Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model

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Bone marrow transplantation (BMT) has considerable potential for the treatment of malignancies, hemoglobinopathies, and autoimmune diseases, as well as the induction of transplantation allograft tolerance. Toxicities associated with standard preparative regimens for bone marrow transplantation, however, make this approach unacceptable for all but the most severe of these clinical situations. Here, we demonstrate that stable mixed hematopoietic cell chimerism and donor-specific tolerance can be established in miniature swine, using a relatively mild, non-myeloablative preparative regimen. We conditioned recipient swine with whole-body and thymic irradiation, and we depleted their T-cells by CD3 immunotoxin-treatment. Infusion of either bone marrow cells or cytokine-mobilized peripheral blood stem cells from leukocyte antigen-matched animals resulted in stable mixed chimerism, as detected by flow cytometry in the peripheral blood, thymus, and bone marrow, without any clinical evidence of graft-versus-host disease (GvHD). Long-term acceptance of donor skin and consistent rejection of third-party skin indicated that the recipients had developed donor-specific tolerance.

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Introduction

Hematopoietic cell transplantation has yet to reach its full potential clinically, primarily because of the severe toxicities associated with the current standard preparative regimens for bone marrow transplantation (BMT). For many patients with hemoglobinopathies, including sickle cell disease and thalassemia, BMT offers the only hope of a cure. For organ-transplant recipients, tolerance induced by BMT would eliminate the need for and avoid the complications of chronic immunosuppression. In this laboratory we have developed methods for achieving transplantation tolerance in mice through the establishment of mixed bone marrow chimerism, a state in which both host and donor elements coexist in the lymphohematopoietic tissues of the recipient (1–3). Mixed chimerism has many potential advantages over fully allogeneic chimerism. These advantages include: (a) the ability to achieve mixed chimerism with less toxic preparative regimens (3–6); (b) the ability to cross MHC barriers; (c) full immunocompetence, even across MHC barriers (7, 8); and (d) a reduced likelihood of graft versus host disease (GvHD) (9–11).

To apply the mixed chimerism approach to patients, large-animal models are needed, both to understand the mechanism of the treatment and to optimize the treatment protocol. Partially inbred miniature swine have been developed in this laboratory as a large-animal, preclinical model for studies of transplantation biology and are very similar to humans in many parameters related to BMT (12–14). One significant obstacle to achieving mixed chimerism without myeloablation in large animals has been the lack of effective in vivo T cell–depleting reagents. Previous studies from our laboratory have shown that without T-cell depletion, bone marrow engraftment could not be achieved in miniature swine unless doses of whole-body irradiation (WBI) exceeded 1000 cGy (13–15). We have recently described the generation of a swine CD3 immunotoxin, pCD3-CRM9, which is extremely effective at depleting mature T cells from the peripheral blood (PB), lymph node, and thymus of miniature swine (16). We have also shown recently that cytokine mobilization and apheresis of miniature swine blood allows the collection of peripheral blood stem cells (PBSC) capable of full hematopoietic reconstitution (manuscript submitted for publication, C. Colby, Bone Marrow Transplantation Service, Massachusetts General Hospital, Boston, Massachusetts, USA). In this report, we test whether stable mixed chimerism and donor-specific tolerance can be established in miniature swine following BMC/PBSC transplantation (BMC/PBSCT) using nonmyeloablative conditioning regimens.

Methods

Animals. Transplant donors and recipients were selected from our herd of partially inbred Massachusetts General Hospital (MGH) miniature swine. The immunogenetic characteristics of this herd and intra-MHC recombinant haplotypes have been described previously (17, 18). Two indwelling central catheters were placed in stem cell donor animals in the right and left external jugular veins, extending well into the superior vena cava. A central catheter was also placed in recipient animals to facilitate cell infusion and frequent blood sampling. The catheters were tunneled subcutaneously, exiting the skin dorsally on the neck.

Bone marrow harvest and infusion. Marrow was harvested with curettes from the proximal humeri and tibiae, from the distal femora, and, if necessary, from the vertebrae following exsanguination of the donor animal. After harvest, the marrow was minced, resuspended in RPMI-1640 with 10 μg DNase, and filtered through sterile nylon mesh before centrifugation. Red blood cells (RBCs) were lysed with ACK buffer (Bio Whittaker, Walkersville, Maryland, USA), and the preparation was washed with HBSS with Ca^{2+} (GIBCO BRL, Gaithersburg, Maryland, USA). The marrow was administered slowly at a cell concentration of 10^8/mL by catheter.

PBSC collection. A stem cell mobilizing regimen consisting of daily treatments with recombinant porcine stem cell factor (pSCF; 100 μg/kg) in combination with recombinant porcine–IL-3 (pIL-3; 100 μg/kg), both from BioTransplant (BTI), Boston Massachusetts, USA; with or without 10 μg/kg recombinant human G-CSF (rhu G-CSF; Amgen, Boulder, Colorado, USA), was administered subcutaneously. Collection of PBSC was achieved by leukapheresis (COBE Spectra Apheresis System, Lakewood, Colorado, USA) beginning on day 5 of cytokine therapy and continuing daily until sufficient numbers of cells were collected. PBSC, either fresh or frozen and quickly thawed, were adjusted to a concentration of 2.0×10^8/mL, and the appropriate volume was infused by catheter over a 15- to 20-minute period. PBSC were administered beginning on day 0.

