Short-Term Administrations of a Combination of Anti–LFA-1 and Anti-CD154 Monoclonal Antibodies Induce Tolerance to Neonatal Porcine Islet Xenografts in Mice

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OBJECTIVE—The objective of this study was to determine whether tolerance to neonatal porcine islet (NPI) xenografts could be achieved by short-term administrations of anti–LFA-1 and anti-CD154 monoclonal antibodies (mAbs).

RESEARCH DESIGN AND METHODS—Diabetic B6 mice received NPI transplants and short-term injections of combined anti–LFA-1 and anti-CD154 mAbs. Mice with long-term islet graft function were treated with depleting anti-CD25 mAb or retransplanted with a second-party NPI. At the end of the study, grafts from mice with long-term islet function were examined. Their spleen cells were characterized and used for in vitro proliferation and adoptive transfer studies.

RESULTS—All mAb-treated NPI recipients maintained normoglycemia for >100 days post-transplantation. Only 5 of 50 mice rejected their grafts before 300 days post-transplantation. Intact islets, foxp3$^+$ immune cells, as well as interleukin (IL)-10 and transforming growth factor (TGF)-β regulatory cytokine transcripts were detected in the NPI xenografts from tolerant mice. A higher percentage of CD4$^+$ T-cell population from these mice expressed regulatory markers, suggesting that tolerance to NPI xenografts may be mediated by T regulatory cells. This was confirmed when tolerant mice treated with depleting anti-CD25 mAb became diabetic. Lymphocytes from tolerant mice inhibited the proliferation of lymphocytes from B6 mice immunized with porcine cells and they displayed limited proliferation when adoptively transferred. All protected B6 mice transplanted with a second-party NPI xenograft maintained long-term normoglycemia even after removal of the first NPI graft-bearing kidney.

CONCLUSIONS—These results demonstrate that tolerance to NPI xenografts can be achieved by transient administrations of combined anti–LFA-1 and anti-CD154 mAb therapy. ***Diabetes*** **59:958–966, 2010**

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Currently, islet transplantation is an alternative treatment for a very select patient population and is unavailable to children with type 1 diabetes. The limitations to the widespread clinical application of this treatment are partly due to the severe shortage of human donor pancreatic tissue (1–3) and the requirement for continuous use of harmful immunosuppressive drugs to prevent rejection of the islet grafts. Neonatal porcine islets (NPIs) are being considered as an alternative source of islets for clinical transplantation. They are easy to maintain in culture and to isolate with abundant yields (4). In addition, NPIs have the inherent ability to proliferate, differentiate, and reverse diabetes in both small (4–6) and large animals (7,8), including the preclinical nonhuman primate model (8).

The short-term administrations of a combination of anti–LFA-1 and anti-CD154 monoclonal antibodies (mAbs) was previously found to be highly effective in preventing NPI xenograft rejection in B6 mice (5,6), suggesting that both adhesion and co-stimulatory pathways of T cell activation are important components of NPI xenograft rejection. The aim of this study was to determine whether interference with adhesion and co-stimulatory pathways by transient administrations of a combination of anti–LFA-1 and anti-CD154 mAbs could induce tolerance to phylogenetically disparate NPI xenografts in mice. Our results show that short-term administrations of this combined mAbs resulted in a robust form of porcine islet xenograft tolerance mediated by T regulatory cells in B6 mice.

RESEARCH DESIGN AND METHODS

Animals. One- to three-day-old Duroc cross-neonatal pigs (>1.5 kg body wt) from the University of Alberta (Edmonton, AB, Canada) were used as islet donors. Six- to eight-week-old male B6 (C57BL/6J, H-2^b) and B6 *rag*$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}/J, H-2^b) mice from Jackson Laboratory (Bar Harbor, ME) were used as recipients of islet transplants. These mice were rendered diabetic by a single intraperitoneal injection of 180 or 175 mg/kg body wt streptozotocin (Sigma, St. Louis, MO) for B6 or B6 *rag*$^{-/-}$ mice, respectively. Blood glucose levels of these mice were measured using a Precision glucose meter (OneTouch Ultra; LifeScan, Milpitas, CA). All mice were fed standard laboratory food and cared for according to the guidelines established by the Canadian Council on Animal Care.

Islet isolation and transplantation. NPIs were isolated and cultured for 7 days in HAM's F10 medium before transplantation as described previously (4,5,9). B6 or B6 *rag*$^{-/-}$ mice that had two consecutive nonfasting blood glucose levels of >20 mmol/l 4–6 days after streptozotocin injection were transplanted with 2,000 NPIs as previously described (5,9). The experiments started with 50 mice, which were monitored for 100 days post-transplantation, then 40 mice were monitored up to 150 days, and 20 mice were monitored up

to 200 days post-transplantation. Finally, 10 mice were monitored up to 250 days post-transplantation, and 8 mice were monitored up to 300 days post-transplantation. Islet engraftment was considered successful when blood glucose levels of these mice reached ≤8.5 mmol/l. Graft rejection was defined as the first of three consecutive days of hyperglycemia (>12 mmol/l) and was confirmed by histological analysis of the graft. Removal of the graft-bearing kidney was performed in randomly selected recipients with long-term graft function (>150 days post-transplantation) to confirm that maintenance of normoglycemia was due to the presence of islet xenograft.

