Mixed chimerism induces donor-specific T-cell tolerance across a highly disparate xenogeneic barrier

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Induction of tolerance is likely to be essential for successful xenotransplantation because immune responses across xenogeneic barriers are vigorous. Although mixed hematopoietic chimerism leads to stable donor-specific tolerance in allogeneic and closely related xenogeneic (e.g., rat-to-mouse) combinations, the ability of this approach to induce tolerance across a highly disparate xenogeneic barrier has not yet been demonstrated. In this study, we investigated the immune responses of murine T cells that developed in mice with pre-established porcine hematopoietic chimerism. Our results show for the first time that induction of porcine hematopoietic chimerism can eliminate the development of antiporcine donor responses in a highly disparate xenogeneic species. Porcine hematopoietic chimeras showed donor-specific nonresponsiveness in the mixed lymphocyte reaction, lack of antidonor IgG antibody production, and acceptance of donor skin grafts. Thus, mixed chimerism is capable of inducing tolerance in a highly disparate xenogeneic combination and may have clinical potential to prevent xenograft rejection. (Blood. 2002;99:3823-3829)

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Introduction

Xenotransplantation provides a possible solution to the severe shortage of allogeneic organs, a major limiting factor in clinical organ transplantation. In view of the ethical issues and impracticalities associated with the use of nonhuman primates, interest has become focused on nonprimates, in particular the pig, as the most suitable organ donor species for humans. However, organ transplants across discordant species barriers are subject to vigorous immunologic rejection.1-4 Transplantation of organs from pigs results in hyperacute rejection in primate recipients due to the presence of anti–Galα1-3Gal natural antibodies in their sera. These antibodies also mediate a delayed form of humoral rejection, acute vascular rejection, if hyperacute rejection is overcome. The cellular immune response to xenografts is also stronger than that to allografts. Thus, tolerance induction is likely to be essential for successful xenotransplantation.

Induction of mixed hematopoietic chimerism by bone marrow transplantation (BMT) leads to stable donor-specific tolerance in allogeneic and closely related xenogeneic (rat-to-mouse) combinations,5-10 but its ability to induce porcine xenograft tolerance has not been demonstrated in any discordant xenogeneic species because of the difficulty in achieving lasting porcine hematopoietic engraftment. Studies using a pig-primate combination have shown prolonged survival of porcine kidney grafts in cynomolgus monkey recipients preinfused with donor marrow cells. However, long-lasting hematopoietic chimerism was not achieved in any of these animals, and all kidney grafts were eventually rejected.11,12 Because donor hematopoietic stem cells may fail to engraft in discordant xenogeneic recipients, even in recipients in which T and B cells are absent,13,14 the rejection of pig kidney grafts in these studies does not distinguish between the failure of donor hematopoietic cells to engraft and an inability of chimerism to induce tolerance across highly disparate species barriers. It has been shown that the persistence of donor cells is essential for maintaining tolerance in mixed chimeras.15-20

Our previous studies showed that lasting porcine hematopoietic chimerism and stem cell engraftment can be induced in nonobese diabetic/severe combined immunodeficiency transgenic (NOD/SCID-Tg) mice expressing pig cytokine transgenes.21 These transgenic mice also demonstrated spontaneous migration of porcine donor antigen-presenting cells (APCs) to an intact recipient thymus, suggesting the possibility of inducing central tolerance in the discordant xenogeneic setting by mixed chimerism. Here we attempted to determine whether or not hematopoietic chimerism is capable of inducing donor-specific tolerance in this pig-to-mouse combination, by comparing the responses of murine T cells that developed in NOD/SCID-Tg mice with or without pre-established porcine chimerism.