Irradiation. WBI was administered on days –4 and –3. Animals were placed in Plexiglas cages and 150 cGy midline WBI was administered at 20 cGy/min from a cobalt 60 source. Twenty-four hours later the animals received a second identical dose of WBI. Thymic irradiation was administered on day –2. Animals received intravenous telazol (0.15 mg/kg) for sedation and were then placed in the supine position on a plastic cradle and secured into position. Irradiation was administered to a field encompassing the thymus, defined by an x-axis, including the width of the jaw, and a y-axis, including the distance from the top of the sternum to the temporomandibular joint. The defined field was irradiated with 700 cGy from a cobalt irradiator source at a rate of 175 cGy/min.

Recipient T-cell depletion. T-cell depletion was achieved using the newly described diphtheria toxin–based swine CD3 immunotoxin, pCD3-CRM9 (16), made by conjugating the diphtheria toxin binding site mutant, CRM9, to the anti-porcine CD3 mAb, 898H2-6-15. On day –2, 0.05 mg/kg pCD3-CRM9 was administered intravenously to recipient animals.

CyA treatment. CyA (Sandimmune, oral solution; Novartis Pharmaceutics, Basel, Switzerland) was administered through a gastric tube at 30 mg/kg per day in divided doses from day –1 to day 30.

Antibodies and flow cytometry. The following antibodies were used to monitor depletion and recovery of cell populations in the pig by flow cytometry after pCD3-CRM9 administration and PBSC infusion: CD1 76-7-4 BALB/c IgG2aK (19); CD3 898H2-6-15 C3H/HEJ IgG2aK (20); CD3a BB23-8E6 IgG1, and CD5 BB6-9G12 IgG1 provided by Mark Pescovitz, Indiana University, Bloomington, Indiana, USA (21). Flow cytometry was performed using a Becton Dickinson FACScan (San Jose, California, USA). Unseparated heparinized PB was distributed into staining tubes (Falcon 2054) at 100 μL/tube and washed twice using 2 mL flow cytometry buffer (HBSS containing Ca^{2+} and Mg^{2+}/0.1% BSA/0.1% NaN_3). Cells were stained with optimal concentrations of the primary mouse anti-pig mAb (either unconjugated or FITC-conjugated) for 30 minutes at room temperature. For indirect staining, goat anti-mouse IgG (heavy and light chain specific) FITC (GAMF; Sigma Immunochemicals, St. Louis, Missouri, USA) was added for an additional 30 minutes at room temperature. For detection of immunotoxin-coated cells, second-step reagent alone was added. To determine the percentage of donor cells among PB cell populations, PB was incubated with the pig monocyte/granulocyte-specific FITC-conjugated SWC3a mAb 74-22-15 (BALB/c, IgG1K) (22) together with the donor-specific biotin-conjugated 1038H-10-9 (B10.PD1, IgMK) mAb specific for swine PAA (pig allelic antigen) (23) followed by streptavidin phycoerythrin (PESA; PharMingen, San Diego, California, USA). RBC were lysed and the cells were fixed using FACS lysing solution (Becton Dickinson) before acquisition. Data were analyzed using Winlist list mode analysis software (Verity Software House, Topsham, Maine, USA).

Preparation of lymphoid cell suspensions for flow cytometry. Biopsy samples of mesenteric lymph node and thymus were placed in Petri dishes containing flow cytometry buffer (HBSS containing 0.1% BSA and 0.1% NaN_3). Cell suspensions were prepared by grinding the tissue between the frosted ends of 2 slides until only fibrous tissue remained. The cells were then passed through FACS mesh before cell counting and staining for flow cytometry. For flow cytometric staining, cells were distributed into tubes at 10^6 cells/tube. Staining was as described above, except no FACS lysing solution was added. Propidium iodide was added immediately before acquisition to exclude dead cells.

Skin grafts. Skin grafts were performed by techniques published previously (24). Briefly, split-thickness skin was harvested from the donor and placed on a deep split-thickness bed on the recipient's dorsal thorax.

Table 1
Summary of miniature swine treated with the nonmyeloablative preparative regimen

Animal no.	SLA	CyA Days –1 through 30	Donor source (× 10⁸/kg)	pCD3-CRM9 Dose, day	Engraftment Thymic chimerism
12534	cc→cc	No	BM (2)	0.05 mg/kg (× 2), days 0, 2	Yes
12674	cc→cc	No	BM (2)	0.05 mg/kg, day –1	Chimerism lost
13235	ac→ac	No	PBSC (20)[A]	0.05 mg/kg, day –2	Yes
13379	ac→ac	No	PBSC (20)[A]	0.05 mg/kg, day –2	Yes
12743	ac→ac	Yes	PBSC (20)[A]	0.05 mg/kg, day –2	Yes
13131	cc→cc	Yes	PBSC (20)	0.05 mg/kg, day –2	Yes
13475	ad	No	None	None	NA

Miniature swine were administered 300 cGy WBI, 700 cGy TI, and pCD3-CRM9, followed by SLA-matched, PAA-mismatched hematopoietic stem cell grafts with or without a 30-day course of CyA. [A]Donor PBSC were stored frozen and thawed before infusion. All other transplants were done using fresh donor cells. BM, bone marrow. NA, not applicable.

