Xenografted Islet Cell Clusters From *INS*LEA29Y Transgenic Pigs Rescue Diabetes and Prevent Immune Rejection in Humanized Mice

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Islet transplantation is a potential treatment for type 1 diabetes, but the shortage of donor organs limits its routine application. As potential donor animals, we generated transgenic pigs expressing LEA29Y, a high-affinity variant of the T-cell costimulation inhibitor CTLA-4Ig, under the control of the porcine insulin gene promoter. Neonatal islet cell clusters (ICCs) from *INS*LEA29Y transgenic (LEA-tg) pigs and wild-type controls were transplanted into streptozotocin-induced hyperglycemic NOD-scid IL2Rγ^{null} mice. Cloned LEA-tg pigs are healthy and exhibit a strong β-cell–specific transgene expression. LEA-tg ICCs displayed the same potential to normalize glucose homeostasis as wild-type ICCs after transplantation. After adoptive transfer of human peripheral blood mononuclear cells, transplanted LEA-tg ICCs were completely protected from rejection, whereas reoccurrence of hyperglycemia was observed in 80% of mice transplanted with wild-type ICCs. In the current study, we provide the first proof-of-principle report on transgenic pigs with β-cell–specific expression of LEA29Y and their successful application as donors in a xenotransplantation model. This approach may represent a major step toward the development of a novel strategy for pig-to-human islet transplantation without side effects of systemic immunosuppression. ***Diabetes*** **61:1527–1532, 2012**

Type 1 diabetes is a chronic metabolic disease associated with development of severe complications (1). It has been shown that type 1 diabetes can be cured by the transplantation of the pancreas or isolated islets of Langerhans. Nonetheless, the success of pancreas and islet transplantation is limited by the shortage of organ donors and the need for systemic immunosuppressive therapy (2) and is therefore restricted to few patients (3).

Limited availability of human donor organs may be overcome by the use of pigs as organ donors. Pig-to-human xenotransplantation faces the problem of strong rejection, predominantly by direct T-cell recognition of pig major histocompatibility complex and indirect T-cell response to xenogeneic antigens presented by the recipient antigen-presenting cells (4).

Recent advances in immunosuppressive therapies provided evidence that transplanted porcine islets can promote the long-lasting cure of diabetes in nonhuman primates (5–7). However, the currently used intensive immunosuppressive regimen in pig islet transplantation may have severe side effects in humans and cannot be transferred into clinical practice. Blockade of the B7/CD28 costimulatory pathway by LEA29Y, a high-affinity variant of the CTLA-4Ig fusion protein (8), has been shown to be effective in clinical trials following kidney transplantation (9,10) and in porcine islet transplantation studies (5,7,11). Thus, local expression of LEA29Y restricted to the transplantation site may represent an innovative approach to protect grafted islets from xenogeneic immune rejection without the side effects of systemic immunosuppression.

Therefore, we chose to generate transgenic pigs expressing LEA29Y specifically in pancreatic β-cells. We demonstrate for the first time the potential of neonatal *INS*LEA29Y transgenic (LEA-tg) islet clusters to normalize blood glucose levels and evaluate the inhibition of human–anti-pig rejection in a humanized NOD-scid IL2Rγ^{null} (NSG) model.

RESEARCH DESIGN AND METHODS

All experiments were approved by the local animal welfare authority. NSG mice were obtained from The Jackson Laboratory. For generation of *INS*LEA-tg pigs, the coding sequence for LEA29Y was cloned into a β-cell–specific expression vector (12) with 1.3-kb upstream regions, exon 1 and intron 1 of the porcine insulin gene, and a poly-adenylation cassette of the bovine growth hormone gene. The vector was completed by linking the *INS*LEA construct to a floxed neomycin resistance cassette (13). Porcine fetal fibroblasts (PFF#14; 1×10^6) were nucleofected (Nucleofector Technology, Lonza, Germany). Stably nucleofected cell clones were used as donors for somatic cell nuclear transfer (14). Embryo transfer was carried out laparoscopically (15). Integration and expression of the transgene was analyzed by Southern blot and immunohistochemistry. Donor piglets for transplantation experiments were generated by recloning, as described previously (13).