Materials and methods

Animals

The NOD/LtSz-SCID/SCID (NOD/SCID) and B10.D2/o-Tg mice that produce porcine cytokines (interleukin 3 [IL-3], granulocyte-macrophage colony-stimulating factor [GM-CSF], and stem cell factor [SCF]) were developed by crossing the transgenic founders with the NOD/LtSz-SCID/SCID (NOD/SCID) and B10.D2/o mice, respectively. B10.D2/o-Tg mice expressing pig cytokine transgenes were purchased from the Jackson Laboratory (Bar Harbor, ME). NOD/SCID-Tg and B10.D2/o-Tg mice that produce porcine cytokines (interleukin 3 [IL-3], granulocyte-macrophage colony-stimulating factor [GM-CSF], and stem cell factor [SCF]) were developed by crossing the transgenic founders with NOD/SCID and B10.D2/o mice for at least 6 generations, respectively. All Tg mice used in this study were identified by tail DNA polymerase chain reaction (PCR) using a primer for cytomegalovirus (CMV) promoter in combination with a primer for porcine IL-3, GM-CSF.
or SCF as described. Mice were housed in a specific pathogen-free microisolator environment and were used at 8 to 13 weeks of age. Partially inbred Massachusetts General Hospital (MGH) miniature swine provided by Dr. David H. Sachs were used as porcine bone marrow donors. All animals were maintained and procedures were performed in accordance with National Institutes of Health guidelines.

**Immune reconstitution**

The NOD/SCID-Tg mice were conditioned with 3 Gy whole body irradiation (WBI), and injected with T- and B-cell-depleted B10.D2/o-Tg mouse bone marrow cells (BMCs; 1-1.5 × 10⁷/mouse) at week 5, 7, or 11 after WBI. To prepare T- and B-cell-depleted BMCs, B10.D2/o-Tg mouse BMCs were first incubated with antimonouse CD4 monoclonal antibody (mAb) GK 1.5 and anti-CD8 mAb 2.43 for 30 minutes, followed by incubation with low-toxicity rabbit complement for 45 minutes, to deplete CD4 and CD8 T cells. Depleted BMCs, B10.D2/o-Tg mouse BMCs (1.5 × 10⁷ intravenously) at week 7 after porcine BMC-reconstituted NOD/SCID-Tg mice. In most mice T cell recovery in NOD/SCID-Tg mice reconstituted with immunocompetent murine stem cells, we followed immune recovery in NOD/SCID-Tg mice reconstituted with T- and B-cell–depleted immunocompetent B10.D2/o-Tg mouse BMCs at various times after 3 Gy WBI. Mice were challenged with skin grafts from allogeneic and xenogeneic donors 2 weeks after B10.D2/o-Tg mouse BMC reconstitution. As shown in Figure 1A, both allogeneic and xenogeneic skin grafts were rejected in NOD/SCID-Tg mice that were reconstituted within 5 to 11 weeks after 3 Gy WBI. Dead cells were excluded by gating out low forward scatter plus high propidium iodide–retaining cells.

**Mixed leukocyte reaction assay**

Mouse splenocytes were prepared and suspended in RPMI 1640 medium supplemented with 15% (vol/vol) controlled processed serum replacement (CPSR-2; Sigma Chemical, St Louis, MO), 2 mM L-glutamine, 0.1 mM nonessential amino acids (Life Technologies, Grand Island, NY), 1 mM sodium pyruvate, 10 U/mL penicillin, 10 μg/mL streptomycin, 1% Hepes buffer, and 10 μM 2-mercaptoethanol. Triplicate wells containing 4 × 10⁵ responders with irradiated (30 Gy) stimulators of swine (1 × 10⁷ PBMCs) or mouse (4 × 10⁵ B10.RII splenocytes) in a total volume of 0.2 mL medium were incubated at 37°C in 5% CO₂. Cultures were pulsed with 1 μCi (3.7 × 10⁶ Bq) [³H]Tdr on days 3 and 4, and harvested 18 hours later with a Tomtec (Wallac, Gaithersburg, MD) automated harvester. Samples were assayed in a Pharmacia LKB (Piscataway, NJ) Betaplate counter and data are expressed as stimulation index (cpm of stimulated culture/cpm of unstimulated [media control] culture).

