogeneic but not to xenogeneic APCs in some cases (29–32), especially in the human anti-mouse (21) and mouse anti-primate (22) species combinations. At present, it is not clear what inter-species interaction limits mouse T-cell recognition of porcine APCs. One possible explanation is that there may be species specificity in a variety of accessory molecule interactions necessary for T-cell activity, as noted for the hyporeactivity seen in the mouse anti-primate direct T-cell response (22). It is also possible

that various cytokines involved in signal transmission between APCs and T-cells might not function across a wide species differences (22). Furthermore, the lack of xenoreactivity by mouse T-cells to porcine antigens could be the result of a lack of positive selection of xenoreactive T-cells due to potentially disparate MHC molecules. However, this latter possibility was refuted as an explanation for low mouse anti-human direct T-cell reactivity (33). Given that in vitro indirect reactivity to porcine cells has been observed in either human (17,39) or mouse (40) T-cells, it may be surprising that this activity was not found in our studies. However, our culture conditions were intentionally biased toward detecting "direct" responses to stimulator cells and not for detecting responder APC-dependent reactivity. Also, most studies suggest that such indirect reactivity is often best detected in primed (or grafted) recipients while primary responses are generally low.

The lack of detectable "direct" reactivity of mouse T-cells to porcine APCs would predict that the major available pathway for porcine antigen recognition by T-cells would be through donor antigens processed and presented by host APCs. The finding that porcine islet xenografts survived indefinitely in C2D hosts is consistent with this concept. An exaggerated role for host MHC class II–restricted antigen recognition in cellular xenograft rejection has been suggested by several studies (11,16,34,35). Our results extend this point by showing that in cases in which direct T-cell reactivity is undetectable, such as the mouse anti-porcine combination, the response in vivo is entirely dependent on "indirect" host MHC class II presentation. This is in contrast to a recent study reported by Yi et al. (23) in which CD8 T-cells were capable of rejecting fetal pig pancreatic islet xenografts in the absence of CD4 T-cells. This discrepancy might be

explained by the difference of porcine tissue and the method used to assess rejection. In their study, rejection was based on histological analysis of porcine islet grafts in which they observed pronounced infiltration of the grafts by CD8 T-cells. In the present study, cellular infiltration of porcine islet xenografts by CD8 T-cells was observed 10–60 days posttransplantation. However, this cellular infiltrate did not result in graft rejection and gradually diminished with all mice remaining euglycemic for more than 100 days posttransplantation. Importantly, the study by Yi et al. also utilized an MHC class II–deficient recipient generated from a different initial source than that used in our study (23).

Interestingly, porcine xenograft rejection appears to closely mirror cellular immunity to extracellular parasites in which CD4 host MHC class II–restricted immunity is a key pathway for host defense (36). It is certainly conceivable that xenograft prolongation in C2D mice is secondary to the corresponding CD4 deficiency seen in these animals (27). However, reconstitution of C2D mice with CD4 T-cells failed to trigger rejection of porcine xenografts despite persistence of the transferred cells in the C2D hosts. Such purified CD4 T-cells rapidly triggered xenograft rejection in MHC class II–bearing hosts and, importantly, we previously found that CD4 T-cells vigorously rejected cardiac allografts in C2D recipients (28), indicating that CD4 T-cells can indeed function in a MHC class II–deficient environment. Thus, host MHC class II–restricted antigen presentation appears to be a rate-limiting pathway for the rejection of porcine xenografts.

While the spontaneous acceptance of porcine xenografts in C2D mice illustrates the significance of host MHC class II–directed immunity in the response, there is another important implication from this result related to CD8 T-cells. Exogenous antigens in some cases are known to gain access to the MHC class I–processing pathway and presented to CD8 T-cells via such "cross-priming" (37,38). Regarding transplantation antigens, this phenomenon of cross-presentation represents an "indirect" host MHC class I–restricted response. Given that C2D mice have both intact CD8 T-cells and MHC class I antigens (27), the failure of such mice to reject porcine xenografts indicates that this potential form of indirect antigen presentation is not sufficient for overt rejection. By inference, the major pathway for indirect xenograft rejection appears to involve the MHC class II pathway rather than the MHC class I pathway. However, peri-islet accumulations of CD8 T-cells were consistently found surrounding porcine xenografts in C2D recipients (not shown), raising the possibility that such cross-presentation to CD8 T-cells may occur, but without apparent significant pathogenic consequences. This is in strong contrast to porcine skin xenograft rejection in which CD8 T-cells can strongly contribute to rejection (11). At present, it is not clear why porcine skin and islet xenograft rejection differ regarding the role of CD8 T-cells in rejection.

