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Xenograft Tolerance and Immune Function of Human T Cells Developing in Pig Thymus Xenografts

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Transplantation of xenogeneic thymus tissue allows xenograft tolerance induction in the highly disparate pig-to-mouse model. Fetal swine thymus (SW THY) can support the generation of a diverse human T cell repertoire that is tolerant of the pig in vitro. We demonstrate that SW THY generates all human T cell subsets, including regulatory T cells (Tregs), in similar numbers as fetal human thymus (HU THY) grafts in immunodeficient mice receiving the same human CD34+ cells. Peripheral T cells are specifically tolerant to the mouse and to the human and porcine donors, with robust responses to nondonor human and pig Ags. Specific tolerance is observed to pig skin grafts sharing the THY donor MHC. SW THY–generated peripheral Tregs show similar function, but include lower percentages of naive-type Tregs compared with HU THY–generated Tregs. Tregs contribute to donor-pig specific tolerance. Peripheral human T cells generated in SW THY exhibit reduced proportions of CD8+ T cells and reduced lymphopenia-driven proliferation and memory-type conversion, accelerated decay of memory-type cells, and reduced responses to protein Ags. Thus, SW thymus transplantation is a powerful xenotolerance approach for human T cells. However, immune function may be further enhanced by strategies to permit positive selection by autologous HLA molecules. The Journal of Immunology, 2014, 192: 000–000.

Xenotransplantation could resolve the supply-demand discrepancy in human organ transplantation, and pigs are considered the most suitable xenogeneic source animal (1–5). However, transplants from pigs are subject to vigorous rejection in primates (5, 6). Although hyperacute rejection owing to anti–Gal1–3Gal (Gal) natural Abs (7) is avoided by using Gal KO pigs (3, 4, 8–10), T cell–dependent Abs recognizing other pig specificities can cause acute humoral rejection (11, 12). T cell–suppressive treatments significantly prolonged porcine xenograft survival in nonhuman primates, but such treatments are highly toxic (9, 10, 13–15). Thus, induction of T cell tolerance is likely to be critical for clinical application of xenotransplantation.

Thymic xenotransplantation can induce robust tolerance across xenogeneic barriers (9, 16–18). In a pig-to-mouse model, murine recipients of fetal swine thymus (SW THY) grafts showed donor-specific unresponsiveness in vitro and specific skin graft acceptance with immunocompetence (19, 20). Porcine thymic grafts can generate diverse and functional human T cell repertoires (21) in mice that are specifically unresponsive to the donor pig in vitro (22, 23). Extension of this approach from the pig to the baboon model has achieved lasting pig kidney xenograft survival in nonhuman primates (9).

We have now used this pig-to–humanized mouse model to demonstrate that SW THY–generated T cells are tolerant to the human HSC donor and the murine recipient and show specific tolerance toward swine leukocyte Ag (SLA)-matched porcine skin grafts. Regulatory T cells (Tregs) develop normally in SW THY and contribute to the donor SLA-specific unresponsiveness. However, human SW THY–generated T cells demonstrate functional alterations in the periphery that may be due to ineffective postthymic interactions with HLA molecules. These results suggest approaches to optimizing immune function when this powerful approach is used to induce xenograft tolerance in human T cells.

Materials and Methods

Animals and tissues

NOD-severe combined immunodeficient (NOD/SCID) mice from Jackson Laboratory (Bar Harbor, ME) were housed in a specific pathogen-free environment. Human fetal thymus (HU THY) and liver (gestational age, 17–20 wk) tissues were obtained from Advanced Biosciences Resource (Alameda, CA). SW THY was harvested from fetuses (60–90 gestational days) of Massachusetts General Hospital SLA-defined miniature swine (provided by Dr. David H. Sachs). Studies were done with approval from Institutional Review Boards and Animal Care and Use Committees.

Human and porcine fetal tissue transplantation

NOD/SCID mice (6–10 wk old) were conditioned with 2.5 Gy total body irradiation. Human (HU) or SW THY plus human fetal liver (FL)
fragments were implanted under the kidney capsule as described previously (22). In some experiments, SW THY fragments were cryopreserved and thawed prior to transplantation as described (24). Fresh or cryopreserved CD34+ human fetal liver cells (FLCs; 1–5 × 10^5), isolated as described (24), were administered i.v.

