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Indirect Recognition of Porcine Swine Leukocyte Ag Class I Molecules Expressed on Islets by Human CD4\(^+\) T Lymphocytes

Barbara Olack,* Partha Manna,* Andrés Jaramillo,† Nancy Steward,* Carol Swanson,* Dana Kaesberg,* Nancy Poindexter,* Todd Howard,* and Thalachallour Mohanakumar,2*†

Xenotransplantation of porcine islets is considered a viable alternative treatment for type 1 diabetes mellitus. Therefore, we characterized human PBL responding to porcine islets both in vitro by coculture and in vivo using SCID mice reconstituted with human PBLs (HuPBL-SCID) and transplanted with porcine islets. T cell lines generated in vitro and graft-infiltrating T cells obtained from HuPBL-SCID mice were CD4\(^+\)-proliferated specifically to porcine islets cultured with autologous APC. This proliferation was abrogated by an anti-human class II Ab. These T cell lines also proliferated to purified swine leukocyte Ag (SLA) class I molecules in the presence of self-APC, indicating that the primary xenoantigens recognized are peptides derived from SLA. This CD4\(^+\) T cell line lysed porcine islets but not splenocytes. CD4\(^+\) T cell clones with Th0, Th1, and Th2 cytokine profiles were isolated. The Th0 and Th1 clones lysed porcine islets, whereas the Th2 clone that secreted a large amount of IL-4 was not lytic. These results demonstrate that human T cells responding to porcine islets are primarily CD4\(^+\) and recognize porcine xenoantigens by the indirect Ag pathway presentation. These activated T cells produce cytokines that lyse islets. Furthermore, we demonstrate that the major porcine xenoantigens recognized are SLA class I molecules. *The Journal of Immunology, 2000, 165: 1294–1299.

Porcine pancreatic islets are considered a viable candidate for clinical transplantation for the treatment of type 1 diabetes mellitus. A thorough characterization of the human immune response to a xenograft is necessary to achieve successful clinical application of xenotransplantation (1). Vascularized xenografts are destroyed as a result of hyperacute rejection caused by preformed natural xenoreactive Abs and complement activation. Currently several approaches have been sought to overcome this problem (2, 3). Xenotransplants may still face destruction by NK cells as well as T cells. Delayed xenograft rejection and a T cell-mediated response against xenografts have been reported (4–6). The T cell-mediated xenograft rejection is thought to be mediated primarily by CD4\(^+\) T cells. However, the mechanism of destruction of the graft by CD4\(^+\) T cells is poorly understood. CD4\(^+\) T cells are capable of mediating delayed-type hypersensitivity reaction, resulting in graft damage because of the production of various cytokines in situ as well as direct cytotoxicity of the graft (7).

In the present study, we characterize human CD4\(^+\) T cells generated against porcine islets both by in vitro stimulation with porcine islets as well as by the use of SCID mice reconstituted with human PBL (HuPBL-SCID) \(^3\) and subsequently transplanted with porcine islets.

Materials and Methods
Reconstitution of SCID mice
CB/17 SCID mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at Washington University School of Medicine in a pathogen-free environment. All animals were 8–12 wk old and had total mouse Ig levels below 30 \(\mu\)g/ml, as measured by ELISA.

Human PBL reconstitution of SCID mice was accomplished as previously described (8). Briefly, human PBL from healthy donors were isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia, Alameda, CA) from heparinized blood of healthy donors. Human PBL (30 × 10^6) were resuspended in a volume of 0.5 ml of PBS (Sigma, St. Louis, MO) and injected i.p. into SCID mice. An additional 15 × 10^6 PBL were cultured in the presence of 100 ng/ml OKT3 (Ortho, Raritan, NJ) in RPMI 1640 medium (Life Technologies, Grand Island, NY), supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 15% heat-inactivated FBS for 2 days at 37°C. OKT3-activated PBL were washed and injected 2 days after the initial injection. Serum was monitored weekly in these mice by ELISA for human IgG. Reconstitution was considered successful when human IgG levels reached >500 \(\mu\)g/ml within 3–5 wk.