Isolation and transplantation of neonatal islet cell clusters into hyperglycemic NSG mice. Islet cell clusters (ICCs) from 1- to 2-day-old recloned LEA-tg and wild-type pigs were isolated as previously described (16) and cultured for 6 days at 37°C in RPMI (Biochrom) with 2% human serum albumin (Octapharm), 1% antibiotic-antimycotic, 10 mmol/L nicotinamide, and 20 nmol/L exendine-4 (Sigma). Insulin content in ICCs was determined by enzyme-linked immunosorbent assay (ELISA) (Millipore) (17). A total of 2,500 clusters per mouse were transplanted under the kidney capsule of streptozotocin-induced diabetic (180 mg/kg; Sigma) NSG mice (blood glucose >350 mg/dL).

Characterization of graft function. Neonatal ICCs require a 6- to 8-week in vivo maturation period until physiological glucose-dependent insulin secretion has developed. Animals with blood glucose levels >300 mg/dL received exogenous insulin subcutaneously (0.5 IU glargine per day). Mice displaying blood

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glucose levels <150 mg/dL for a period of 5 days were considered normoglycemic. Intraperitoneal glucose tolerance testing (IPGTT) was performed 10 days later using 2 g glucose/kg body weight (18). Porcine serum insulin was determined by ELISA (Mercodia) that displayed no cross-reactivity with mouse insulin. Serum LEA29Y concentrations were determined by sandwich ELISA using 1 μg/mL monoclonal anti–human-CTLA-4 antibody (Beckmann Coulter) and horseradish peroxidase–conjugated polyclonal rabbit anti-human IgG (Dako).

Analyses in humanized mice. To analyze human anti-pig immune response, 20×10^6 human peripheral blood mononuclear cells (hPBMCs) from one donor were transferred intraperitoneally into transplanted normoglycemic NSG mice, as described previously (19). The remaining PBMCs were cultured in X-VIVO 20 medium (CambrexBio Science) supplemented with porcine splenocyte cell lysate (cytosolic fraction corresponding to 15×10^6 splenocytes) added at day 0 and day 3 to activate T cells directed against porcine antigens. After 6 days of culture, 2.5×10^6 primed hPBMCs were injected intravenously.

Reconstituted mice were monitored daily for the reoccurrence of hyperglycemia. The investigation period was limited to 29 days because of the development of graft-versus-host disease. Animals displaying severe hyperglycemia (blood glucose levels >350 mg/dL in two consecutive measurements) were killed ahead of schedule, whereas normoglycemic mice underwent IPGTT at day 27. To exclude endogenous β-cell regeneration, the graft of normoglycemic mice was removed at day 28 by uninephrectomy. From each animal, blood and specimen for fluorescence-activated cell sorter (FACS) analysis (FACS Canto; BD Biosciences) and immunohistochemistry (kidney and liver) were taken. Spleen and bone marrow cells were stained using the following fluorochrome-labeled monoclonal antibodies: mouse CD45-FITC, human CD45-APC, or matched isotype antibodies (eBioscience).

Immunohistochemical analyses. Pig organs and graft-bearing kidneys were processed as described previously (20). Serial paraffin sections were stained with guinea pig anti-insulin (1:500), rabbit anti-human CD3 (1:100), rabbit anti-human IgG (recognizing the COOH-terminal part of LEA29Y; 1:50), mouse anti-human CD4 (1:20) (Dako), rabbit anti-human CD8 (1:80; Vector), and rabbit anti-human CD45 (1:400; antibodies-online Inc.). As secondary antibodies, horseradish peroxidase–conjugated anti-guinea pig IgG, anti-rabbit IgG, biotinylated anti-rabbit IgG or anti-mouse IgG (Dako), and alkaline phosphatase–conjugated anti-guinea pig IgG (Southern Biotech) were used. Chromogens included Fuchsin+Substrate Chromogen (Dako) or 3,3′-diaminobenzidine (DAB) (Kem-En-Tec Diagnostics) (20).

Data presentation and statistical analysis. Data represent means and SEM. Statistical analyses were performed using the Student *t* test or Log-rank test (diabetes reoccurrence). *P* values <0.05 were considered significant.