**Flow cytometry**

Human hematopoietic chimerism was assessed by flow cytometry (FCM) as described (24). Fluorochrome-labeled mAbs purchased from BD Pharmingen (San Diego, CA) were used: anti-mouse CD45 and Ter119, anti-human CD4, CD8, CD14, CD19, CD45, CD3, CD45RA, CD45RO, CD62L, CD127, HLA-DR, CCR7, FOXP3, anti-pig CD2, and isotype control mAbs. FCM was performed with exclusion of dead cells and murine erythroid cells as described (24).

For intracellular staining, cells were washed, surface Ag stained, and then fixed and permeabilized with the FOXP3 Staining Buffer Set (eBioscience, San Diego, CA), followed by incubation with anti-human FOXP3. Cells were washed in permeabilization buffer and resuspended in PBS.

**In vitro T cell responses**

Splenocytes and lymph node (LN) cells were harvested from humanized mice and mononuclear cells isolated by Ficoll separation. Human T cells were enriched (purity 90%) by depletion of anti-mouse CD45 and Ter119 microbeads (Miltenyi Biotec, Auburn, CA) followed by T cell purification (Pan T cell isolation kit II; Miltenyi Biotec). Responders T cells (10^5 per well) were cultured in U-bottom, 96-well plates in AIM-V medium (Invitrogen, Carlsbad, MO) supplemented with 10% human AB serum, 1% HEPES buffer, and 1 × 10^-5 M 2-ME (Sigma-Aldrich, St. Louis, MO). Irradiated human allogeneic or swine xenogeneic PBMCs (3000 cGy, 10^5 cells per well) stimulators were cocultured for 5 d at 37°C in 5% CO2. Irradiated NOD/SCID mouse splenocytes or human FL donor-derived dendritic cells (DCs) were also used as stimulators. Human FL donor-derived DCs were generated from CD34 FLCs by culture for 3 d in rhGM-CSF (50 ng/ml) and recombinant human stem cell factor (recombinant human stem cell factor [rhSCF]; 50 ng/ml); followed by 3 d in rhGM-CSF (100 ng/ml), rhSCF (50 ng/ml), and rhTNF-α (1 ng/ml); then 6 d in rhGM-CSF (100 ng/ml), rhIL-4 (20 ng/ml), and rhTNF-α (0.2ng/ml); and an additional day in rhGM-CSF (100 ng/ml), rhIL-4 (20 ng/ml), TNF-α (20 ng/ml), and PGE2 (3 μg/ml). Cytokines were purchased from R&D Systems (Minneapolis, MN). Cells were harvested after 16 h of incubation with 1 μCi of [3H]thymidine, and [3H]thymidine incorporation was measured as described previously (25). Data are presented as mean of [3H]thymidine incorporation in triplicate cultures or as stimulation index (mean cpm of the indicated coculture divided by that of responder cells alone).

To determine the possible role of Tregs in donor SLA-specific unresponsiveness, enriched human T cells from splenocytes and LNs were depleted of human CD25+ cells by MACS Microbeads (Miltenyi Biotec) and cultured in U-bottom 96-well plates as described above.

**Skin grafting**

Split thickness (2.3 mm) skin from donor SLA-matched and mismatched Massachusetts General Hospital miniature swine was grafted on the lateral thoracic wall 12 wk after THY transplantation. Skin grafts were evaluated for activity following transplantation in NOD/SCID mice with untreated (white bars; n = 29) versus HU THY in combination with human FL CD34 cells (white bars; n = 17). The figure shows combined results of two experiments. (D) Peripheral pig CD2+ detection following transplantation in NOD/SCID mice with untreated (white bars; n = 35) and cryopreserved/thawed (black bars; n = 19) fetal porcine thymus/human liver grafts plus human CD34 cells. (E) Reduced CD8+CD4+ T cell ratio detected among PBMCs of SW/HU (black bars; n = 19) compared with HU/HU mice (white bars; n = 19) 16 wk after THY/LIV/FL CD34 transplantation. The figure shows combined results of two experiments in which the SW thymus graft had been fresh or cryopreserved at the time of implantation. *p < 0.05, **p < 0.01, ***p < 0.001 two-way ANOVA Bonferroni test or Mann–Whitney test.
daily from day 7 until 4 wk, then once every 3 d and considered rejected when <10% of the graft remained viable.