Grafts were examined daily until rejection occurred. Rejection was determined macroscopically and defined as diffuse cyanosis and induration of the graft.

Results

Optimizing the dosage and timing of pCD3-CRM9 administration to allow stable hematopoietic cell engraftment in miniature swine. Table 1 summarizes all animals treated with this nonmyeloablative protocol. The dosage and timing of pCD3-CRM9 administration was optimized to allow engraftment in these animals with minimal toxicity. The initial dosing regimen (given to animal 12534) was chosen based on previous experience using this immunotoxin to deplete swine T cells in vivo without any other conditioning. Maximal depletion occurred 2 days following the second dose when pCD3-CRM9 was administered in 2 split doses of 0.1 mg/kg, each 2 days apart (16).

Although T-cell depletion was best with this dosage and timing, the risk of complications because of peripheral polyneuropathy caused by the toxin component of the immunotoxin was increased when doses of 0.1 mg/kg or greater were used. Animal 12534 was given 2 doses of 0.05 mg/kg pCD3-CRM9 on days 0 and 2, for a total dose of 0.1 mg/kg. This animal developed respiratory complications because of immunotoxin-induced polyneuropathy and was sacrificed on day 33. The dose of pCD3-CRM9 was therefore reduced to 0.05 mg/kg in 1 dose to avoid neurotoxicity in miniature swine. Animal 12674 received 1 dose of 0.05 mg/kg pCD3-CRM9 12 hours before bone marrow cell infusion. No neuropathy developed, but this animal failed to stabilize clinically and had severe platelet (PLT) consumption. Chimerism was lost by 40 days after BMT. All subsequent animals were given 1 dose of 0.05 mg/kg pCD3-CRM9 48 hours before PBSC infusion to allow maximal host T-cell depletion before donor cell infusion. All PBSC-transplanted animals receiving 0.05 mg/kg pCD3-CRM9 on day 2 (4/4) successfully engrafted with no complications of neuropathy. Figure 1 is a schematic diagram of this nonmyeloablative preparative regimen for hematopoietic cell transplantation, which was used in all subsequently transplanted animals. Two PBSC-transplanted animals were treated with a 30-day course of CyA and 2 additional animals were treated with the same preparative regimen without CyA (Table 1). All PBSC-transplanted animals (4/4) successfully engrafted regardless of whether or not they were given a short course of CyA. The addition of CyA to this nonmyeloablative regimen was not necessary, therefore, for stable engraftment across minor histocompatibility barriers in miniature swine.

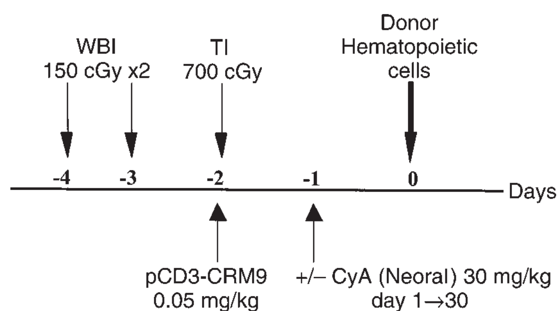

Figure 1
Schematic representation of nonmyeloablative preparative regimen used to establish hematopoietic mixed chimerism in miniature swine.

Effect of the preparative regimen on PB T-cell depletion. T-cell depletion was monitored daily by FACS from the first day of WBI until infusion of donor cells on day 0 in all animals, except animal 12534. Figure 2a shows the absolute number of T cells remaining in the PB each day, as determined by staining with 2 different T-cell specific mAbs, BB23-8E6 (CD3a) and BB6-9G12 (CD5) and by multiplying the percentage of positive cells by the white blood cell count as determined by complete blood cell count. A comparison was made of animals receiving pCD3-CRM9 and 1 control animal (animal 13475) receiving an identical conditioning regimen without pCD3-CRM9. In all animals treated with immunotoxin, T-cell numbers reached less than 0.2% of the pretreatment level in the PB by day 0. Absolute numbers of T cells remaining in PB on day 0 ranged from 4 (animal 13235) to 112 (animal 12674) T cells per microliter of blood (Figure 2b). All remaining T cells were coated with immunotoxin on day 0 as detected by staining with a FITC-conjugated goat anti-mouse Ig-specific reagent (data not shown). The control animal without pCD3-CRM9 had 30- to 150-fold more T cells remaining in the PB (593 T cells/μL) than suc-

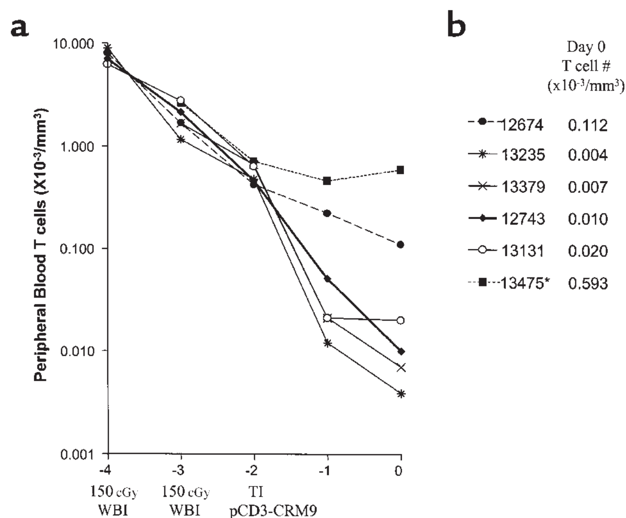

Figure 2
PB T-cell depletion. (**a**) Graph of the absolute number of T cells remaining in PB each day during the nonmyeloablative preparative regimen. *Animal 13475 received the same preparative regimen, except without T-cell depletion. (**b**) Chart showing the actual number of T cells detected in the PB immediately before donor cell infusion on day 0.

cessfully engrafted animals treated with pCD3-CRM9 (4–20 T cells/μL).