**Quantitation of antipig IgG and total IgG**

Indirect immunofluorescence staining of donor swine PBMCs was used to detect antiporcine donor antibodies. Frozen PBMCs prepared from the porcine BM donors were thawed, washed with FACS buffer, and 10⁶ cells were stained for 30 minutes at 4°C with 2 or 10 μL undiluted mouse serum followed by incubation with FITC-conjugated rat antimouse IgG1, IgG2a, IgG2b, or IgG3 mAb (Pharmingen) for 30 minutes at 4°C. Cells were washed with FACS buffer after each incubation and were analyzed on a FACScan (Becton Dickinson). The serum levels of antipig antibody are presented as median fluorescence intensity (MFI). The serum levels of total murine IgG were measured by enzyme-linked immunosorbent assay (ELISA) and calibrated with purified mouse IgG.

**Results**

**Administration of T- and B-cell–depleted immunocompetent mouse BMCs led to development of functional T and B cells in NOD/SCID-Tg mice**

Previous studies have shown that injection of immunocompetent murine hematopoietic stem cells gives rise to functional T and B cells in SCID mice. Because demonstration of porcine chimerism prior to immune reconstitution is essential to distinguish failure of porcine marrow engraftment from a failure of tolerance induction, we attempted to establish murine lymphopoiesis after stable porcine chimerism had first been confirmed. To determine the optimal timing for giving immunocompetent murine stem cells, we followed immune recovery in NOD/SCID-Tg mice reconstituted with T- and B-cell–depleted immunocompetent B10.D2/o-Tg mouse BMCs at various times after 3 Gy WBI. Mice were challenged with skin grafts from allogeneic and xenogeneic donors 2 weeks after B10.D2/o mouse BMC reconstitution. As shown in Figure 1A, both allogeneic and xenogeneic skin grafts were rejected in NOD/SCID-Tg mice that were reconstituted within 5 to 11 weeks after WBI with B10.D2/o-Tg mouse BMCs, whereas skin grafts were permanently accepted by NOD/SCID-Tg mice that did not receive B10.D2/o-Tg mouse BMCs. FACS analyses demonstrated the presence of substantial numbers of mouse T (CD4⁺, CD8⁺) and B (surface IgM⁺) cells in the blood and spleen of B10.D2/o-Tg mouse BMC-reconstituted NOD/SCID-Tg mice. In most mice T and B cells became detectable in the WBCs by 2 to 3 weeks and gradually increased to a stable level within 10 to 15 weeks after
We next investigated whether or not murine T cells that developed in porcine hematopoietic chimeras could be specifically tolerated to the porcine BM donor (Figure 2). Porcine hematopoietic chimeras were prepared by injection of porcine hematopoietic cells into 3 Gy WBI-conditioned NOD/SCID-Tg mice as previously described. These chimeras and NOD/SCID-Tg mice that did not receive porcine cell transplantation were then reconstituted with T- and B-cell-depleted B10.D2/o-Tg mouse BMCs 7 weeks later. Skin grafting was performed 2 weeks after infusion of B10.D2/o-Tg mouse BMCs to assess tolerance. Consistent with the results shown in Figure 1A, both allogeneic third-party and porcine skin grafts were permanently accepted by NOD/SCID-Tg mice that did not receive B10.D2/o-Tg mouse BMCs (Figure 3A), whereas they were both rejected by NOD/SCID-Tg mice that did receive B10.D2/o-Tg mouse BMCs (Figure 3B). However, specific acceptance of porcine donor skin grafts was observed in 5 of 8 B10.D2/o-Tg mouse BMC-reconstituted porcine hematopoietic chimeras (ie, animals in which significant porcine chimerism was detected in the WBCs 1 week prior to murine BMC reconstitution; Figure 3C). Although rejection of porcine donor skin grafts occurred in 3 porcine hematopoietic chimeras, rejection in 2 of
these 3 mice was delayed. Porcine donor skin rejection in these porcine hematopoietic chimeras might be due to incomplete tolerance, or skin-specific antigens, or both, because all of these mice showed a specific lack of antiporcine donor responses in mixed lymphocyte reaction (MLR; see below). In contrast, skin grafts from the porcine BM donor were rejected in B10.D2/o-Tg mouse BMC-reconstituted NOD/SCID-Tg mice with poor porcine chimerism (ie, mice with undetectable or < 1.5% WBC chimerism at 1 week prior to murine BMC reconstitution). Both B10.D2/o-Tg mouse BMC-reconstituted porcine hematopoietic chimeras and NOD/SCID-Tg mice with poor porcine chimerism rejected all-ge neic third-party skin grafts (Figure 3C), demonstrating immune function in animals that accepted porcine skins.