Transplantation of porcine islets and isolation of graft-infiltrating lymphocytes (GIL)
Porcine islets were isolated from outbred Yorkshire sows by the Islet Isolation Core Facility of the Diabetes Research and Training Center of Washington University. Approximately 3000 islet equivalents were transplanted under the kidney capsule of the reconstituted mice as previously described (9). Two to three weeks after transplantation, the mice were sacrificed and the GIL were recovered by excising the site of the transplant from the kidney. The parenchyma was then minced into small pieces and incubated in media containing 20 U human rIL-2/ml and 5% T-Stim (Collaborative Biomedical Products, Bedford, MA). Cultures were maintained weekly by the addition of irradiated (3000 rads) porcine islets or splenocytes as an Ag source and autologous irradiated PBL as APC. After 6 wk of culture, the

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\(^3\) Abbreviations used in this paper: HuPBL-SCID, SCID mice reconstituted with human PBLs; GIL, graft-infiltrating lymphocyte; SI, stimulation index; CML, cell-mediated lympholysis; SLA, swine leukocyte Ag.
antiporcine T cell cell line was cloned by limiting dilution at 1 cell/well with the autologous, irradiated, EBV-transformed lymphoblastoid cell line (1 × 10^5) as feeder cells and relevant porcine splenocytes (1 × 10^5) as Ag in RPMI 1640 medium supplemented with 20 U/ml rIL-2.

**In vitro generation of human T cells and clones against porcine islets**

Four separate T cell lines were generated using 10 × 10^4 human PBL from healthy donors cultured with 100 islet-equivalent porcine islets in RPMI 1640 medium supplemented with 20 U/ml human rIL-2 and 5% T-Stim. Cultures were maintained by weekly addition of relevant porcine islets as Ag and autologous irradiated PBL as APC. After 3 wk of culture, the cell lines were cloned by limiting dilution at 1 cell/well with irradiated autologous PBL (1 × 10^5) as feeder cells (10) in the presence of 0.025% PHA (Difco Laboratories, Detroit, MI) in RPMI 1640 supplemented with 50 U/ml rIL-2.

**Phenotype identification**

The phenotype of cells populations in both the in vivo and in vitro studies was identified by flow cytometric analysis using anti-human CD3, CD4, and CD8 mAbs conjugated with peridinin chlorophyl protein, PE, and FITC, respectively (Becton Dickinson, San Jose, CA) (10). Briefly, 5 × 10^6 cells were incubated for 30 min at 4°C in a 1:50 dilution of Ab in PBS. The cells were then washed (three times) and analyzed using a FACScan with Lysis II software (Becton Dickinson).

**Swine Leukocyte Ag (SLA) class I isolation and purification**

HIC class I was isolated and purified following the procedure of Naziruddin et al. (11). Briefly, HIC class I molecules were affinity purified from detergent extracts of 10 g of spleen harvested from z/z haplotype of miniature swine, using two mAbs against porcine SLA class I framework determinants, 7,34.1 and 74.11.10 (American Type Culture Collection, Manassas, VA). Concentrations of 5 μg/ml and 10 μg/ml were used as an Ag source for proliferation assays.

**Mixed lymphocyte islet reaction**

Proliferation of T cell lines was done by mixed lymphocyte islet reaction using 4 × 10^4 responder cells (GILs or PBL) and stimulator cells (1–2 porcine islets/well or 1 × 10^4 porcine splenocytes) in presence of irradiated autologous APC (2 × 10^5 cells/well). Cells were incubated at 37°C for 4 days. During the last 18 h of culture, each well was pulsed with 1 μCi [3H]thymidine (1 × 10^5 Ci/well). Cells were then washed onto glass fiber filters, and radioactivity was assessed by β-scintillation counting. Results of replicate cultures are reported as a stimulation index (SI), which was calculated using the following formula: SI = (mean cpm of experimental cultures)/mean cpm of control cultures). Ab blocking experiments were done using W6/32 anti-HLA class I and Kula2 anti-HLA class II mAbs at 5 μg/ml added at the initiation of culture.

