

Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates

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Cell-based diabetes therapy requires an abundant cell source. Here, we report reversal of diabetes for more than 100 d in cynomolgus macaques after intraportal transplantation of cultured islets from genetically unmodified pigs without Gal-specific antibody manipulation. Immunotherapy with CD25-specific and CD154-specific monoclonal antibodies, FTY720 (or tacrolimus), everolimus and leflunomide suppressed indirect activation of T cells, elicitation of non-Gal pig-specific IgG antibody, intragraft expression of proinflammatory cytokines and invasion of infiltrating mononuclear cells into islets.

Understanding the pathways involved in xenograft rejection will aid the rational design of interventional protocols. Elimination of the galactose α -1,3-galactose (Gal) epitope prevented hyperacute rejection of pig-to-nonhuman primate (NHP) solid-organ xenografts¹. In contrast, the Gal epitope was expressed on fewer than 5% of adult pig islet endocrine cells, and very few donor islet endothelial cells survived pretransplant islet culture^{2,3}. Consequently, cultured xenografts from genetically wild-type pigs transplanted intraportally to NHPs seemed to undergo primarily cellular rejection mediated by T cells and macrophages, rather than Gal-specific antibody-initiated hyperacute rejection⁴. These and other observations^{5,6} led us to hypothesize that immunosuppressive therapy targeting T cells would allow functional survival of intraportally transplanted wild-type porcine islets in diabetic NHPs.

To test this hypothesis, we transplanted adult porcine islets (25,000 islet equivalents/kg; cultured for 48 h) from genetically unmodified pigs intraportally into 12 streptozotocin-diabetic, immunosuppressed, fully heparinized cynomolgus macaques (**Supplementary Methods, Supplementary Fig. 1 and Supplementary Table 1** online). Macaques

were killed at the time of rejection, killed because of morbidity or followed until death or for up to 6 months after transplant. All recipients became porcine C-peptide positive, normoglycemic and insulin independent after transplant. Functional islet xenograft survival in recipients given basiliximab for induction (therapy given in the peritransplant period only) and FTY720 plus the sirolimus derivative everolimus for maintenance immunosuppression (group A; $n = 3$) was 24, ≥ 39 and 45 d. By adding maintenance immunosuppression with human CD154-specific human monoclonal antibody ABI793 (ref. 7; group B; $n = 4$), we prolonged islet xenograft survival to 47, 54, ≥ 73 and ≥ 187 d. By adding ABI793 and leflunomide (group C; $n = 5$), we prolonged islet xenograft survival to ≥ 68 , ≥ 111 , ≥ 140 , ≥ 145 and ≥ 158 d. Causes for killing of normoglycemic recipients were infection ($n = 3$), weakness ($n = 2$), neuritis ($n = 1$) and completion of 6-month post-transplant follow up ($n = 1$). One normoglycemic recipient died of pulmonary embolism. At 100 d after transplant, islet xenograft survival rates were 0% (group A), 50% (group B) and 100% (group C; overall log-rank test, $P = 0.0003$; group A versus C with Bonferroni correction, $P = 0.017$). Average basal porcine C-peptide levels in all islet xenograft recipients in our study was 0.67 ± 0.67 ng/ml during the normoglycemic post-transplant period. For the duration of graft function, average post-transplant blood glucose levels were 146 ± 89 mg/dl in group A, 112 ± 71 in group B and 112 ± 37 in group C. Histologic analysis showed numerous intrahepatic islets, without signs of rejection, in normoglycemic macaques killed up to 187 d after transplant (**Fig. 1 and Supplementary Fig. 2 and Supplementary Table 2** online). Adverse events (**Supplementary Table 3** online) included thromboembolic lesions, found in eight of nine macaques from groups B and C and probably related to ABI793 therapy⁷.

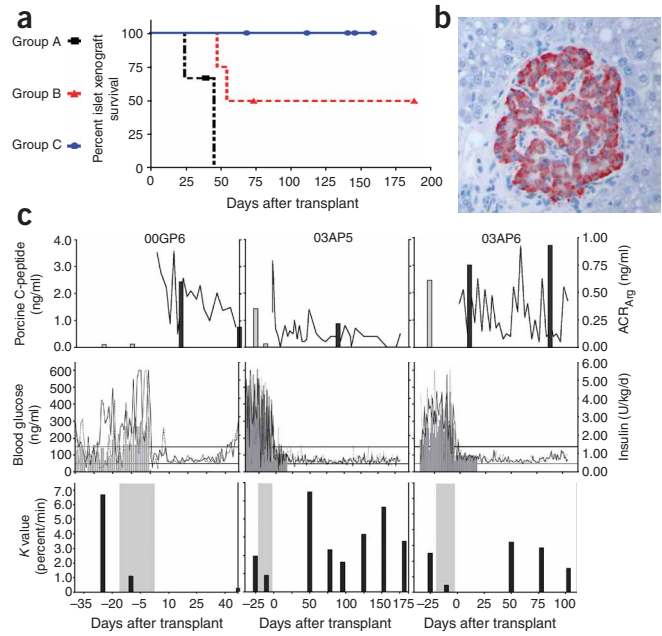
Our study shows that prolonged functional adult pig-to-NHP islet xenograft survival is achievable without elimination of donor Gal epitope or manipulation of recipient Gal-specific antibody. Also, in recipients undergoing rejection, we observed no increases in Gal-specific IgG or IgM antibody levels after transplant, no Gal-specific (isolectin B4) staining (**Fig. 2a,b and Supplementary Fig. 3** online) and no IgG and IgM staining with associated C9 complement deposition (data not shown) on islets. Therefore, unlike in whole-organ xenotransplants, Gal-specific antibodies do not seem to have a major role in the rejection of pig islet xenografts in NHPs.