Lack of antiporcine donor MLR responses or antiporcine donor IgG production in B10.D2/o-Tg mouse BMC-reconstituted porcine hematopoietic chimeras

Prolongation of porcine donor skin grafts was accompanied by donor-specific nonresponsiveness in MLRs. MLR assays were performed after skin grafting, because mice do not mount primary antipig MLR responses.27,28 As shown in Figure 4, NOD/SCID-Tg mice that did not receive B10.D2/o-Tg mouse BMCs (group I) showed no MLR responses to either porcine donor or third-party donor, whereas responses against both stimulators were measurable in NOD/SCID-Tg mice reconstituted with B10.D2/o-Tg mouse BMCs (group II). However, MLR responses against the porcine donor were undetectable in B10.D2/o-Tg mouse BMC-reconstituted porcine hematopoietic chimeras, including the 3 mice that rejected the porcine donor skin graft (Figure 4A, group III). In contrast, antiporcine donor MLR responses were detected in B10.D2/o-Tg mouse BMC-reconstituted NOD/SCID-Tg mice with poor porcine chimerism (Figure 4A, group IV). Anti–third-party MLR responses were detected in both porcine hematopoietic chimeras and NOD/SCID-Tg mice with poor porcine chimerism, and their levels were comparable to those in B10.D2/o-Tg mouse BMCRestricted NOD/SCID-Tg mice that did not receive porcine cells (Figure 4B).

Sera were collected for measuring antiporcine donor IgG antibodies and total murine IgG. Similar to immunodeficient NOD/SCID-Tg mice that did not receive B10.D2/o-Tg mouse BMCs, antidonor swine IgG antibodies were undetectable in B10.D2/o-Tg mouse BMC-reconstituted porcine hematopoietic chimeras that accepted the porcine donor skin graft (Figure 5A). In contrast, the production of all isotypes of antiporcine donor IgG antibodies was stimulated in B10.D2/o-Tg mouse BMC-reconstituted NOD/SCID-Tg mice that rejected porcine donor skin grafts (Figure 5A). Because no difference in serum levels of total murine IgG was detected between mice that accepted or rejected porcine donor skin (Figure 5B), the absence of antiporcine donor IgG production in porcine hematopoietic chimeras reflects tolerance to the porcine hematopoietic donor. Previous studies have shown that antipig IgG antibodies are only produced after exposure to pig antigens, and their production is T cell dependent.25 Thus, these results confirmed tolerance of murine T cells to the porcine BMC donor in B10.D2/o-Tg mouse BMC-reconstituted porcine hematopoietic chimeras.