**Cell-mediated lympholysis (CML)**

CML activity was measured using a standard 51Cr release assay with an E:T ratio of 25:1 for T cell bulk lines or 5:1 for T cell clone. Trypsin-dispersed relevant porcine islets and porcine splenocytes were incubated with 250 μCi of 51Cr for 120 and 60 min, respectively, at 37°C. T cells were incubated with labeled targets for 4 h in 96-well round-bottom plates. The supernatants were then harvested and assessed for 51Cr concentration. Results are expressed as a percent specific lysis, which was calculated using the following formula: % specific lysis = [(experimental cpm - spontaneous release)/(maximum lysis - spontaneous release)] × 100. Spontaneous release was determined by incubation of target cells in medium alone, and the maximum lysis was determined by incubation of target cells in 0.1% Triton X-100.

**Assay for cytokine-mediated cytotoxicity**

Cytokine-mediated cytotoxicity was measured by culturing 2 × 10^6 T cell clones (effectors) plus 10 porcine islets (Ag) in the top section of a transwell culture system (Costar, Cambridge, MA). The bottom section contained a designated target cell: 15 porcine or human hand-picked islets, 1 × 10^5 lymphoblastoid cell line or kidney cell line, 1 × 10^6 PHA-blasted porcine lymphoid cells, 1 × 10^5 porcine endothelial cell line, or 1 × 10^5 human breast cancer cell line (HL-100) or lung cancer cell line (HTB-57). The cells were cultured at 37°C in RPMI 1640 medium supplemented with 20 U/ml human rIL-2. After 48 h, the top section of the transwell was removed, and supernatant was harvested. Targets were stained with fluorescein diacetate and propidium iodide, and percent viability was determined under a fluorescent microscope.

**FIGURE 1.** In vitro proliferative response of GIL to relevant and third-party porcine Ag both alone and in the presence of autologous APC. Mouse IgG is used as an isotype Ab control. Results are expressed as the mean ± SD and are representative of four separate experiments with baseline counts of 75 cpm for T cells alone and 209 cpm for T cells and APC.

**Cytokine measurement**

Cytokine concentration in the supernatant was measured by ELISA. TNF-α and IL-4 were determined using the Quantigen (PharMingen, San Diego, CA) kit, and IFN-γ was measured by a matched Ab pair Sample-Pak (Endogen, Woburn, MA).

**Results**

**Isolation and characterization of CD4+ human T cells and clones from HuPBL-SCID mice transplanted with porcine islets.**

Five GIL T cell lines were successfully developed from 10 mice that were reconstituted and transplanted. Haplotypes of human donors were as follows: (A30, 3 B60, 65 Cw3, 8 DR0102,0104), (A2, 29 B62,- Cv3,- DR0101,0404)×2, (A2,- B37, 44 Cvw,- DR4, 10)×2. Phenotypic evaluation showed greater than 97% CD4+ T cells with less than 2% CD8+ T cells. CML assays performed on GIL T cell lines showed no specific lysis of relevant or third-party targets. Clones obtained from the T cell lines after limiting dilution were all CD4+.

GIL T cell lines showed significant (SI > 80) proliferation to both relevant (autologous to transplanted islets) and third-party (other outbred) porcine Ag only in the presence of autologous APC (Fig. 1). This proliferation was blocked by the addition of the Kula2 mAb, indicating Ags expressed on porcine-stimulating cells were processed and presented by self-HLA class II molecules. Addition of control mouse IgG showed no blocking of the proliferation.

GILs derived from the transplanted mice were tested for their proliferative response to porcine Ag. Fig. 2 shows that the GIL responded to both porcine islets as well as purified SLA αβ class I molecules isolated from porcine splenocytes only in the presence of self-APC, and this proliferation was significantly blocked by the addition of the Kula2 mAb.