The reliance on host MHC class II for rejection in vivo only partially correlated with the degree of direct T-cell reactivity in vitro. Of interest is the finding that even phylogenetically related rat islet xenografts enjoyed marked prolongation in C2D recipients. In vitro results indicated that the inherent direct response of mouse T-cells, including purified CD8 T-cells, to rat hematopoietic stimulator cells is comparable to the corresponding response to allogeneic cells. Also, purified mouse CD8 T-cells mount robust proliferative and cytotoxic responses to rat stimulator cells. Despite such in vitro reactivity, however, rat islet xenografts nevertheless demonstrate considerable reliance on host MHC class II for overt rejection. This is in contrast to the rapid rejection of islet allografts in either the wild-type or the C2D recipients. The reason for this lack of correlation between such in vitro reactivity and xenograft rejection in vivo is not clear. However, this result raises the possibility that the requirements for CD8-mediated islet xenograft rejection in vivo differ and/or are more stringent than those necessary for islet allograft rejection. This has important implications for other xenograft situations. For example, human CD8 T-cells can clearly respond in vitro to porcine MHC class I swine leukocyte antigens in vitro (17). However, as illustrated by results of rat-to-mouse xenografts, this in vitro reactivity may not necessarily be predictive of a pathogenic response in vivo. Thus, even in cases of documented inter-species CD8 T-cell xenoreactivity, the response in vivo may involve unexpectedly high reliance on indirect CD4 T-cell–dependent reactivity. Preliminary studies using mouse or rat islet parenchymal cells as CTL targets in vitro did not demonstrate a clear difference in sensitivity to CD8 T-cell killing in vitro. That is, primed CD8 T-cells demonstrated extremely low direct killing activity against either mouse or rat islet cells (Z.A.J., unpublished observations). Thus, the exact nature of CD8 T-cell interaction with either allogeneic or xenogeneic islet cells requires future clarification.

Interestingly, although the T-cell response is generally considered more violent toward porcine islet xenografts than for allografts, this pathway actually appears to be more limited in scope. That is, while islet allograft rejection has both MHC class II–dependent and –independent mechanisms of rejection, the response to porcine xenografts appears to be greatly reliant on this indirect MHC class II–restricted pathway. However, despite being more finite in nature, this indirect anti-xenograft response is extremely virulent. An important issue requiring future investigation is the inherent reason(s) for the pronounced indirect response to islet xenografts. It is possible that both the repertoire and affinity of T-cells for processed xenogeneic antigens is greater than those generated in response to allogeneic antigens. Thus, a diminished role for direct donor MHC-restricted xenoreactive may correspond with an increased array of T-cells responding to phylogenetically diverse peptide epitopes presented on host APCs. Clear testing of this concept will require a better understanding of the specific xenograft-associated antigens targeted by this pathway. Alternatively, phylogenetically diverse donor-recipient combinations may invoke unexpected innate immune responses. For example, inter-species recognition may include unforseen donor-associated molecular pattern recognition events akin to pathogen recognition that result in heightened activation of innate immune elements. The relative contribution of an increased number or affinity of indirect xenograft-derived antigens and/or altered innate immune activation leading to exaggerated host MHC class II–restricted immunity to

xenografts remains to be clarified. Importantly, it is also not clear whether cellular pathways that predominate xenograft responses in mice will readily translate to responses found in large animal models or in humans.

Taken together, results show a hierarchy in the requirement for the indirect pathway of antigen recognition in the rejection of islet grafts. Porcine islet xenografts are markedly more dependent on the host MHC class II–restricted indirect pathway than either xenogeneic WF rat or BALB/c allogeneic islet grafts. This indicates that the relative requirement for host MHC class II–restricted antigen presentation varies greatly according to the donor species. Thus, different anti-rejection strategies may need to be developed to optimally target allograft versus xenograft responses.