Porcine hematopoietic cells have a competitive disadvantage in murine recipients

Despite the persistence of donor-specific tolerance in B10.D2/o-Tg mouse BMC-reconstituted porcine hematopoietic chimeras, FACS
BMT. Percentages of antipig pan-tissue mAb levels of porcine chimerism in recipient BM at week 12 after NOD/SCID-Tg mouse BMT, and weeks 2, 5, 8, and 12 after NOD/SCID-Tg mouse BMT. (B) Percentages of antipig pan-tissue mAb-positive cells in recipient WBCs at 1 week before (6 weeks after porcine BMT) subsequent injection of NOD/SCID-Tg mouse BMCs. (A) Percentages of c-kit+ and c-kit- porcine BMCs with or without subsequent infusion of immunodeficient mouse BMCs. It has been demonstrated that NOD/SCID mice lack functional T, B, and natural killer cells.29 NOD/SCID-Tg mice were treated with 3 Gy WBI followed within 4 to 8 hours by porcine BMT. Some of these mice were subsequently reconstituted with 5 × 10^6 NOD/SCID-Tg mouse BMCs at week 7 after porcine BMT. Although porcine chimerism in the WBCs declined rapidly at early times in porcine BM recipients, probably due to the death of injected mature porcine cells,21 all NOD/SCID-Tg mice that received only porcine cells (ie, without subsequent murine BMT) maintained long-term (>19 weeks) chimerism (Figure 6). However, injection of NOD/SCID-Tg mouse BMCs at week 7 following porcine BMT completely eliminated pre-established porcine hematopoietic chimerism, despite the inability of these cells to reconstitute lymphopoiesis and to mediate immunologic rejection (Figure 6). Together, these results indicate that the loss of porcine chimerism in B10.D2/o-Tg mouse BMC-reconstituted porcine hematopoietic chimeras, which are tolerant to the porcine donor, was likely due to the competitive effect of infused B10.D2/o-Tg mouse BMCs, but not immunologic rejection.

Discussion

In this study we observed that murine T cells developing in porcine hematopoietic chimeras show specific nonresponsiveness to porcine donors, as evidenced by the acceptance of donor skin grafts and lack of antidonor MLR or antidonor IgG production. The capacity to respond to allogeneic antigens indicates that tolerance to the porcine donor has been achieved in these mice. This was further supported by the observation that T cells developing in similarly treated mice without porcine chimerism responded to both porcine donor and allogeneic antigens. It is not clear from the present study whether or not tolerance achieved in this model is swine leukocyte antigen allele specific, because xenoreactivity to a porcine third-party control was not assessed. The capacity of hematopoietic chimerism to induce T-cell tolerance results in large part from the ability of donor cells to induce intrathymic clonal deletion of maturing donor-reactive thymocytes,17,18,30,31 resulting in the generation of a T-cell repertoire that is tolerant of the hematopoietic cell donor. Elimination of antidonor swine responses of murine T cells developing in porcine hematopoietic chimeras suggests that the intrathymic clonal deletion of porcine donor-reactive murine T cells occurs in highly disparate xenogeneic species. Previous studies suggested that the repopulation of host thymus with donor antigen-expressing cells is critical for the deletion of donor-reactive T cells and the maintenance of stable tolerance in mixed chimeras.15-20 Thus, the host thymus-homing capacity of donor cells would be an important determinant for tolerance induction by mixed chimerism across discordant xenogeneic barriers. Although cross-species incompatibilities in adhesion molecules may limit the thymic homing of donor cells in discordant xenogeneic recipients,32-35 such defects have not been detected in the highly disparate pig-to-mouse combination. We have previously shown that long-term (>20 weeks) repopulation of host thymus with porcine class II+ cells can be achieved in NOD/SCID-Tg mouse recipients of porcine BM (without subsequent mouse BMT).21 In the present study, thymus was prepared at week 24 or week 27 after porcine BMT and analyzed for porcine chimerism by immunohistochemical staining. Despite the fact that transplantation of mouse BMCs into porcine chimeras leads to chronic elimination of porcine chimerism through competition (Figure 6), repopulation of host thymus with porcine class II+ cells was maintained for over 24 weeks in 1 of 5 B10.D2/o-Tg mouse BMC-reconstituted porcine hematopoietic chimeras that accepted porcine donor skin grafts (data not shown).