RESULTS

Generation of *INS*LEA29Y transgenic (LEA-tg) pigs. The *INS*LEA29Y expression construct (Fig. 1*A*) was used for nucleofection of porcine fetal fibroblasts, and stable cell clones were pooled for nuclear transfer (13). Cloned embryos (n = 216) were transferred to three synchronized gilts, resulting in two pregnancies with nine born piglets including two stillborn animals. Seven of eight genotyped piglets were transgenic, each representing a unique founder, as demonstrated by Southern blot analysis (Fig. 1*B*). Four of these animals were killed at the age of 3 months for immunohistochemical staining of different organs (Fig. 1*C*). Transgenic pigs displayed a strong LEA29Y staining in the pancreatic islets (Fig. 1*C*). Recloned animals served as islet donors or were raised for future breeding purposes. These pigs are fertile, have no signs of opportunistic infections, and exhibit normal blood glucose levels.

Differentiation and maturation of transplanted ICCs. Mice transplanted with wild-type ICCs (Tx, wt; n = 5) and mice transplanted with ICCs from LEA-tg pigs (Tx, LEA-tg; n = 5) (insulin content 3.2 ± 0.9 ng/μg protein) returned to normoglycemia after 51 ± 7 and 43 ± 7 days, respectively (Fig. 2*A*). Normoglycemic mice of both transplantation groups exhibited a comparable restored glucose tolerance (area under the curve of glucose during IPGTT: Tx, wt: 12,121 ± 1,303; Tx, LEA-tg: 11,310 ± 719) with similar