WBC and PLT level of animals undergoing nonmyeloablative regimen. Figure 3 shows the WBC and PLT level of animals treated with the nonmyeloablative preparative regimen for hematopoietic cell transplantation. The WBC and PLT level of animals given BMT (animals 12534 and 12674) is shown separately from that of those receiving PBSCT (animals 13235, 13379, 12743, 13131). WBC dropped below 1000/μL within 1 week of the start of the preparative regimen for all animals except animal 13131 (Figure 3a), then recovered within 2 to 3 weeks after transplant in most animals. PLT dropped below 100,000/μL in the first 3 weeks and recovered in most animals within 2 to 3 weeks (Figure 3b). Multiple PLT infusions were necessary during the time of low PLT and cytopenia. Animal 12674 never fully stabilized clinically and was sacrificed on day 40 because of severe PLT consumption and failure to recover WBC.

Detection of multilineage chimerism in PB. Chimerism was detected by flow cytometry using our pig allelic antigen (PAA) mAb, which recognizes a PAA present on swine leukocytes (23). Donor and recipient animals were selected as PAA$^+$ and PAA$^-$, respectively, to facilitate chimerism detection. Figure 4 shows the percentage of donor cells detected in the PB following either BMT (Figure 4a) or PBSCT (Figure 4b). Five out of 6 animals successfully engrafted with the protocol in Table 1 showed high levels of myeloid chimerism. Most animals showed initially low levels of lymphoid chimerism, but this gradually increased over time (Figure 4b). Although the proportion of donor myeloid cells appears to decrease slightly over the first 200 days, myeloid chimerism remains stable long-term, as shown for animal 13235 and animal 13131 in Figure 4b. The 1 animal that failed to engraft (animal 12674) showed very poor T-cell depletion compared with the other animals. Animal 12674 had 112 T cells remaining per microliter of blood, whereas all 4 successfully engrafted animals had only 4–20 T cells/μL remaining on day 0. The unstable clinical course observed in animal 12674 may have been a consequence of an host-versus-graft reaction resulting in graft rejection, possibly combined with a GvHD reaction leading to failure of host hematopoietic recovery (see discussion). All other animals recovered without any evidence of GvHD. Although the level of T-cell depletion was not determined for animal 12534, we can presume that it was very good because the dose of pCD3-CRM9 was twice that used in all of the other animals. Unlike all other engrafted animals in this regimen, animal 13131 showed a high percentage of donor cells in the lymphocyte lineage even at the earliest time point during the first week.

Detection of a high level of donor cell chimerism in the thymus of successfully engrafted animals. The level of thymic chimerism was determined by FACS staining of thymocyte suspensions prepared from thymic biopsy tissue using the donor-specific PAA mAb. Figure 5 shows the percentage of PAA$^+$ donor cells detected in the thymus at times of thymic biopsy for each animal. All animals except 12674 had very high levels of chimerism detected in the thymus. The thymic chimerism level was consistent with the level of myeloid chimerism in each animal and may reflect the level of hematopoietic progenitor and stem cell engraftment. Thymocyte development appeared phenotypically normal following mixed chimerism establishment, and donor cells could be detected in thymocytes at all stages of development (Figure 6, a and b). Figure 6a, parts 1,2, and 3, show, respectively, forward scatter (FSC) versus CD1, FSC versus CD3, and FSC versus PAA profiles before and 72 days after PBSCT in animal 13235. The distribution of immature and mature thymocyte populations was identical before and after transplantation (Figure 6a, parts 1 and 2). Donor-specific PAA could be detected in all size fractions of thymocytes after, but not before PBSCT (Figure 6a, part 3). Figure 6b confirms that donor PAA$^+$ thymocytes were present among the immature CD1-positive thymocytes (upper graph, quadrant 2), and among the mature CD3-high thymocytes (lower graph, quadrant 2). A high level of donor cell chimerism (> 20% PAA$^+$) could also be detected in the bone marrow of all successfully engrafted animals (data not shown). However, the actual level of bone marrow chimerism is difficult to quantify in this model, because PAA is not expressed on 100% of bone marrow cells in naïve PAA$^+$ animals as it is in all other lymphoid tissues (23).

Prolongation of donor skin graft survival times in mixed chimeric animals. Skin grafts were placed on the four long-term stable mixed chimeras (animals 13235, 13379, 12743, and 13131) at least 60 days after PBSCT.