Adoptive transfer

Eighteen to 20 wk after transplantation, human cells were prepared from spleens and peripheral LNs of humanized mice. Naïve T cells enriched using anti-CD45RO microbeads (>97% CD45RO+) and labeled with CFSE (Molecular Probes, Eugene, OR), and 2 × 10^6 cells were transferred into secondary recipient mice that previously received fetal CD34 cells from the same donor without a THY graft and therefore contained autologous (to the T cells) APCs without T cells as described previously (26). Fourteen days later, LN and spleen cells were harvested from secondary recipients, pooled from each individual mouse, and analyzed as described (26).

T cell decay study

To analyze decay of HU or SW THY-generated T cells, unilateral nephrectomy of the kidney bearing the graft was performed.

Tetanus responses of humanized mice

Sixteen weeks after transplantation, THY-grafted mice were immunized with 100 μl tetanus toxoid (TT; 1 flculation unit per mouse; Sanofi Pasteur, Swiftwater, PA), or PBS in controls, emulsified in 100 μl CFA (Sigma-Aldrich) s.c. on the back and boosted with 100 μl TT (or PBS) in 100 μl IFA (Sigma-Aldrich) 2 and 5 wk later. Three weeks after reboost, splenocytes and LNs were harvested and human cells were enriched as described above, to purity >90%. Responder human cells were cocultured with a neoantigen-, chicken Ig- (10 μg/ml, Sigma-Aldrich), a recall Ag, TT-pulsed human FL donor-derived DCs (1 μg/ml, EMD Millipore, Billerica, MA), or medium alone for 5 d at 37°C in 5% CO₂. Cells were harvested after 16 h of incubation with 1 μCi [3H]thymidine. [3H]Thymidine incorporation was measured with a β-counter. All data are shown as mean stimulation index.

In vitro suppression assay

Human T cells of HU or SW THY–grafted mice were immunized with 100 μl tetanus toxoid (TT; 1 flculation unit per mouse; Sanofi Pasteur, Swiftwater, PA), or PBS in controls, emulsified in 100 μl CFA (Sigma-Aldrich) s.c. on the back and boosted with 100 μl TT (or PBS) in 100 μl IFA (Sigma-Aldrich) 2 and 5 wk later. Three weeks after reboost, splenocytes and LNs were harvested and human cells were enriched as described above, to purity >90%. Responder human cells were cocultured with a neoantigen-, chicken Ig- (10 μg/ml, Sigma-Aldrich), a recall Ag, TT-pulsed human FL donor-derived DCs (1 μg/ml, EMD Millipore, Billerica, MA), or medium alone for 5 d at 37°C in 5% CO₂. Cells were harvested after 16 h of incubation with 1 μCi [3H]thymidine. [3H]Thymidine incorporation was measured with a β-counter. Data are expressed as actual cpm. In self-stimulated control cultures, responder cells were incubated with irradiated autologous PBMCs. Data are shown as mean of [3H]thymidine incorporation in triplicate cultures or as stimulation index that was calculated by dividing the stimulated response with the mean [3H]thymidine incorporation with autologous stimulators. Statistical analysis

Statistical analyses were performed with PRISM 4.0 (GraphPad, San Diego, CA). Data in bar graphs are expressed as mean ± SEM. Student t test, Mann–Whitney U test, or ANOVA were used to compare groups. Pearson’s correlation coefficient test was used to analyze curve regression; p < 0.05 was considered statistically significant.

Results

Multilineage human immune reconstitution in humanized mice with a porcine thymus

We compared the kinetics of human T cell reconstitution in sublethally irradiated NOD/SCID mice given a SW or autologous HU THY graft plus a HU FL graft along with i.v. injected CD34 cells isolated from the same human FL. These mice are termed SW/HU and HU/HU mice, respectively. As shown in Fig. 1A, similar human peripheral blood chimerism was detected, with similar percentages of human CD44^hi, CD19^hi, and CD14^hi cells. However, CD3^hi cell recovery was slightly reduced in SW/HU compared with HU/HU mice when all five experiments were combined (Fig. 1A). This difference was apparent, however, only in experiments involving fresh SW THY tissue (Fig. 1B) and disappeared when the graft was cryopreserved and thawed prior to implantation (Fig. 1C). In mice that received a fresh SW THY graft, a significantly greater proportion of peripheral pig CD2^hi T cells was detected (Fig. 1D).