Eleven T cell clones were generated from GIL. As indicated earlier, all clones produced were of CD4 phenotype. Seven of the 11 clones developed from GIL showed significant proliferation (SI > 4) to both relevant and third-party porcine Ag only when autologous APC were present in the culture, similar to the results obtained from T cell lines (Fig. 3).
Development and characterization of T cell line and clones by in vitro stimulation of human PBL with porcine islets

Four different human antiporcine islet T cell lines were generated against different outbred pig islets. Haplotypes of donors were as follows: (A2, B37, Cw-, DR7, 10) and (A2, B35, Cw4, DR4, 9). Two cultures contained only CD4+ T cells, whereas the remaining two showed 12% and 15% CD8+ T cells. Fig. 4 shows the proliferative response of a representative T cell line in response to porcine Ag. This response was also dependent on the presence of autologous APC and was blocked by the KuLa2, the anti-class II mAb. This result, along with the data obtained from GILs generated from HuPBL-SCID mice transplanted with porcine islets, indicates a predominant role for indirect Ag presentation of xenoantigens for the generation of human antiporcine immune response.

T cell clones were isolated from the in vitro T cell lines, and all 24 recovered clones were CD4+. Four clones were further characterized. All four clones proliferated significantly to porcine islets in the presence of self-APC (Fig. 5). Clone no. 33 substantially proliferated to relevant porcine islets with a SI of 40 times that of the other clones. These results again indicate that porcine Ags are presented by human APC to the human T cells. In general, human islets failed to stimulate three of the four clones. Proliferation was seen in clone no. 18 with and without APC when human islets were used as Ag (Fig. 5). It is likely that some of the human and porcine Ags may cross-react because clone no. 18 demonstrated some proliferation with human islets as the Ag source.

Cytotoxicity of human T clones to porcine islets

Human T cell clones were tested for their ability to lyse porcine islets by standard CML assay. All of the clones were CD4+ and were tested at an E:T ratio of 5:1. Seventeen of the 24 clones demonstrated >8% lysis of porcine islet target with six of the clones showing lysis of porcine islet targets >10% (Fig. 6). Initial testing of other porcine and human cell types for lysis by the same clones demonstrated no lysis (data not presented). This suggests that cytotoxicity of the islets may be mediated by cytokine-induced lysis, considering the high susceptibility of pancreatic islets to cytokine-mediated damage (12, 13).

Cytokine-mediated killing of target cells

To understand the mechanism of killing of porcine islets by CD4+ T cells clones, experiments were designed utilizing a transwell culture system in which effectors and targets are separated by a filter barrier and therefore are not in direct contact. The T cell clones were cultured with porcine islets in the top section of the transwell culture system with the bottom section containing the different targets. After 48 h, the target cells were analyzed for viability. Fig. 7A shows that clones #20 and #18 significantly lysed the porcine as well as human islets. In addition, there was no cytotoxicity for the other human or porcine targets previously described by any of the three clones (no lysis was observed by clone #33). It is of interest that in some cases, even if APCs were not added to the top of the transwell at the time of performing the assay, these clones produced cytokines that lysed the islets on the bottom portion of the transwell. We feel that these clones, which were stimulated with porcine islets in the presence of APC on a weekly basis, remain in an activated state, resulting in continued production of cytokines. Analysis of the culture media during the cytotoxicity studies revealed the cytokine profile of each of the three clones investigated. Fig. 7B shows that clone #18 produced high concentrations of NF-α (150 pg/ml) and IFN-γ (110.9 pg/ml) and negligible IL-4 (6.6 pg/ml). Clone #20 produced moderate...
amounts of IL-4 (85.5 pg/ml) and IFN-γ (53 pg/ml) and low levels of TNF-α (14.2 pg/ml), and clone #33 produced extremely high amounts of IL-4 (532 pg/ml), moderate levels of TNF-α (79.4 pg/ml), and very low quantities of IFN-γ (12.4 pg/ml). Preliminary results of TNF-α blocking show no killing of islets in the presence of anti-TNF-α (data not shown). These data are consistent with clone #18 being of Th1 phenotype, clone #20 being of Th0 phenotype, and clone #33 being of Th2 phenotype. These results also indicate the high susceptibility of both human and porcine islets for cytokine-mediated damage by IFN-γ.