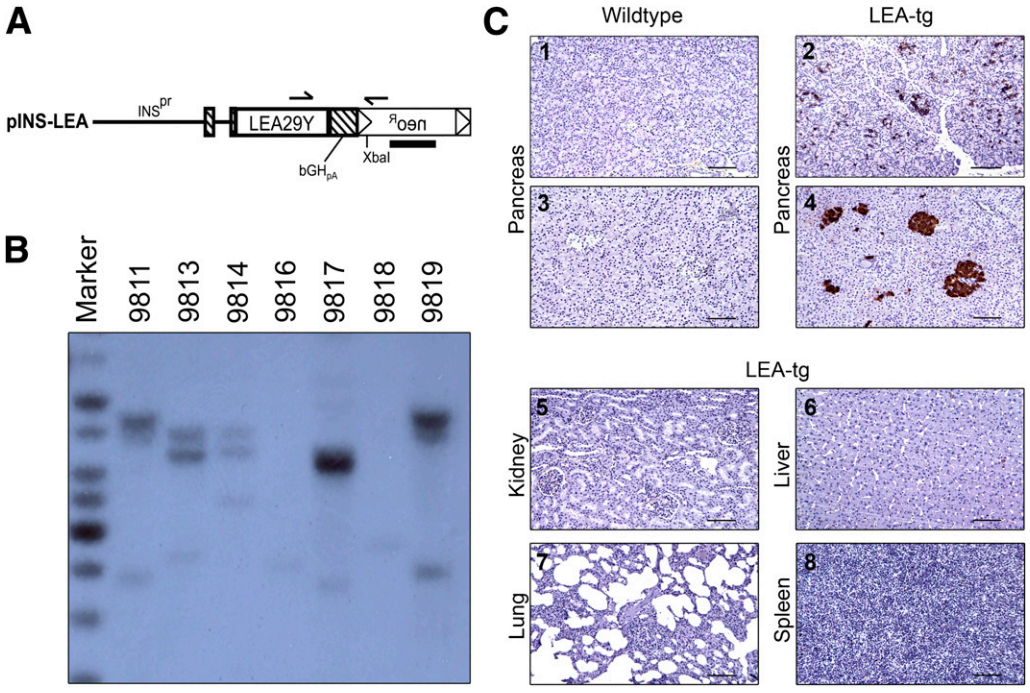

FIG. 1. Generation of *INS*LEA29Y transgenic (LEA-tg) pigs. *A*: The vector consisted of a 1.3-kb regulatory sequence from the porcine *INS* gene, the LEA29Y coding sequence, and the poly-adenylation box from the bovine *GH* gene. Regulatory sequences are depicted as lines, whereas exonic structures are boxed. Untranslated regions are shaded. The selection cassette provides resistance to neomycin. Binding sites for primers are indicated as arrows, and the probe for Southern blot hybridization is shown as a bold line. *B*: Southern blotting of seven founders was performed on *Xba*I-digested genomic DNA with a probe binding to the neomycin resistance cassette. *C*: Immunohistochemical staining for LEA29Y on tissue sections from a neonatal transgenic pig (aged 2 days, pancreas, C2), an adult founder animal (age 3 months; pancreas, liver, lung, kidney, and spleen, C4, 5–8), and from age-matched wild-type control pigs (pancreas, C1, 3). Scale bar: 100 μm. (A high-quality digital representation of this figure is available in the online issue.)

glucose-responsive porcine insulin secretion (Fig. 2*B*). Immunohistochemical staining of the subcapsular graft revealed maturation of the transplanted ICCs toward a strongly insulin-expressing endocrine tissue in both transplantation groups with LEA29Y transgene expression restricted to the grafts of Tx, LEA-tg mice (Fig. 2*C* and *D*). LEA29Y concentrations in the plasma of normoglycemic Tx, LEA-tg mice were 270 ± 24 ng/mL.

***INS*LEA29Y expression prevents reoccurrence of hyperglycemia.** The finding that transgenic ICCs had strong LEA29Y expression and were able to normalize blood glucose levels raised the question whether these ICCs were protected from graft rejection after reconstitution with human PBMCs. The proportion of human CD45$^+$ cells in the spleen and bone marrow was comparable in both transplantation groups (Fig. 3*A*), and histological examination revealed identical mononuclear cell infiltration in various tissues reflecting graft-versus-host disease in both transplantation groups (Supplementary Fig. 1). However, at day 14 after adoptive transfer of PBMCs, blood glucose levels were significantly elevated in Tx, wt as compared with Tx, LEA-tg mice (204 ± 63 mg/dL vs. 58 ± 2 mg/dL; $P < 0.05$). Life-table analysis revealed that reoccurrence of hyperglycemia, defined by blood glucose levels >250 mg/dL, was absent in Tx, LEA-tg mice ($n = 5$), whereas four of five animals (80%) transplanted with wild-type ICCs became hyperglycemic within the observation period of 29 days ($P < 0.05$) (Fig. 3*B* and *C*). One Tx, wt mouse remained normoglycemic throughout the observation period, with preserved glucose tolerance at day 27. In three of four hyperglycemic mice, porcine serum insulin was below the detection limit. In contrast, in all mice transplanted with LEA29Y transgenic ICCs, the area under the glucose and insulin curve during IPGTT was comparable before and 27 d after transfer of human PBMCs (Fig. 3*D*). After removal of the graft-bearing kidney, all of the diabetes-free animals returned to hyperglycemia (Fig. 3*B*). Histological examination of the graft bearing kidney from wild-type mice which developed hyperglycemia revealed a massive infiltration of ICCs with human mononuclear cells (CD3$^+$, CD45$^+$, CD4$^+$, and CD8$^+$ cells) and reduced insulin staining. In contrast, in all Tx, LEA-tg mice, ICCs appeared preserved and T-cell infiltration was restricted to the surrounding tissue and almost absent within islet clusters (Fig. 4).