Despite the overall similar peripheral human T cell reconstitution, the CD8:CD4 ratio was significantly reduced in SW/HU compared with HU/HU mice, regardless of whether the implanted SW THY graft had been fresh or cryopreserved (Fig. 1E).

Donor-specific xenotolerance of human T cells in SW/HU mice in vitro and in vivo

Previously, we demonstrated donor-specific xenogenic tolerance of SW THY–generated human thymocytes (22). We assessed the tolerance of peripheral T cells in SW/HU mice. We compared the
reactivity of splenic and LN T cells toward allogeneic human PBMCs, DCs generated from the fetal liver of the original human donor, donor SLA-matched and third party pig PBMCs, and the recipient mouse strain (Fig. 2A). Although human T cells in SW/HU and HU/HU mice showed robust in vitro alloresponses, self-tolerance toward DCs from the human CD34 cell donor and to the NOD/SCID recipient mouse was observed in both groups. Furthermore, T cells in the SW/HU but not HU/HU mice showed specific tolerance to the porcine thymus donor, demonstrating unresponsiveness to donor SLA-matched PBMCs, with strong responses to unrelated pig PBMCs (Fig. 2A). In contrast, T cells from the HU/HU mice showed similarly strong xenoresponses to both SLA haplotypes.

After T cell reconstitution, the animals received donor SLA-matched and -mismatched skin grafts. As shown in Fig. 2B, four of five SW/HU mice accepted their thymus donor SLA-matched skin graft for >60 d, and three of these four rejected SLA-mismatched porcine grafts. All HU/HU control mice rejected xenogeneic skin grafts. As expected, unreconstituted NOD/SCID mice failed to reject porcine skin.

Comparable human thymocyte subsets and Treg development in HU and SW grafts

We compared thymocyte numbers and phenotypes in SW and HU grafts generated with the same human FL donor. As shown in Fig. 3A, similar, large numbers of human thymocytes were generated in both sets of grafts with normal percentages of double-positive and CD4 and CD8 single-positive thymocytes (Fig. 3A; Supplemental Fig. 1A), as described (21). Thus, the reduced CD8:CD4 ratio in the periphery of SW/HU compared with HU/HU mice cannot be explained by reduced CD8 cell development in the SW/HU mice. Human natural Tregs were present in both sets of grafts, with similar proportions and numbers of FOXP3+ cells among CD4 SP thymocytes (Fig. 3A; Supplemental Fig. 1A).

Reduced proportions of naive Tregs in periphery of SW/HU compared with HU/HU mice

Significantly reduced percentages of human FOXP3+CD25+CD127- Tregs were detected among CD4+ T cells of SW/HU compared with HU/HU mice in two of three experiments (Fig. 3B; Supplemental Fig. 1B–D), whereas no difference was seen in a

**FIGURE 3.** Comparison of thymocyte subsets and peripheral Treg phenotypes in SW/HU and HU/HU mice. (A) Similar cellularity and normal subset distribution as well as Treg percentages of single positive CD4 thymocytes were detected in porcine (black bars; n = 3) versus human fetal thymus grafts (white bars; n = 3) implanted with fetal liver 18 wk earlier in NOD/SCID mice that also received HU FL CD34 cells. (B) Reduced percentages of human FOXP3+CD25+CD127- Tregs were detected among the CD4+ T cells in PBMCs of SW/HU compared with HU/HU mice in two of three experiments 16 wk after THY/LIV/FL CD34 transplantation. The indicated number of mice was analyzed in each experiment. ***p < 0.001 Mann–Whitney U test. (C) Sixteen weeks after THY/LIV/FL CD34 transplantation, HU/HU and SW/HU mice were bled, and the proportions of CD45RO+ and HLA-DR+ cells among human FOXP3+CD25+CD127- CD4+ cells in PBMCs were compared. The figures show combined results of three independent experiments. Data are expressed as means ± SEM. *p < 0.05, **p < 0.05, ***p < 0.001, compared in the indicated combinations (two-way ANOVA, Bonferroni test.).