**Discussion**

Organ transplantation today is limited by the number of available human donors. One appealing alternative is the use of xenogeneic organs. For a number of ethical and physiological reasons, the swine has attracted interest as the most appropriate potential xenogeneic organ donor (14). It is generally believed that porcine islet transplantation may pioneer clinical human xenotransplantation. Human and porcine insulin are chemically and functionally very similar. Insulin produced from the porcine pancreas has been successfully used in humans for many years.

Knowledge of porcine islet rejection mechanisms mediated by human T lymphocytes is necessary for successful long-term xenograft survival. During graft rejection, mononuclear cells infiltrate the graft, and an array of cytokines is produced that is known to have deleterious effects on the transplanted organ. This process is likely to be more significant in islet allograft rejection because it is known that islets are highly susceptible to cytokine-mediated damage (13). Cytokines such as TNF-α, IFN-γ, and IL-4 have been shown to regulate the expression of various cell-surface molecules involved in immune responses, such as adhesion molecules and MHC class I and II Ags of the transplanted organs (15).

Studies have shown that human T cell response against porcine Ags is at least as strong as the alloresponse and that both CD4+ and CD8+ T cells play a role in this process (16, 17). It has also been proposed that the xenoresponse is primarily a CD4+ T cell-dependent process and that indirect Ag presentation of xenointermediates plays an important part in this response (18). However, the mechanism by which CD4+ T cells destroy the xenograft is not yet clear.

**FIGURE 4.** Human T cells generated from PBL after coculturing with porcine islets showed significant proliferative response to relevant porcine islets in the presence of autologous APC. Mouse IgG is used as an isotype Ab control. Results are expressed as the mean ± SD and are representative of four separate experiments.

**FIGURE 5.** Human CD4+ T cell clones proliferate to both porcine and human islets in the presence of autologous APC. Results were expressed as the mean ± SD and are representative of three similar experiments. Note that clone #33 significantly proliferated to the porcine islets in the presence of APC with an SI of 404.

**FIGURE 6.** Cytotoxic activity of CD4+ T cell clones derived from an in vitro-produced T cell line against porcine islet cells. Results are averages of triplicate cultures.

**FIGURE 7.** Lysis of both porcine and human islets and its correlation to human cytokine production (clones #18 and #20). Protection of islet viability correlates to secretion of large amounts of IL-4 by Th2 type clone (clone #33). Results are expressed as mean ± SD and are representative of two separate experiments. A, Lysis of cells; B, cytokine production.
poorly understood (19). Although it is generally assumed that cytokines play a major role in this process, the results reported here indicate that cell-mediated cytotoxicity of porcine islets by CD4+ T cells is primarily due to Th1-type cytokines.

Graft-infiltrating CD4+ T cells derived from the porcine islet-transplanted HuPBL-SCID mice were primarily CD4+ within 3 wk of culture. Culture conditions were general and did not promote one phenotype over another. GIL CD4+ T cells proliferated when porcine Ag was provided as a stimulus in conjunction with autologous APC (Fig. 1). It is not surprising that all of the CD4 T cell clones tested needed self-APC because islets normally do not express MHC class II Ags. Work by Bretzel et al. (20) states that MHC class II is expressed only on vascular endothelium, macrophages, and monocytes and not on porcine islet cells. In addition, Pavlovic et al. (21) and Swift et al. (22) indicate the absence or lack of impact of MHC class II expression on islets with regard to T cell stimulation, even with the addition of high-dose IFN-γ and/or TNF-α. However, it was surprising that the major porcine Ag recognized by these GILs is MHC molecule, because the human CD4+ T cells not only proliferated when porcine islets were given as Ag but also when purified SLA class I molecules were given as Ag (Fig. 2). This strongly indicates that SLA molecules present on porcine xenografts are the major Ags that are recognized by human T cells and that indirect presentation of these molecules constitutes a major pathway of xenorecognition. Recent studies both in animal models and in human solid organ transplantation have shown that indirect Ag presentation of peptides derived from mismatched MHC class I and II molecules plays an important role in chronic allograft rejection (23–25). Results presented in this study indicate that a similar mechanism may also play a role in xenograft rejection. Human CD4+ T cell clones generated in vitro against porcine islets demonstrated specificity identical with those obtained by GILs. APC were needed for the proliferation, and the MHC class II restriction of these T cell clones were further documented in this study by the inhibition of proliferation by the Kua2 mAb.