Our study also suggests that islet xenograft rejection in the pig-to-NHP model is cell mediated. Our immunopathologic analysis of livers obtained from macaques that experienced graft failure showed both peri- and intraislet infiltration by CD4⁺ and CD8⁺ T cells, by macrophages (**Fig. 2c,d**) and, occasionally, by CD20⁺ B cells. We rarely observed neutrophils; natural killer cells and eosinophils were also absent. Notably, our demonstration of cell-mediated rejection on days 24 and 45 after transplant in group A macaques on a regimen

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Figure 1 Functional and histologic islet xenograft survival. **(a)** Kaplan-Meier estimates of functional islet xenograft survival rates in groups A, B and C plotted over time. Symbols indicate seven euthanasia censors and one death censor. **(b)** Porcine islet in recipient liver (O3AP5) on day 187 after transplant. Insulin-specific staining; original magnification, $\times 40$. **(c)** Pre- and post-transplant metabolic results in three xenograft recipients (00GP6, group A; O3AP5, group B; O3AP6, group C). Upper panels show weekly serum porcine C-peptide levels (left y-axis) as well as acute macaque and porcine C-peptide responses (ACR) to arginine (70 mg/kg intravenously; right y-axis). Macaque C-peptide responses (gray bars) were detectable before but not after induction of diabetes. Porcine C-peptide responses (black bars) became and remained detectable after porcine islet xenotransplants. Middle panels show glycemic control and insulin blood glucose. Black lines, morning blood glucose; gray lines, evening blood glucose. Exogenous insulin doses (U/kg/d) are represented by gray bars. Insulin was administered per protocol through post-transplant day 21 in groups B and C but not in group A. Islet xenotransplants restored normoglycemia and insulin independence for 45, ≥ 187 and ≥ 111 d in the three recipients depicted, respectively. Lower panels show the glucose-disposal rates (*K* values). *K* values were in the diabetic range (< 1.0) after streptozotocin injection; mostly nondiabetic glucose disposal rates were documented after transplant (*K* values 1.5–5.5, as compared with levels of 2.0–2.5 in healthy, nondiabetic macaques before diabetes induction).



that prevented islet allograft rejection—in cynomolgus macaques⁸—suggests that islet xenograft rejection involves cellular mechanisms not operative, or at least not predominant, in islet allograft rejection. Specifically, the increases in serum pig-specific IgG non-Gal antibodies (Supplementary Table 4 online) as well as the highly significant increases in the number of circulating, indirectly activated, interferon (IFN)- γ -secreting, donor-reactive T cells (Fig. 2e,f), both of which we saw only in macaques that rejected islet xenografts (two macaques each in groups A and B, none in C), indicate incomplete suppression

of indirect pathway immune recognition and of effector pathways in macaques experiencing islet xenograft rejection.

The involvement of the direct pathway of T-cell activation has been suggested in islet xenograft rejection⁹, but other evidence indicates that the indirect pathway is the most crucial immune-recognition pathway in islet xenotransplantation^{10,11}. The restriction of indirectly activated CD4⁺ and CD8⁺ T cells to host major histocompatibility (MHC) molecules precludes cognate, T-cell receptor-mediated engagement of transplanted cells, thereby limiting