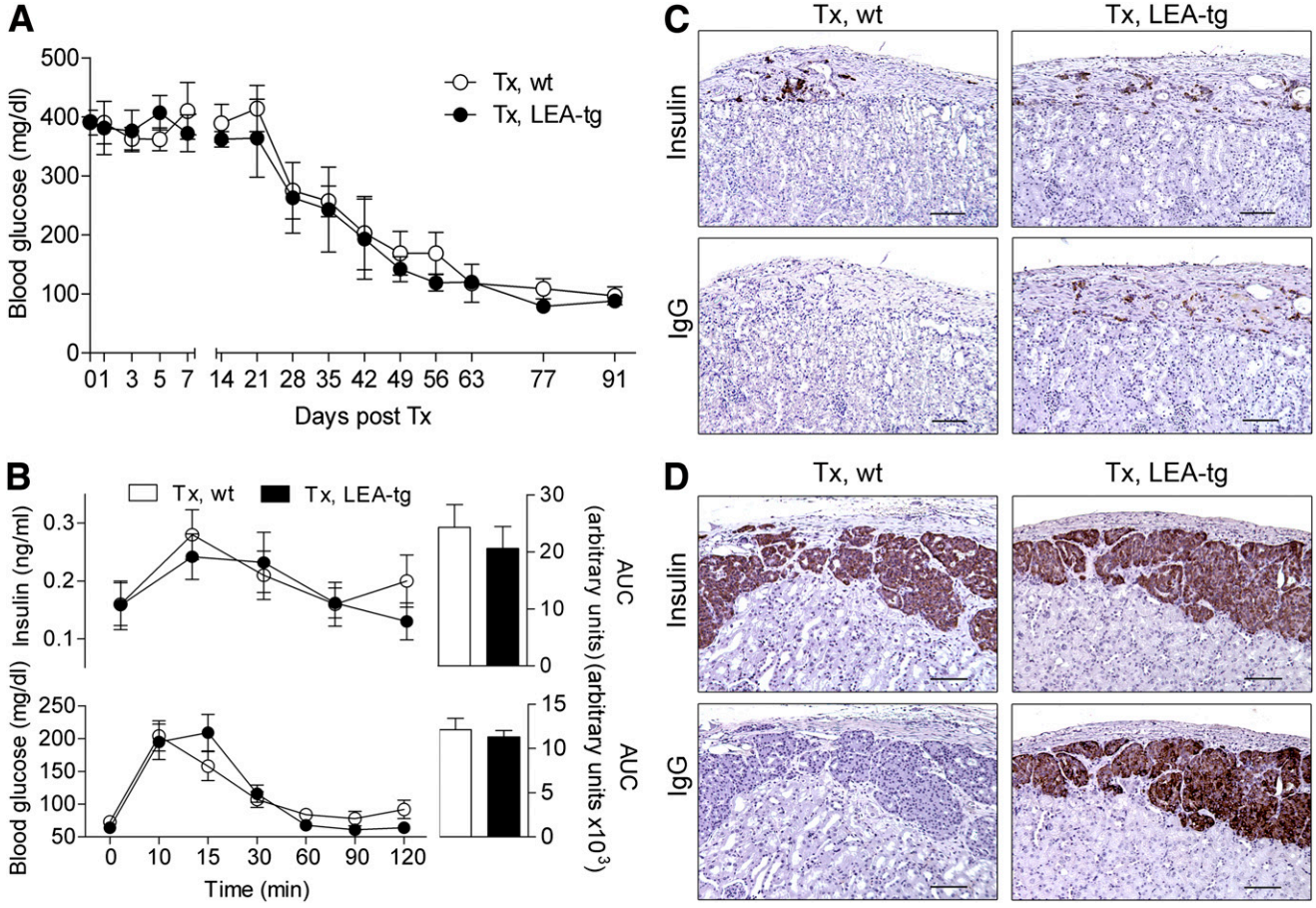

FIG. 2. Grafted LEA-tg ICCs display physiological β-cell function. Course of blood glucose levels after transplantation (*A*), IPGTT (performed 10 days after the development of normoglycemia (*B*), and immunohistochemistry of grafted ICCs (7–9 days after transplantation [*C*] and 4.0–4.5 months after transplantation [normoglycemic animals] [*D*]) in mice transplanted with wild-type (Tx, wt) and in mice transplanted with LEA-tg (Tx, LEA-tg) ICCs. Mice of both transplantation groups developed stable normoglycemia (*A*) and restored glucose tolerance (*B*, *bottom*), by porcine insulin secretion (*B*, *top*). The area under the curve (AUC) for glucose and insulin (*B*) during IPGTT was comparable in both transplantation groups. *C*: Immunohistochemical staining of serial sections from the transplantation site against insulin and IgG revealed insulin/LEA29Y expression in a minor proportion of ICCs a few days after transplantation. *D*: In contrast, after the development of normoglycemia, the transplanted cells have differentiated into a widespread insulin-positive stained tissue in both transplantation groups with LEA29Y transgene expression restricted to the grafted ICCs from transgenic pigs. Scale bar: 100 μm. $n = 5$ for each transplantation group. (A high-quality digital representation of this figure is available in the online issue.)