Based on their cytokine production, CD4+ T cells have been grouped into three kinds. Th1 cells primarily produce IL-2, IFN-γ, and TNF-α and regulate cellular immune response to viral and intracellular pathogen infections. Th2 cells primarily secrete IL-4, IL-5, IL-10, and IL-13 and regulate humoral immune responses. Th0 clones are defined as a subset that produce both Th1 and Th2 cytokines and that are often considered as a precursor cell. Immune responses to antigenic stimuli are polarized with respect to the types of T cells that mediate these responses. For example, type 1 diabetes mellitus is thought to be mediated by the Th1 subset, whereas the Th2 cells may play a protective role (26). Allograft rejection is often associated with the Th1 response, whereas the long-term graft survival has been shown to be associated with a Th2 response (27, 28). In the present study, CD4+ T cell clones derived after coculturing normal human PBL with porcine islets exhibit three cytokine profiles consistent with Th1, Th0, and Th2 phenotypes. Clone #18 was strongly cytotoxic to the pig as well as to the human islets and exhibited a characteristic Th1 cytokine profile producing large amounts of TNF-α and IFN-γ with no IL-4 secretion. The Th0 type clone, clone #20, produced intermediate levels of TNF-α, IFN-γ, and IL-4. This clone also lysed both human and porcine islets. However, the Th2 type clone (clone #33) did not show any cytotoxicity against the target, and it secreted large quantities of IL-4, an intermediate amount of TNF-α, and no IFN-γ. Many studies have identified that graft acceptance is associated with preferential inhibition of Th1 cells and activation of Th2 cells (29, 30). The potential central role of IL-4 in tolerance induction and maintenance has been proposed to be related to its ability to regulate the commitment of Th0 cells to the Th2 phenotype and to inhibit the development and function of Th1 cells. Note that clone #33 showed a 40-fold SI compared with that of the other clones in the proliferation assay, supporting the fact that IL-4 increases T cell differentiation. Our data, based on a limited number of clones analyzed, supports such mechanisms in porcine islet rejection. These results indicate that cytokines secreted by Th1 clones may cause damage to porcine islets similar to what has been reported for islet allograft rejection (12). However, Th2 clones producing IL-4 are protective to the xeno islets, and results from clone #33 demonstrate that it can neutralize the effect of TNF-α because no islet lysis was detected. Therefore, a balance of various cytokines in situ is significant in determining islet survival.

Several studies have shown that indirect presentation of antigeneic peptide by host APC constitute a pathway leading to the xenograft rejection (19). Our laboratory has demonstrated that human CD8+ T cells stimulated by porcine endothelial cells are capable of recognizing porcine Ag presented by porcine MHC class I molecules in a manner similar to an allogeneic response (16). This may be due to a close homology between porcine and human class I Ag at the molecular level. Further work from our laboratory has also shown that human CD4+ T cells recognize xenogenic proteins of porcine endothelial cells mostly by the indirect pathway as in the context of self-APC involvement (17). Results presented in this report using porcine islets agree with our earlier findings and clearly point out the important role of CD4+ T cells in the rejection of islet xenografts. Our results have also shown that the primary xenogenic Ag recognized is indeed the SLA class I molecule. This is most likely because of the fact that the porcine islets used in both our in vivo and our in vitro studies were cryopreserved and/or cultured for a period of time, and therefore MHC class II-expressing cells may have been depleted. However, if one performs transplantations of fresh islets, it is likely that peptides derived from SLA class II molecules may also be taken up and presented by self-APC. We are currently investigating this possibility. Also, we are currently identifying the epitopes of the SLA class I molecule recognized by the human T cells and by the restricting elements of the human APCs.

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