To limit the potential for engraftment failure to interfere with the evaluation of the ability of porcine hematopoietic chimerism to induce tolerance in mice, we used a unique model, in which porcine BM recipients maintained immunodeficiency until stable porcine hematopoietic chimerism had been established. Thus, this study assessed only the ability of porcine hematopoietic chimerism to induce tolerance of newly developing, but not mature, murine T cells, and established the principle that such tolerance can be achieved. Studies are in progress to use immunocompetent porcine cytokine transgenic mice to determine what level of immunosuppression is required for induction of porcine hematopoietic chimerism and whether or not mixed chimerism can lead to tolerance in mice with incomplete immunosuppression. Because it is unknown whether it will be clinically feasible and necessary to completely suppress the host cellular and humoral immunity to the level of SCID mice in humans, these studies would be important in determining the applicability of mixed hematopoietic chimerism to clinical xenotransplantation.

Previous studies in a rat-to-mouse BMT model have shown that host hematopoietic stem cells have a competitive advantage that limits xenogeneic donor hematopoietic repopulation.56,37 Here we demonstrate that the competitive advantage for host hematopoiesis is even stronger in pig-to-mouse combination. Injection of immunodeficient NOD/SCID mouse BMCs into porcine hematopoietic chimeras led to complete eradication of porcine hematopoietic chimerism (Figure 6). The disadvantage for porcine hematopoiesis in mice is likely to be the cause for the loss of porcine hematopoietic chimerism in porcine

![Figure 6. Competitive advantage for murine hematopoiesis over porcine hematopoiesis in NOD/SCID-Tg mice. (A) Percentages of antipig pan-tissue mAb-positive cells in recipient WBCs at 1 week before (6 weeks after porcine BMT) subsequent injection of NOD/SCID-Tg mouse BMCs. (B) Percentages of c-kit+ and c-kit- cells (stained by pig SCF) cells are shown. Each symbol represents an individual animal.](image-url)
hematopoietic chimeras following injection of B10.D2/Tg mouse BMCs, in which donor-specific tolerance was persistent. The maintenance of tolerance in these mice might be due to the persistence of “micro” hematopoietic chimerism. However, it is unclear if such tolerance would be permanently stable. Because active donor hematopoiesis is needed to provide a consistent supply of donor antigens to ensure the ongoing central deletion of donor-reactive T-cell clones, development of strategies overcoming the nonimmunologic factors that limit donor hematopoiesis would be important for xenograft tolerance induction through hematopoietic chimerism. Incompatibilities between the donor and host in adhesion molecules and hematopoietic cytokines, which are required for adequate homing, stromal interaction, self-renewal, and differentiation of hematopoietic stem cells, are important nonimmune factors limiting donor hematopoietic engraftment in discordant xenogeneic species. Previous studies in both murine and primate models have shown that donor cytokines can improve the engraftment of porcine hematopoietic cells across highly disparate xenogeneic barriers. We have recently observed that the use of mAbs that specifically block the adhesion of host but not xenogeneic hematopoietic stem cells to BM stroma could overcome the competitive advantage of host hematopoiesis and thereby facilitate the induction of porcine hematopoietic chimerism in xenogeneic recipients (our unpublished data; April 2001).

In this report, we demonstrate that mixed chimerism induces donor-specific tolerance in a highly disparate xenogeneic combination, pig-to-mouse. We have recently observed that implantation of fetal human thymus and liver fragments into NOD/SCID-Tg mice with pre-established porcine chimerism led to the development of human T cells in these mice. Interestingly, human T cells generated in these chimeras did not reject porcine cells and stable triple hematopoietic chimerism (human/swine/mouse) was maintained until killed at week 20 after transplantation (our unpublished data, October 2000). Additional studies are clearly necessary, but these results suggest that induction of mixed chimerism may also overcome the rejection of porcine xenografts by human T cells.

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