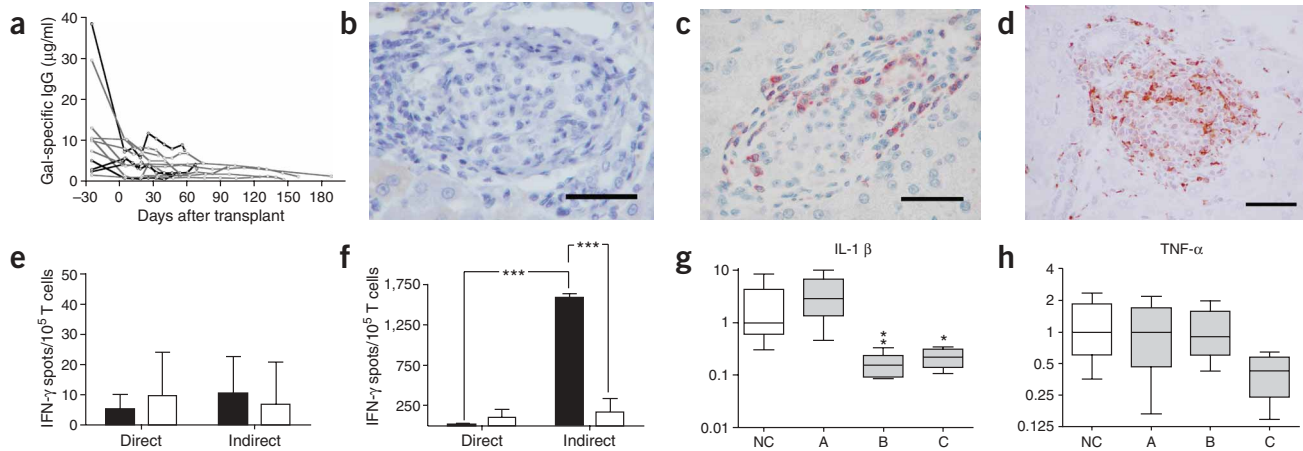


Figure 2 Immune responses to islet xenografts. **(a)** Lack of increase in post-transplant Gal-specific IgG serum levels reactive with Gal type VI (Supplementary Methods online) in rejecting (gray lines) and nonrejecting (black lines) macaques. **(b)** No Gal-specific (isolectin B4) staining on porcine islets undergoing rejection (O2CP4; day 21 after transplant; original magnification, $\times 40$). Scale bar, 50 μm . **(c,d)** T-cell (CD3-specific; **c**) and macrophage (HAM56-specific; **d**) infiltration of islets undergoing rejection (00GP6; day 47 after transplant; original magnification, $\times 40$). Scale bar, 50 μm . **(e)** Similarly low pretransplant frequencies for directly and indirectly stimulated, donor-reactive, IFN- γ -secreting, circulating T cells in rejecting (black bars) and nonrejecting (white bars) macaques. **(f)** Significant post-transplant increase in the frequency of indirectly activated T cells in rejecting (black bars) but not in nonrejecting macaques (white bars; $***P < 0.001$, ANOVA). Higher frequencies of indirectly (versus directly) stimulated IFN- γ -producing T cells in rejecting macaques ($***P < 0.001$). **(g,h)** Transcripts encoding proinflammatory cytokines normalized to β_2 -microglobulin in liver specimens, per qPCR. Specimens were obtained from 9 untreated, normal control macaques (NC) and in livers bearing islet xenografts from 11 recipients on three different immunosuppressive protocols (groups A, B and C). Excluded from our analysis were extremely high levels of mRNA encoding cytokines in two group B recipients experiencing infection. Results are given as fold change differences, on a \log_2 scale, as compared with expression in NC macaques and presented as whisker plots for the 25th and 75th percentile, median and maximum and minimum values. **(g)** Significantly lower relative IL-1 β transcripts as compared with tissue from NC in livers from group B and C macaques ($*P < 0.05$, $**P < 0.01$; Tukey test for multiple comparisons). **(h)** Suppressed intra-graft TNF- α transcripts in group C recipients.

immune-destruction mechanisms resulting from this activation pathway to antibody and inflammatory responses that may exceed typical allograft responses¹². Here we show that CD154-specific antibody therapy, known to be very effective in blocking indirect pathway activation¹³, interfered with elicitation of noncognate effector functions that depend on indirectly activated CD4⁺ T cells (Fig. 2e,f). The prolonged xenograft survival that we observed in our group B and C recipients was associated with undetectable non-Gal pig-specific IgG antibodies, with suppressed intragraft expression of the gene encoding interleukin (IL)-1 β (Fig. 2g), and with the absence of any invasion by infiltrating mononuclear cells into islets.

Leflunomide is known to suppress tumor necrosis factor (TNF)-induced tissue injury¹⁴ and, in rat xenotransplant models, to suppress xenoreactive antibody responses and intragraft expression of proinflammatory cytokines¹⁵. Our results are consistent with leflunomide-mediated inhibition of noncognate effector functions (Fig. 2h and Supplementary Table 4 online), yet further studies are needed to determine the efficacy of leflunomide and its mechanism of action in islet xenotransplantation.

Intragraft expression of mRNA encoding granzyme B, and to a lesser extent, mRNA encoding perforin and FasL, was suppressed in the livers of recipients that were exposed to high CD154-specific antibody trough concentrations (Supplementary Fig. 4 online). But the overall pattern of intragraft mRNA expression of these cytolytic effector molecules was not different between treatment groups, and did not correlate with xenograft survival (Supplementary Fig. 4 online). Together, the results indicate that prolonged islet xenograft survival in groups B and C was probably linked to the ability of the immunosuppressive treatment to inhibit indirect priming of donor-specific cells and noncognate effector functions rather than its ability to suppress the expression of cytolytic effector transcripts.

We conclude that prolonged pig islet xenograft survival is possible in immunosuppressed NHPs without targeting Gal epitopes or Gal-specific antibodies. Rejection, if manifest, involves indirect pathway activation of T cells and participation of noncognate effector mechanisms. The immunosuppression we used inhibited those pathways; however, the associated severe morbidity—particularly CD154-specific antibody-induced thromboembolic events⁷—preclude

its clinical use. Therefore, more selective targeting of indirect immune recognition and effector pathways is important for advancing islet xenotransplantation.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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