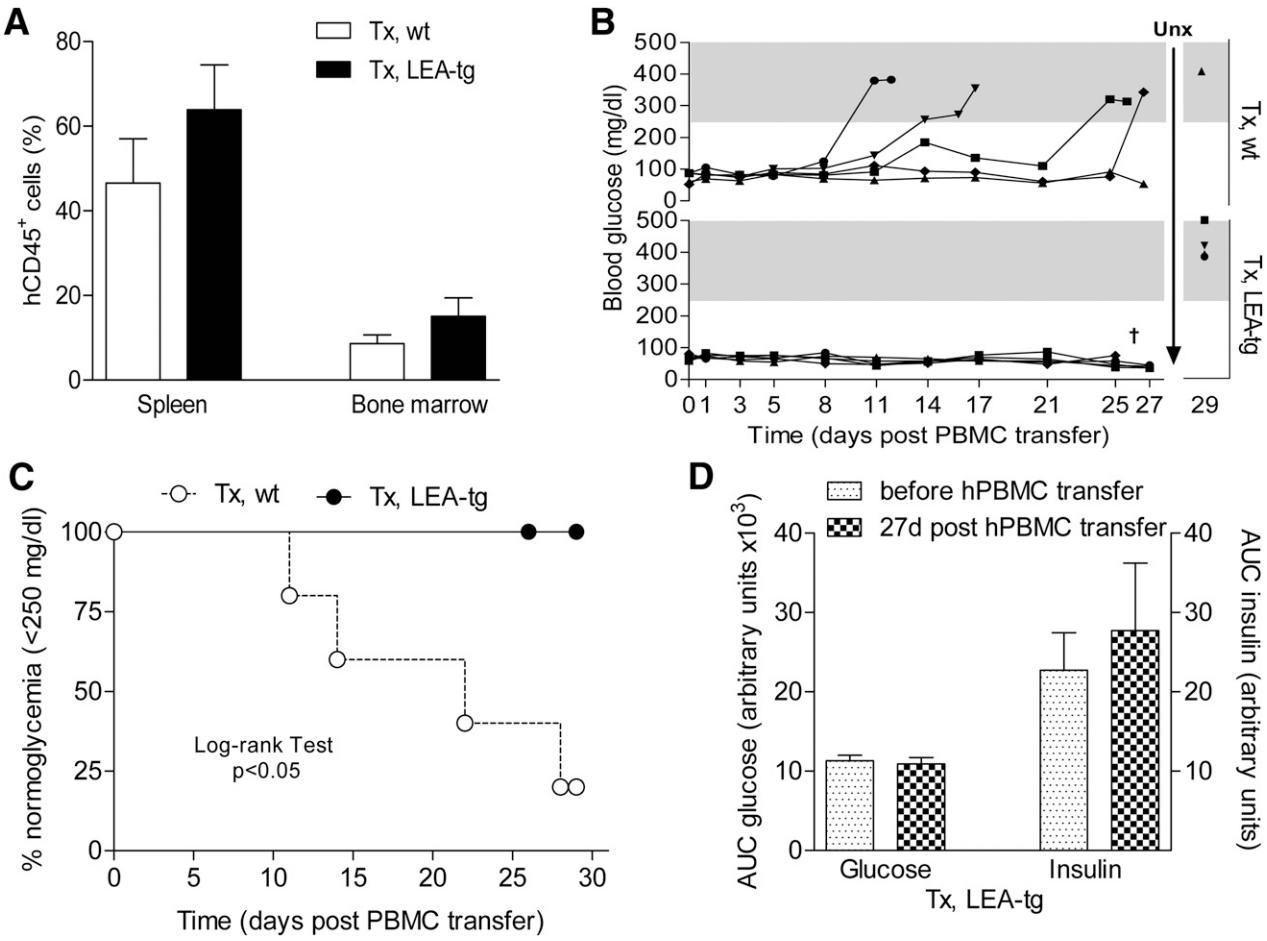

FIG. 3. LEA29Y expression prevents reoccurrence of hyperglycemia after transfer of human PBMCs. *A*: Engraftment of human PBMCs (as indicated by FACS staining for human CD45⁺ cells in both spleen and bone marrow cells) did not significantly differ between mice transplanted with wild-type (Tx, wt) and in mice transplanted with LEA-tg (Tx, LEA-tg) ICCs. *B*: Four of five Tx, wt mice, but no Tx, LEA-tg mice, developed hyperglycemia within 28 days after human PBMC transfer. After removal of the graft-bearing kidney (uninephrectomy, Unx) of normoglycemic animals, all mice returned to severe hyperglycemia, indicating the absence of endogenous β-cell regeneration. *C*: Life-table analysis revealed a significantly ($P = 0.016$) higher proportion of hyperglycemia reoccurrence in Tx, wt as compared with Tx, LEA-tg mice. *D*: Furthermore, the area under the curve (AUC) of glucose and insulin during the IPGTT was unchanged before and 27 days after the transfer of human PBMCs in Tx, LEA-tg mice. $n = 4–5$ animals for each transplantation group. †One animal died at day 26 as a result of graft-versus-host disease.

DISCUSSION

Major obstacles in pig-to-human islet transplantation are the strong xenogeneic immune response and the severe adverse effects of the required intensive immunosuppressive regimen. To overcome these limitations, we developed an islet donor animal that provides a local immunosuppressive environment within transplanted islets of Langerhans.

The LEA-tg pigs generated in this study express high levels of LEA29Y, specifically in the β-cells, with no signs of β-cell dysfunction or systemic immunosuppression, such as increased susceptibility to opportunistic infections. This is in contrast to transgenic pigs with ubiquitous porcine CTLA-4Ig expression, which were immune compromised and died of