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third experiment. In normal, adult human PBMCs, most FOXP3+ Tregs are characterized by the CD45RA–CD45RO+ “memory” phenotype and ∼40% express HLA-DR (Fig. 3C; Supplemental Fig. 1E). However, 16–18 wk after thymus grafting, the percentage of “memory”-type cells in HU/HU and SW/HU mice was significantly reduced compared with normal adult humans, consistent with the younger immune systems in the animals (Fig. 3C). The reduced Treg proportions in SW/HU mice, when present, reflected only reductions in naive-type Tregs, with no difference from HU/HU mice in the proportions of CD4+ cells that were memory-type Tregs (Fig. 3C; Supplemental Fig. 2).

SW THY–generated Tregs participate in donor-specific unresponsiveness

To examine the role of peripheral Tregs in maintaining donor SLA-specific tolerance in SW/HU mice, we assessed responses of human splenic and LN T cells depleted of CD25+ Tregs (Fig. 4A). Alloreactivity and xenoreactivity of T cells from two of two HU/HU mice tested was significantly increased in vitro after Treg depletion. In contrast, in vitro T cell responses from two SW/HU mice toward allogeneic PBMC did not change significantly after Treg depletion (Fig. 4). However, depletion of Tregs revealed a modest proliferative response toward donor SLA-matched (SLAdd) pig stimulators that was statistically significant in one animal, but just missed significance in the other (p = 0.06), suggesting a role for Tregs in the donor-specific unresponsiveness of peripheral T cells in SW/HU mice.

Suppressive function of peripheral Tregs in SW/HU and HU/HU mice

We purified CD25− and CD25+ human CD4+ cells from the spleens and LNs of SW/HU and HU/HU mice to examine Treg function. CD25+CD4+ cells were added back in various numbers to CD25−CD4+ cells from the same spleens in allogeneic MLRs. Peripheral Tregs isolated from SW/HU mice showed generally similar suppressive activity as Tregs isolated from HU/HU mice, but was

FIGURE 4. Treg suppression and participation in SLA-specific unresponsiveness. (A) To examine peripheral T reg participation in SLA-specific unresponsiveness, enriched human CD4+ T cells from splenocytes and lymph nodes from two SW/HU and two HU/HU mice were depleted of human CD25+ cells by MACS microbeads and cultured with irradiated stimulator cells as indicated. Data are expressed as mean of triplicates ± SEM. Titrated numbers of Tregs were added to assess suppression of alloseponses by CD25+CD127+CD4+ cells sorted and pooled from spleens and LNs of three HU/HU mice and four SW/HU mice. The average cpm values of CD25+CD4+ cells from HU/HU mice in the presence of CD25+CD127+CD4+ cells from the indicated group (HU/HU mice; open circles, SW/HU mice; closed circles) in response to allogeneic human PBMC stimulators—(B) representative of two similar experiments—or upon stimulation with anti-CD3/-CD28 (C) is shown. Data are expressed as mean ± SEM. *p < 0.05, Student t test, comparing cpm of CD25-depleted (closed bar) versus undepleted (open bar) CD4+ T cells to the indicated stimulators in each mouse.

FIGURE 5. Proliferation assay with Ag stimulation 1 wk after boost with IFA plus TT. HU/HU and SW/HU mice immunized with CFA plus TT were boosted with IFA plus TT twice and assessed for proliferative responses 3 wk later. Human T cells isolated from spleen and lymph nodes by depletion of mouse CD45+ and Ter119+ cells and magnetic cell sorting for human T cells, served as responders. Responder cells were cultured with indicated stimulators. The mean stimulation index values are shown for HU/HU and SW/HU mice immunized with PBS or tetanus toxoid (n = 3, each). Data are expressed as mean ± SEM. ***p < 0.001, comparing response to chicken Ig-pulsed DC (two-way ANOVA, Bonferroni test). One representative result of two experiments is shown.
slightly lower for SW/HU compared with HU/HU Tregs at lower Treg dilutions (Fig. 4B). Upon stimulation with anti-CD3/CD28, the level of suppression of nonspecific T cell responses was similar for Tregs from SW/HU and HU/HU mice (Fig. 4C).