The day of rejection for skin grafts is shown in Table 2. All animals showed either indefinite acceptance or significant prolongation of donor skin relative to third-party swine leukocyte antigen–matched (SLA-matched) control skin. Figure 7 shows skin graft appearances for animal 13379, which showed the smallest difference in rejection times of control versus donor skin grafts. Donor skin (Figure 7b) was completely intact with no significant difference from self skin (Figure 7a) at 41 days after skin grafting and remained intact up to 50 days, whereas both third-party SLA-matched skin grafts (Figure 7, c and d) were completely rejected by day 41 (Figure 7). Slight prolongation of SLA-matched third-party skin grafts was observed in 3 out of 4 animals receiving the nonmyeloablative regimen, regardless of whether or not CyA was included in the regimen (Table 2). CyA levels were undetectable in all animals at the time of skin grafting. One of 2 animals receiving the nonmyeloablative regimen without CyA rejected third-party SLA-matched skin promptly at the expected time of 10 days. In one animal on which both third-party SLA-matched and SLA-mismatched skin was placed, the SLA-mismatched skin was rejected promptly as expected (10 days), whereas the SLA-matched skin graft survival was slightly prolonged (24 days).

Table 2
Skin graft survival times in mixed chimeric animals

Animal no.	Donor SLA-matched	Third-party
13235	45	10, 10
13379	50	31, 41
12743	>50	24, 10[A]
13131	>235	22, 30

[A]SLA-mismatched third-party skin.

Discussion

In this report, we demonstrate, we believe for the first time, the successful establishment of stable mixed chimerism and donor-specific tolerance in miniature swine. Chimerism was established using a relatively mild nonmyeloablative conditioning regimen, similar to that developed initially in rodents. To extend treatment regimens developed in rodents to the clinic, large-animal models are needed, both to understand the mechanism and to optimize the treatment protocol. A critical component for successful engraftment using the nonmyeloablative regimen developed in rodents is effective T-cell depletion of the recipient (25, 26). Complete and specific T-cell depletion in large animals, however, has been much more difficult to achieve (27, 28). In this report, we describe the successful establishment of stable mixed chimerism in miniature swine using a nonmyeloablative preparative regimen that includes T-cell depletion with the newly described immunotoxin, pCD3-CRM9 (16). The availability of our newly described PAA mAb, which is capable of distinguishing a non-MHC allelic marker, PAA, present on

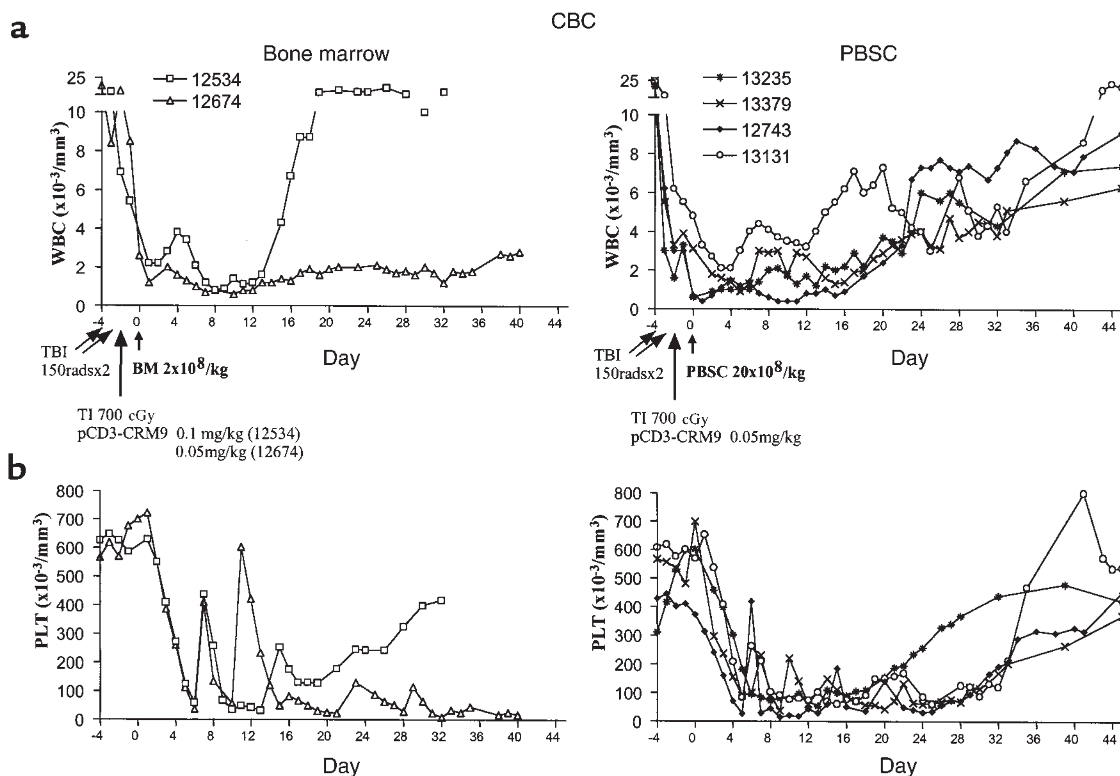

Figure 3
WBC and PLT counts during engraftment after nonmyeloablative preparative regimen. (**a**) WBC and (**b**) PLT.

pig leukocytes, greatly facilitates chimerism detection in this model (23).