infections (21). To assess the in vivo β-cell function and the immunomodulatory potential of LEA-tg islets, ICCs were transplanted into NSG mice, an established model for studying human immunity (22,23). After an in vivo maturation period, which is required for immature ICCs to develop physiological insulin secretion (16), mice of both transplantation groups developed complete restoration of glucose homeostasis. These findings, together with the strong, colocalized graft staining for insulin and LEA29Y, indicate that LEA29Y expression in β-cells does not interfere with β-cell development and function. Previous transplantation studies in rats and nonhuman primates using high doses of belatacept for systemic immunosuppression also have shown that costimulatory blockade by LEA29Y does not exert any adverse effects on β-cell function (5,11).

After adoptive transfer of a human immune system, we observed that 80% of Tx, wt animals developed hyperglycemia, whereas all Tx, LEA-tg mice were protected from graft rejection and showed preserved β-cell function. The development of hyperglycemia after xenograft removal indicated that glucose homeostasis was completely maintained by graft-derived porcine insulin secretion, excluding the possibility of endogenous β-cell regeneration. In Tx, LEA-tg mice, human lymphocyte accumulation was observed in the periphery of the transplantation site and in the kidney, but LEA29Y-tg ICCs were protected from infiltration. Thus, our study shows for the first time that local expression of LEA29Y results in a prolonged islet xenograft function, supporting the hypothesis that inhibition of costimulation is able to modulate allo- and xenoimmunity (6,7). These data are in line with findings from Zhai et al. (24), demonstrating a prolonged survival of adenoviral vector–transduced pig islets expressing porcine CTLA-4Ig. LEA29Y