Comparison of Ag-specific T cell responses in SW/HU and HU/HU mice

We immunized SW/HU and HU/HU mice with tetanus toxoid (TT) and measured proliferative responses. Human T cells from SW/HU mice demonstrated markedly weaker responses to TT compared with HU/HU mice (Fig. 4). No responses to a neoantigen (chicken IgG) were detected in either group (Fig. 5).

Suboptimal homeostatic maintenance of human T cells developing in porcine thymus

In HU/HU mice, we previously demonstrated that naive human T cells undergo expansion and conversion to the effector/memory phenotype upon transfer to a T cell deficient environment containing autologous APCs (26). Using the same approach, we compared T cells developing in SW versus HU THY. Naive CD45RO⁻ human T cells from spleens and LNs of HU/HU and SW/HU mice were sorted and adoptively transferred into NOD/SCID mice that previously received FL CD34 cells from the same human donor but no thymus graft. Fourteen days later, T cell recovery in adoptive recipients was strongly correlated to the level of human chimerism at the time of transfer, as previously observed (26) (Fig. 6A). However, recovery of human CD4 and CD8 T cells that were generated in SW THY was significantly reduced compared with that of T cells transferred from HU/HU mice (Fig. 6A, 6B).

Rapidly proliferating human T cells convert to the memory phenotype when transferred to T cell–deficient humanized mice (26). Similar numbers of slowly proliferating CD4 and CD8 cells were recovered from both groups, and these consisted largely of naive-type cells (Fig. 6C, 6D). Among the rapidly proliferating subset of CD4⁺ T cells recovered from adoptive recipients, the majority were central memory and effector memory cells. However, the numbers of these cells were significantly lower among CD4 cells transferred from SW/HU compared with HU/HU mice (Fig. 6D). Rapidly dividing CD8⁺ T cells generated in SW/HU

![Image](https://via.placeholder.com/150)

FIGURE 6. Suboptimal maintenance and homeostasis of human T cells generated in a porcine thymus graft. Naive SW and HU THY–generated human T cells were labeled with CFSE and transferred into T cell–deficient secondary recipient mice that previously received fetal CD34 cells from the same donor. Adoptive recipient mice were sacrificed 14 d after transfer, and recovery of transferred cells was analyzed with FCM. Correlations between human chimerism levels in T cell–deficient adoptive recipients at the time of transfer and recovered total (left panel), CD4 (middle panel), and CD8 T cell numbers (right panel) are shown 14 d after transfer. (A) Recovered cell number was calculated from the total recovered cell number, and the proportion of each T cell subset in spleen and LNs. (B) The total number of each cell type was calculated from the total recovered cell number and the proportion of each human T cell subset among total recovered cells determined by FCM (n = 4 mice in each group). Data are expressed as mean ± SEM. *p < 0.05, ***p < 0.001, comparing indicated combinations (two-way ANOVA, Bonferroni test). (C) Naive human T cells isolated from the peripheral lymphoid tissues of HU/HU (top panel) or SW/HU (bottom panel) mice were CFSE-labeled and transferred into T cell–deficient recipient humanized mice. Pooled splenocytes and LN cells from each individual recipient mouse were analyzed phenotypically with FCM 14 d after transfer. FCM plots showing CFSE intensity and expression of indicated markers of recovered CD4⁺ and CD8⁺ T cells from one representative recipient mouse are shown. The rapidly and slowly proliferating populations are defined by the left and right bars, respectively, above the histograms showing CFSE intensity. (D) Phenotypic characteristics of rapidly or slowly proliferating population of CD4 (top panel) or CD8 (bottom panel) T cells recovered in T cell–deficient recipient humanized mice. Naive, central memory (CM), effector memory (EM), and EM cells that re-express CD45RA (EMRA) subpopulations were defined as CD45RA⁺CCR7⁺, CD45RA⁻CCR7⁺, CD45RA⁻CCR7⁻, and CD45RA⁺CCR7⁻, respectively. Data are expressed as mean ± SEM. Results are shown from one representative experiment (n = 4 mice in each group) of two experiments. ***p < 0.001, comparing indicated combinations (two-way ANOVA, Bonferroni test).
mice largely failed to convert to the memory phenotype upon adoptive transfer to T cell–deficient recipients, in contrast to those generated in HU/HU mice (Fig. 6C, 6D).