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REFERENCES

1. Shizuru JA, Gregory AK, Chao CTB, Fathman CG: Islet allograft survival after a single course of treatment of recipient with antibody to L3T4. *Science* 237:278–280, 1987
2. Hao L, Wang Y, Gill RG, Lafferty KJ: Role of the L3T4+ T-cell in allograft rejection. *J Immunol* 139:4022–4026, 1987
3. Yamamoto H, Monden M, Kawai M, Uenaka A, Gotoh M, Mori T, Sakurai M, Shiku H, Nakayama E: The role of CD8+ and CD4+ cell in islet allograft rejection. *Transplantation* 50:120–125, 1990
4. Wolf LA, Coulombe M, Gill RG: Donor antigen-presenting cell-independent rejection of islet xenografts. *Transplantation* 60:1164–1170, 1995
5. Markmann JF, Bassiri H, Desai NM, Odorico JS, Kim JI, Koller BH, Smithies O, Barker CF: Indefinite survival of MHC class I-deficient murine pancreatic islet allografts. *Transplantation* 54:1085–1089, 1992
6. Coulombe M, Yang H, Wolf LA, Gill RG: Tolerance to antigen-presenting cell-depleted islet allografts is CD4 T-cell dependent. *J Immunol* 162:2503–2510, 1999
7. Osorio RW, Ascher NL, Jaenisch R, Freise CE, Roberts JP, Stock PG: Major histocompatibility complex class I deficiency prolongs islet allograft survival. *Diabetes* 42:1520–1527, 1993
8. Gill RG, Coulombe M: Rejection of pancreatic islet xenografts does not require CD8+ T-lymphocytes. *Transplant Proc* 24:2877–2878, 1992
9. Desai NM, Bassiri H, Kim J, Koller BH, Smithies O, Barker CF, Naji A, Markman JF: Islet allograft, islet xenograft, and skin allograft survival in CD8$^+$ T lymphocyte-deficient mice. *Transplantation* 55:718–722, 1993
10. Gill RG: Direct and indirect pathways of immunity to pancreatic islet transplants. *Transplant Rev* 12:85–95, 1998
11. Chitilian HV, Laufer TM, Stenger K, Shea S, Auchincloss HJ: The strength of cell-mediated xenograft rejection in the mouse is due to the CD4+ indirect response. *Xenotransplantation* 5:93–98, 1998
12. Pierson RN III, Winn HJ, Russell PS, Auchincloss H: Xenogeneic skin graft rejection is especially dependent on CD4+ T-cells. *J Exp Med* 170:991–996, 1989
13. Gill R, Wolf L, Daniel D, Coulombe M: CD4 T-cells are both necessary and sufficient for islet xenograft rejection. *Transplant Proc* 26:1203, 1994
14. Wecker H, Winn H, Auchincloss HJ: CD4+ T-cells, without CD8+ or B lymphocytes, can reject xenogeneic skin grafts. *Xenotransplantation* 1:8–16, 1994
15. Widmer MB, Bach FH: Allogeneic and xenogeneic response in mixed leukocyte cultures. *J Exp Med* 135:1204–1208, 1972
16. Gill RG: The role of direct and indirect antigen presentation in the response to islet xenografts. *Transplant Proc* 24:642–643, 1992
17. Yamada K, Sachs DH, DerSimonian H: Human anti-porcine xenogeneic T-cell response: evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. *J Immunol* 155:5249–5256, 1995
18. Yamada K, Sachs DH, DerSimonian H: Direct and indirect recognition of pig class II antigens by human T-cells. *Transplant Proc* 27:258–259, 1995
19. Murray AG, Nelson RC, Rayat GR, Elliott JF, Korbutt GS: Neonatal porcine islet cells induced human CD4+, but not CD8+ lymphocytes proliferation and resist cell-mediated cytolytic injury in vitro. *Diabetes* 48:1713–1719, 1999
20. Warrens AN, Simon AR, Theodore PR, Sykes M: Human-porcine receptor-ligand compatibility within the immune system: relevance for xenotransplantation. *Xenotransplantation* 6:75–78, 1999
21. Alter BJ, Bach FH: Cellular basis of the proliferative response of human T-cells to mouse xenoantigens. *J Exp Med* 171:333–338, 1990