Although we observed some neurotoxicity in miniature swine when using the diphtheria-based immunotoxin at doses of 0.1 mg/kg or greater, we were able to overcome this problem by using lower doses. It is possible that irradiation given in this protocol may increase the risk of neuropathy in miniature swine. No neuropathy was observed in animal 12674 following the reduction to 1 dose of 0.05 mg/kg pCD3-CRM9 12 hours before donor bone marrow infusion. This animal failed to engraft, however, and never fully stabilized clinically. Administration of pCD3-CRM9 too close to BMT may have resulted in insufficient host T-cell depletion by day 0 and also may have led to partial in vivo T-cell depletion of donor T cells, possibly contributing to HvGD and the animal's clinical instability. Based on our experience using this immunotoxin in combination with WBI and thymic irradiation (TI; Table 1), we have determined that a dose of 0.05 mg/kg pCD3-CRM9 administered 2 days before PBSC transplantation allows sufficient T-cell depletion in this protocol to enable successful engraftment with minimal toxicity. It should be pointed out that peripheral neuropathy has never been observed in rhesus monkeys receiving anti-rhesus CD3-CRM9 at doses ranging between 0.13–0.20 mg/kg (29–32).

Initial attempts at establishing mixed chimerism in miniature swine using the newly developed swine T cell–depleting reagent pCD3-CRM9 were made using bone marrow as the donor stem cell source. With the successful development of PBSC technology in our laboratory, we are now consistently using cytokine-mobilized PBSC as a source of donor hematopoietic cells for transplantation. Two major advantages of using PBSC over BM as the donor cell source are that the donor can

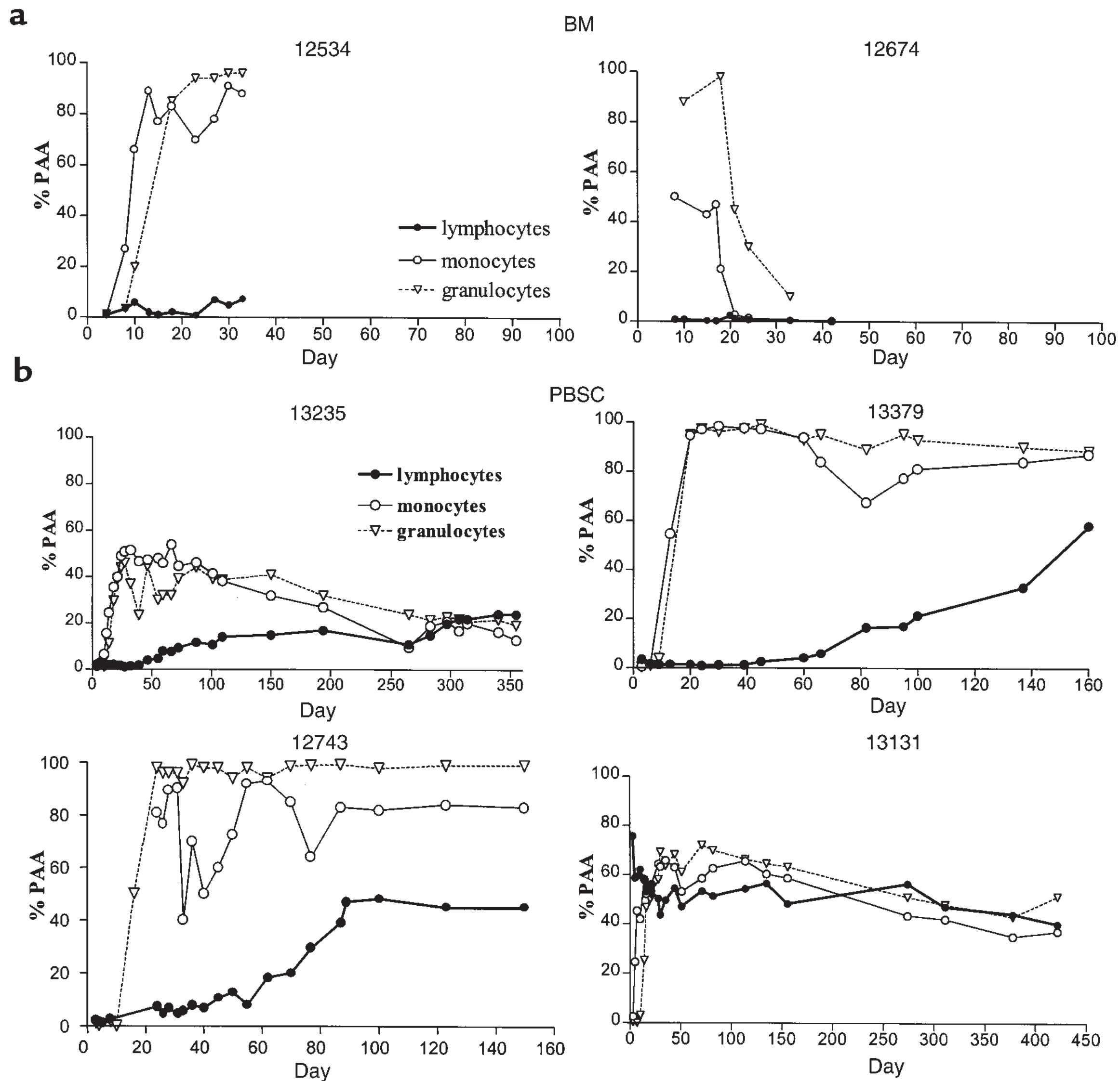

Figure 4
Percent of donor PAA+ cells detected in the PB after (**a**) BMT and (**b**) PBSCT in miniature swine treated with the nonmyeloablative preparative regimen.

be kept alive for future skin/organ transplantation into the recipient and that very large numbers of donor hematopoietic cells can be collected by multiple leukapheresis procedures.