**Decay of T cells is accelerated in SW/HU mice after graftectomy**

Twenty-two weeks after transplantation, T cell decay in SW/HU and HU/HU mice was compared after thymic graftectomy. As shown in Fig. 7A, CD4+ T cell decay in SW/HU mice was significantly greater after 2 wk compared with that in HU/HU mice in graftectomized but not control mice. This difference between SW/HU and HU/HU mice only achieved significance for memory-type and not for naïve-type CD4+ T cells (Fig. 7B, 7C). However, both naïve and memory-type CD4 cells showed significantly greater decay in graftectomized compared with control SW/HU mice, whereas neither subset showed a significant difference between graftectomized and control HU/HU mice in the 2-wk period of follow-up. These results demonstrate clearly that maintenance of both naïve and memory CD4 subsets was more dependent on continuous thymic graft output in SW/HU than in HU/HU mice. CD8 T cells were barely detectable in either group 2 wk after thymectomy.

**Discussion**

In this study, we report that SW THY supports the development of functional human T cells and Tregs from human FL CD34 cells. These T cells show robust alloresponses and xenoresponses with specific immunologic tolerance in vivo and in vitro toward the discordant xenogeneic THY donor and to the murine recipient, with self-tolerance to the human donor. However, human T cells generated in SW THY exhibit an increased CD4+:CD8 ratio, defects in homeostasis, and reduced responses to protein Ags compared with those generated in autologous human thymus grafts. Pig xenotransplantation could overcome the human organ shortage; however, tolerance approaches are most likely necessary to prevent the strong immune responses to xenoidgens. SW THY can support human thymopoiesis, leading to donor-specific unresponsiveness in vitro (22, 23) and normal TCR diversity (21). To our knowledge, we have demonstrated for the first time xenogeneic tolerance of a human immune system by the stringent criterion of long-term acceptance of SLA-matched xenogeneic skin grafts. Acceptance of xenogeneic porcine skin is restricted to skin sharing the SLA Ags of the thymus donor, because SLA-mismatched grafts were rejected. Thus, central tolerance induced by thymic xenografting leads to xenograft tolerance. The donor-matched skin graft rejection observed in one of five SW/HU mice might reflect reactivity to minor histocompatibility Ags expressed by the skin graft donor that were not shared by the fetal THY donor, because these animals were inbred only at the SLA.

T cell tolerance induction to donor porcine Ags in the thymus graft most likely reflects negative selection by porcine thymic epithelial cells and APCs that persist in the graft (22). The unresponsiveness to recipient mouse Ags likely reflects central clonal deletion due to the presence of murine APCs, which we have detected in SW THY (22). However, the unresponsiveness to donor porcine Ags may also have involved positive selection of Tregs that suppress anti-donor responses, as depletion of Tregs revealed some anti-pig donor MLR responsiveness in peripheral T cells of SW/HU mice. Clonal deletion of donor-specific thymocytes may be incomplete because of the expected eventual replacement of SW hematopoietic APCs with human and mouse APCs in the graft, resulting in partial reliance on Tregs to maintain tolerance.

We show in this study that peripheral human T cell reconstitution can occur just as efficiently from porcine as from human THY grafts. Consistent with our previous observations for HU THY (24), cryopreservation and thawing of SW THY reduced the level of peripheral porcine T cell reconstitution, permitting faster T cell reconstitution from the i.v. injected human CD34 cells. Thus, creation of open niches by thymocyte depletion accelerates...
thymopoiesis from peripheral stem cells. Collectively, our data argue against any major species barriers to human thymopoiesis in porcine thymic grafts.