22. Moses RD, Winn HJ, Auchincloss HJ: Evidence that multiple defects in cell-surface molecule interactions across species differences are responsible for diminished xenogeneic T-cell responses. *Transplantation* 53:203–209, 1992
23. Yi S, Feng X, Hawthorne W, Patel A, Walters S, O'Connell PJ: CD8+ T-cells are capable of rejecting pancreatic islet xenografts. *Transplantation* 70:896–906, 2000
24. Nicolls MR, Colulombe M, Yang H, Bolwerk A, Gill RG: Anti-LFA-1 therapy induces long term islet allograft acceptance in the absence of IFN-γ or IL-4. *J Immunol* 164:3627–3634, 2000
25. Korbutt GS, Elliott JF, Ao Z, Smith DK, Warnock GL, Rajotte RV: Large scale isolation, growth, and function of porcine neonatal islet cells. *J Clin Invest* 97:2119–2129, 1996
26. Hoffman R, Langrehr J, Billiar T, Curran R, Simmons R: Alloantigen-induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. *J Immunol* 145:2220–2226, 1990
27. Grusby MJ, Johnson RS, Papaioannou VE, Glimcher LH: Depletion of CD4$^+$ T-cells in major histocompatibility complex class II–deficient mice. *Science* 253:1417–1420, 1991
28. Pietra BA, Wiseman A, Bolwerk A, Rizeq M, Gill RG: CD4 T-cell-mediated cardiac allograft rejection requires donor but not host NHC class II. *J Clin Invest* 106:1003–1010, 2000
29. Wilson DB, Nowell PC: Quantitative studies on the mixed lymphocyte interaction. IV. Immunological potentiality of the responding cells. *J Exp Med* 131:391–407, 1970
30. Nielsen HE: Reactivity of lymphocytes from germfree rats in mixed leukocyte culture and in graft-versus-host reaction. *J Exp Med* 136:417–425, 1972
31. Asantila T, Vahala J, Toivanen P: Response of human fetal lymphocytes in xenogeneic mixed lymphocyte culture: phylogenetic and ontogenetic aspects. *Immunogenetics* 1:272–290, 1974
32. Woolnough J, Misko I, Lafferty KJ: Cytotoxic and proliferative lymphocyte responses to allogeneic and xenogeneic antigens in vitro. *Aust J Exp Bio Med Sci* 57:467–477, 1979
33. Kalinke U, Arnold B, Hammerling GJ: Strong xenogeneic HLA response in transgenic mice after introducing an α3 domain into HLA B27. *Nature* 348:642–644, 1990
34. Moses RD, Pierson RN III, Winn HJ, Auchincloss JH: Xenogeneic proliferation and lymphokine production are dependent on CD4+ helper T-cells and self antigen-presenting cells in the mouse. *J Exp Med* 172:567–575, 1990
35. Murphy B, Auchincloss H Jr, Carpenter CB, Sayegh MH: T-cell recognition of xeno-MHC peptides during concordant xenograft rejection. *Transplantation* 61:1133–1137, 1996
36. Finkelman FD, Shea-Donohue T, Goldhill J, Sullivan CA, Morris SC, Madden KB, Gause WC, Urban JF Jr: Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annu Rev Immunol* 15:505–533, 1997
37. Kurts C, Heath WR, Carbone FR, Allison J, Miller JFAP, Kosaka H: Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med* 184:923–930, 1996
38. Brossart P, Bevan MJ: Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* 90:1594–1599, 1997
39. Olack B, Manna PP, Jaramillo A, Steward N, Swanson C, Kaesberg D, Poindexter N, Howard T, Mohanakumar T: Indirect recognition of porcine swine leukocyte Ag class I molecules expressed on islets by human CD4+ T lymphocytes. *J Immunol* 165:1294–1299, 2000
40. Olack BJ, Jaramillo A, Benshoff ND, Kaleem Z, Swanson CJ, Lowell JA, Mohanakumar T: Rejection of porcine islet xenografts mrediated by CD4+ T-cells activated through the indirect antigen recognition pathway. *Xenotransplantation* 9:393–401, 2002