Stable mixed chimerism has been demonstrated after bone marrow transplants in lethally and sublethally irradiated DLA-identical littermate dogs (33, 34). The addition of immunosuppressive drugs, including CyA, following transplantation was determined to be necessary to maintain stable chimerism in the dog model, which did not include recipient T-cell depletion or TI. Our studies using miniature swine demonstrate that CyA administration is not necessary for successful engraftment across minor histocompatibility barriers. The addition of pCD3-CRM9 to the regimen in miniature swine may provide sufficient T-cell depletion to allow engraftment without additional immunosuppression. Although these studies show that CyA administration is not necessary for successful engraftment across minor histocompatibility barriers in miniature swine, our preliminary studies suggest that CyA may still be valuable for GvHD prophylaxis when MHC barriers are crossed.

These studies in miniature swine provide the first evidence for donor-specific tolerance induced by the mixed chimerism approach in a large-animal model. Donor skin graft survival was significantly prolonged relative to third-party SLA-matched and SLA-mismatched skin. The eventual rejection of donor skin in some tolerant animals is likely because of skin-specific antigens, because these animals nevertheless maintained peripheral and thymic mixed chimerism after donor skin graft rejection. Despite the induction of tolerance by donor PBSC, it is possible that skin-specific antigens for which tolerance had not been induced in the recipient by PBSC were present on the donor skin graft. Animal 13131, tested in the same way, showed permanent donor skin survival. These data are consistent with what is known about skin-specific antigens in mice (35–37). The slight prolongation of third-party SLA-matched skin in 3 of 4 animals may be due to sharing of minor histocompatibility antigens between the PBSC donor and/or recipient and the SLA-matched skin graft donors. Another possibility is that this nonmyeloablative regimen causes some degree of immunoincompetence, resulting in slight prolongation of minor-mismatched skin, but still allowing normal rejection of SLA-mismatched skin. In 2 of the 3 animals that showed delayed rejection of third-party skin, donor-specific tolerance was clearly demonstrated, through indefinite donor skin graft survival (13131) or through consistent donor-specific hyporesponsiveness as assessed in vitro by cell-mediated lympholysis (CML) assays, even after rejection of donor skin (13379). These data will be presented in a subsequent article on the subject of skin-specific antigens in miniature swine (Y. Fuchimoto, manuscript in preparation).

Three of 4 animals showed a delayed increase in donor cell chimerism among the lymphocyte lineage during the first 2–3 months, compared with the high level of chimerism detected in the thymus at the time of the earliest biopsy (3–4 weeks). Consistent with the percentage of chimerism as shown in Figure 4b, the absolute number of donor lymphocytes present in the PB does not increase significantly until 30–60 days following transplantation (data not shown). These data suggest that it takes approximately 30–60 days for the thymus to regenerate and start functioning following TI and T-cell depletion. The existence of host-type lymphocytes in the PB during this time is most likely because of an expansion of residual mature host T cells remaining after conditioning, rather than recent emigration from the thymus. The donor cell product infused into the 1 animal with an immediate high level of lymphoid chimerism (animal 13131) had a very high percentage of T cells (65%) compared with that found in the products infused into the other 3 animals (15–20%) (data not shown). Persistence of mature donor T cells from the PBSC inoculum may explain the early high level of lymphoid chimerism detected in this animal.

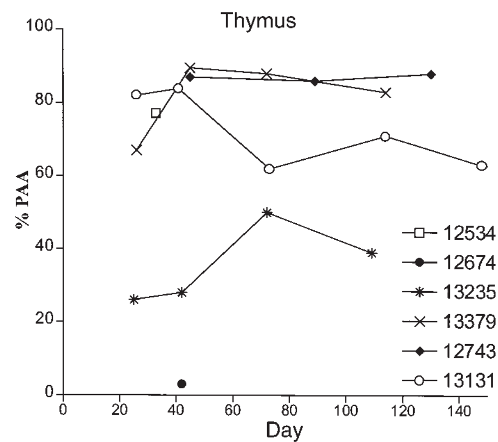

Figure 5
Percent of donor PAA$^+$ thymocytes detected in thymic biopsy samples taken from miniature swine after nonmyeloablative preparative regimen and hematopoietic cell transplantation.

Although the relative percentage of donor cells among the myeloid populations decreased slowly over the first 200 days in the 2 long-term animals, this level has now remained stable for at least 150 days. We do not believe that this decrease was because of chronic rejection, but rather because of a slow approach to equilibrium, because the animals remained tolerant to the donors (data to be presented in a future communication). Thus, for example, animal 13131, which showed the greatest proportional decline in myeloid chimerism, has subsequently accepted a heart transplant from the PBSC donor for over 300 days without any immunosuppression (J. Madsen, personal communication).