By studying thymectomized, T cell–depleted wild-type, knock-out, and TCR transgenic mice receiving SW THY, we previously showed that pig and mouse MHCs mediate negative selection of mouse T cells developing in SW THY, but only pig MHC mediates positive selection (19, 27–29). Application of these principles to the SW/HU model would imply that human, pig, and mouse APCs present in SW THY grafts (22) contribute to negative selection, explaining the highly specific, three-species tolerance observed in SW/HU mice, with tolerance to the mouse recipient strain in vitro and in vivo (the animals did not develop GVHD) and specific unresponsiveness to the human and the porcine donors.

Significant T cell recall responses to tetanus immunization were demonstrated in SW/HU mice, although at reduced levels compared with HU/HU mice. The lack of positive selection on human thymic MHCs can render SW THY–generated T cells less efficient at recognizing Ag presented on autologous human HLA molecules. However, studies in the pig THY to immunocompetent mouse model showed that cross-reactivity of murine T cells generated in SW THY allowed mouse class II–restricted responsiveness and protective responses to opportunistic infection (18, 27). A normal, diverse human T cell repertoire is generated in SW THY grafts (21). Further study is needed to elucidate the effects of development in a pig thymus on protective human T cell responses.

The exclusive positive selection by porcine thymic epithelium in SW/HU mice could explain the reduced CD8 T cell percentages, accelerated memory cell decay, and reduced lymphopenia–driven expansion and CD8 cell memory differentiation in the periphery of these mice compared with HU/HU mice. In mice, the same MHC/peptides mediating positive selection in the thymus regulate T cell homeostasis, proliferation, and survival in the periphery (30–35). The absence of SW APCs in the periphery of SW/HU mice may therefore result in defects in human T cell homeostasis. Reduced memory conversion was apparent only among rapidly proliferating CD8 T cells, but both CD4+ and CD8+ cells showed less accumulation of the rapidly proliferating populations. These cells, which presumably respond to cognate environmental Ags (36, 37), could represent a smaller proportion of the repertoire of SW/HU mice because of limited cross-reactivity with autologous HLA/peptides following positive selection on porcine MHC (38, 39). Accelerated decay of memory CD4 T cells following graftectomy in SW/HU compared with HU/HU mice was consistent with our previous studies in thymectomized immunocompetent mice receiving SW THY (40). Whereas naive T cells require TCR signaling and IL-7 for survival, memory CD4 and CD8 T cell homeostasis in mice is largely maintained through IL-7 and IL-15 (37). However, MHC class II molecules do play a role in homeostasis of memory-phenotype CD4 T cells (41). The rapid disappearance of CD8 cells after graftectomy in both SW/HU and HU/HU mice suggests that this subset has poor peripheral survival, possibly reflecting insufficient cytokine signaling, and is highly dependent on continued thymic output for its maintenance. HLA-DR* Tregs have greater suppressive function than their HLA-DR– counterparts (42). CD45RO* Tregs have been activated (43), are functionally differentiated, and require TCR stimulation to exert suppression in vitro (43, 44). The similar numbers in the thymus and the similar function of human Tregs in the HU/HU and SW/HU mice, coupled with the reduced naive Treg numbers in the periphery of SW/HU compared with HU/HU mice, suggest that peripheral survival of naive Tregs in SW/HU mice might be limited by the absence of the same peripheral MHC/peptide complexes as those encountered on thymic epithelial cells (TECs). The experiment-to-experiment variability in observing this difference might reflect different degrees of cross-reactivity between different combinations of human and porcine MHC genotypes in each experiment. Furthermore, human APCs migrating into the SW thymus may contribute to Treg–positive selection as described (45), resulting in Tregs that interact efficiently with human HLA/peptide complexes in the periphery.

In conclusion, we demonstrate that human T cells generated in porcine thymus grafts are functional, self-tolerant, and specifically tolerant toward the porcine donor. Functional Tregs develop normally in the porcine thymus and contribute to tolerance. However, human T cells generated in porcine thymus grafts demonstrate immune defects that might be attributable to suboptimal post-thymic interactions with human APCs following positive selection on porcine MHC. For clinical application of this tolerance approach, methods of optimizing T cell function following development in SW THY are needed. These methods could include cotransplantation of human TECs to add an element of positive selection on autologous TECs, which was effective in the pig thymus to nude mouse transplant model (28, 46–49), or the use of HLA transgenic porcine source animals.

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Disclosures

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