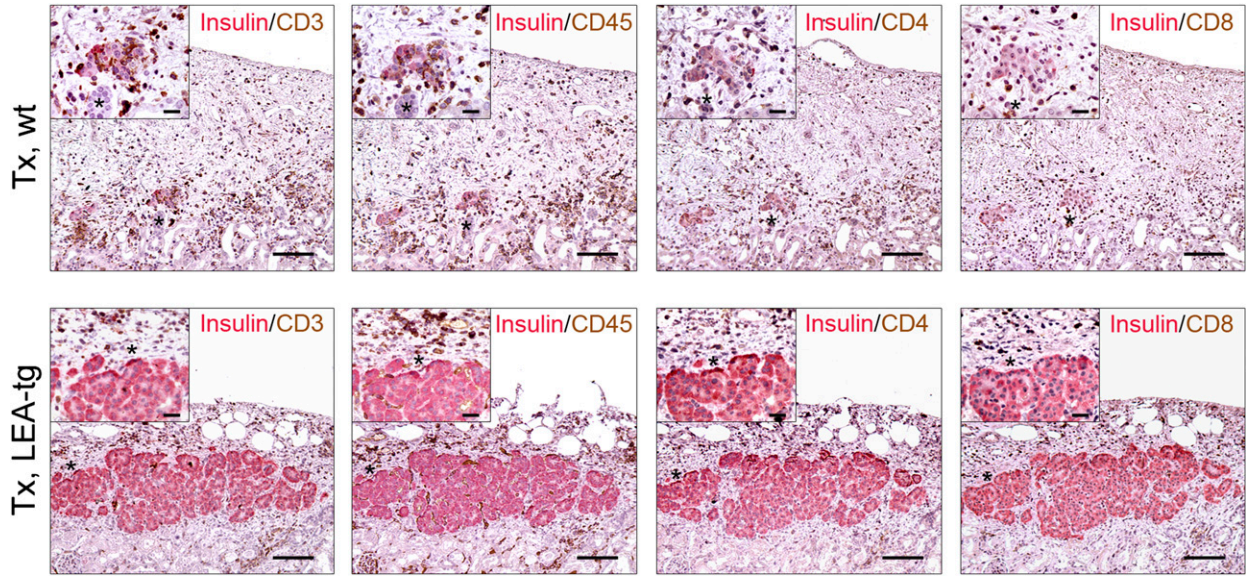

FIG. 4. LEA29Y expressing ICCs are almost completely preserved from mononuclear cell infiltration. Characteristic insulin (red) and CD3$^+$, CD45$^+$, CD4$^+$, and CD8$^+$ cell (brown) staining pattern of serial sections from the transplantation sites of a mouse transplanted with wild-type ICCs (Tx, wt; rejection at day 12 after PBMC transfer) vs. an animal with LEA29Y transgenic ICCs (Tx, LEA-tg, day 29 post PBMC transfer). In Tx, wt only few ICCs were detectable with vast T-cell (CD3$^+$, CD4$^+$, and CD8$^+$) and CD45$^+$ cell infiltration in the graft region. In contrast, Tx LEA-tg ICCs appeared completely preserved with T-cell and leukocyte accumulation restricted to the subcapsular area (day 29 after Tx). The localization of tissue sections shown in the insets is marked by an asterisk. Scale bar: 100 μm, insets: scale bar 20 μm. (A high-quality digital representation of this figure is available in the online issue.)

serum concentrations in recipients of LEA-tg ICCs were ~100–150 times lower as compared with systemic LEA29Y treatment in clinical trials (belatacept, BMS-224818), suggesting that graft protection is primarily mediated by local and not systemic LEA29Y immunomodulatory effects.

In conclusion, the present proof-of-principle study demonstrates that the availability of transgenic pigs expressing LEA29Y in β-cells may represent a major step forward to overcome the immunological barrier to islet xenotransplantation. Additional transplantation studies using larger groups of mice with a stably transferred human immune system (22) will be conducted to investigate the long-term effects of LEA29Y transgenic islets on xenogeneic graft rejection.

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No potential conflicts of interest relevant to this article were reported.

N.K. and E.W. researched data (generation and characterization of transgenic pigs) and wrote the manuscript. L.v.B. and J.S. researched data (isolation of ICCs, transplantation, FACS, and immunohistochemical analyses) and wrote the manuscript. A.B. and B.K. researched data (characterization of transgenic pigs and pig pancreas extraction) and reviewed and edited the manuscript. M.O. and K.L. researched data (ELISA, animal monitoring, and FACS analyses) and reviewed and edited the manuscript. M.K. researched data (cloning and recloning of transgenic pigs and SCNT) and reviewed and edited the manuscript. M.T. researched data (large animal surgery) and reviewed and edited the manuscript. H.N. researched data (SCNT) and reviewed and edited the manuscript. N.H. and R.W. researched data (immunohistochemical analyses) and reviewed and edited the manuscript. J.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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