In this paper, we have demonstrated, we believe for the first time, the establishment of stable multilineage mixed chimerism and tolerance in miniature swine. The ability to achieve mixed chimerism and tolerance with-

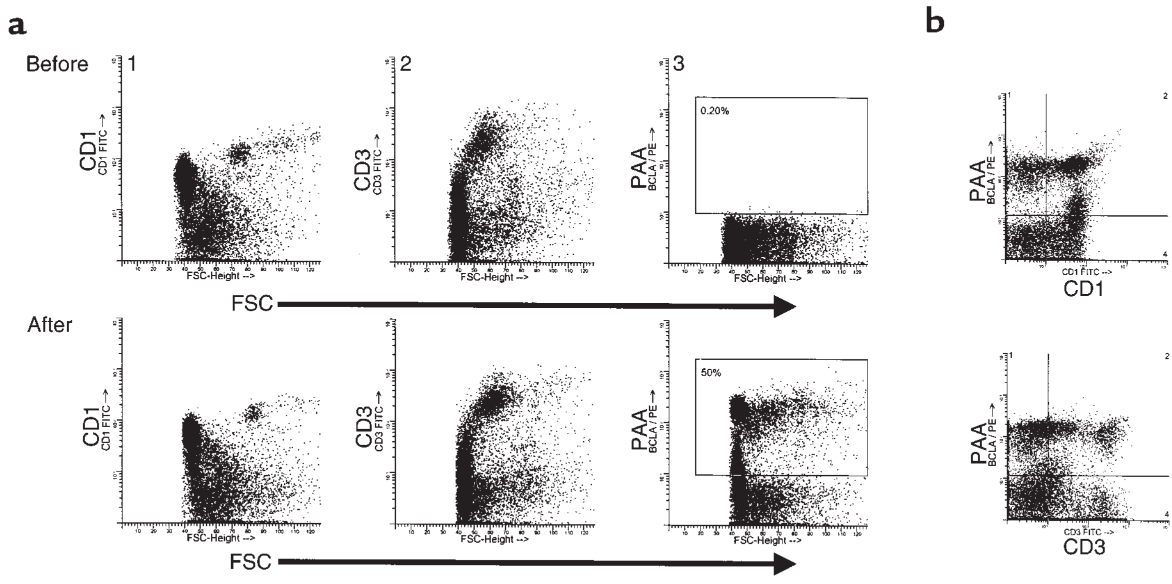

Figure 6
Thymic regeneration and mixed chimerism establishment after nonmyeloablative preparative regimen. (**a**) FACS dot plot representation of different size (FSC) fractions of (1) immature (CD1⁺); (2) mature (CD3⁺); and (3) donor-type (PAA⁺) thymocytes before and 72 days after PBSCT in animal 13235. (**b**) Two-color FACS analysis of thymocytes from animal 13235 on day 72 showing donor PAA+ thymocytes present in both the immature (CD1⁺) and mature (CD3⁺) fractions.

out myeloablation makes this an attractive protocol for potential clinical application. The relatively mild preparative regimen should make this approach appropriate for the treatment of patients with nonmalignant diseases, such as hemoglobinopathies, as well as for the induction of tolerance for organ transplantation, avoiding the need for chronic immunosuppression. Immediate clinical applications for this mixed chimerism approach include establishment of chimerism from an HLA-matched sibling as treatment of a nonmalignant disease and kidney transplantation following chimerism establishment from an HLA-matched living related donor. Although a slightly increased cancer risk has long been associated with exposure to irradiation (38, 39), the benefits of avoiding long-term immunosuppression are likely to outweigh the risk of cancer because of low-dose WBI in this model. Future challenges include the elimination of WBI in this protocol, the use of MHC-mismatched donors, and the development of a protocol suitable for use in cadaveric organ recipients. Experiments are currently in progress in our laboratory to establish mixed chimerism across MHC barriers and to reduce toxicity of the protocol even further by eliminating WBI. These experiments will be the subject of a subsequent article.

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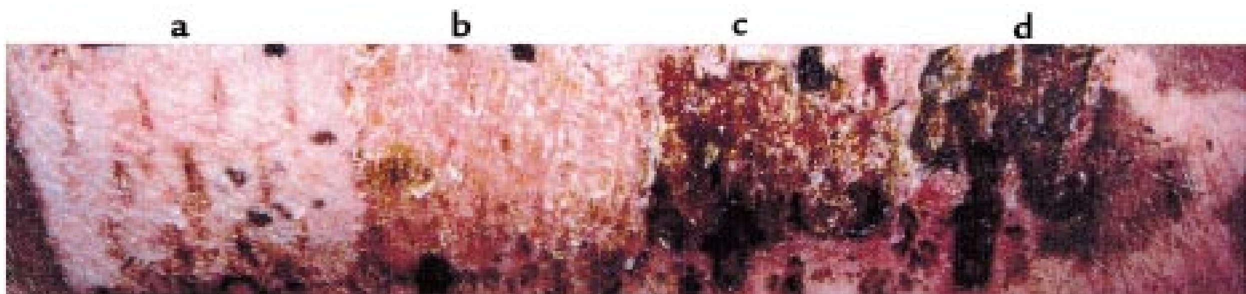

Figure 7
Day 41 after grafting of self skin (**a**), PBSC donor skin (**b**), and 2 third-party SLA-matched skin grafts (**c** and **d**) placed on animal 13379 82 days after PBSCT. Black rectangular areas are the sites where skin biopsies were taken.

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