Monoclonal antibody therapies. Anti–LFA-1 mAb (KBA; rat IgG2a) was prepared as ascites and administered intraperitoneally (i.p.) at 200 μg on days 0, 1, 7, and 14 post-transplantation. Anti-CD154 mAb (MR-1, hamster IgG; BioExpress, West Lebanon, NH) was administered intraperitoneally at 250 μg on days −1 and 1 and two times a week for 4 weeks post-transplantation. Anti-CD25 mAb (PC6.1.5.3, rat IgG1, BioExpress) was injected intraperitoneally at 500 μg on 0, 2, 4, and 6 days postadministration starting at 150 days post-transplantation.

Oral glucose tolerance test. At 150 days post-transplantation, an oral glucose tolerance test was performed in randomly selected B6 and B6 *rag*$^{-/-}$ mice transplanted with NPI that maintained long-term graft function as well as in naïve B6 mice (control group) following our previously described method (4).

Immunohistological analysis. The presence of insulin-producing β-cells and immune cells in the grafts were examined following our published protocol (5,9). For foxp3 staining, rat anti-mouse foxp3 antibody (1:25 dilutions; eBioscience, San Diego, CA) and biotinylated goat anti-rat IgG antibody (1:200 dilutions; Southern Biotechnology Associates, Birmingham, AL) were used as primary and secondary antibody, respectively.

RNA extraction and RT-PCR analysis. mRNA was extracted from NPI xenograft-bearing kidneys using Trizol reagent following the manufacturer's protocol (Invitrogen, Burlington, ON, Canada). cDNA was constructed from 1 μg total RNA using Superscript RNase H$^-$ Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol, and 1 μl cDNA was amplified for 35 cycles using *Taq*DNA Polymerase (Invitrogen). The PCR conditions and the primer sequences were similar to what we have previously published (10). Positive control includes thymus cDNA from naïve B6 mice, whereas the negative control includes kidney from these mice and water in place of experimental cDNA. GAPDH primers (housekeeping gene) ensured the integrity of cDNA, and all the primer pairs spanned at least one intron to make sure that no genomic DNA was detected during the amplification.

Detection of anti-porcine IgG antibodies by flow cytometry. The effect of combined mAb therapy on the humoral immune responses of B6 and reconstituted B6 *rag*$^{-/-}$ mice with NPI xenografts was determined by measuring the levels of mouse anti-porcine IgG antibodies in the serum samples of these mice using flow cytometry following our published method (5,9). Controls for this experiment include porcine spleen cells alone and porcine spleen cells incubated with the secondary antibody in the absence of mouse serum.

Characterization of lymphocytes by flow cytometry. Spleens from B6 and reconstituted B6 *rag*$^{-/-}$ mice transplanted with NPI were harvested on the day of rejection or at the end of the study. Spleen cells were isolated (11) and aliquots of 1×10^6 spleen cells were incubated for 30 min at 4°C with fluorescent conjugated antibodies (1:100 dilutions; eBioscience, San Diego, CA) specific for particular lymphocyte markers. Fluorescence histograms were created using a BD FACS Calibur flow cytometry machine (BD Biosciences Pharmingen, Mississauga, ON, Canada) and were used to determine the percentage of positive cells labeled with the corresponding antibodies. Controls for this experiment include spleen cells from tolerant or naïve B6 mice that were or were not incubated with any of the antibodies.

In vitro proliferation assays. The in vitro proliferation of T-cells (5×10^5) from naïve or B6 mice transplanted with NPI was determined after stimulation with Con A (10 μg/ml, Sigma), anti-CD3ε mAb (10 μg/ml, eBioscience), or irradiated (2,500 rad) donor neonatal porcine spleen cells (3×10^5 cells) following our published protocol (11). After 1–6 days (for Con A and anti-CD3ε mAb stimulation assays) and 3, 4, and 5 days of culture for mixed lymphocyte reaction assays, T-cell proliferation was determined by pulsing the cells of primary culture with 1 μCi [^{3}H]-thymidine/well for 18 h. Cells were then harvested onto glass microfiber filters (Wallac, Turku, Finland), and counts per minute (CPM) per sample were detected using the Wallac MicroBeta TriLux luminescence counter (PerkinElmer, Waltham, MA).

Adoptive transfer experiments. At 150 days post-transplantation, spleen cells from naïve or NPI transplanted B6 mice were either left unlabeled or labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's protocol (Molecular Probes, Eugene, OR). Fifty million of these cells in a total volume of 200 μl saline were injected into the peritoneum of naïve or NPI transplanted B6 *rag*$^{-/-}$ mice. Blood glucose levels of these mice were monitored three times a week for 60 days post–cell injection. At the time of rejection or at 60 days post–cell injection if mice remained normoglycemic, NPI xenografts were harvested and were examined for the presence of insulin-positive cells as well as immune cells as described above. The blood glucose levels of B6 *rag*$^{-/-}$ mice that maintained normoglycemia for 60 days post–cell injection were measured after removal of the kidney bearing the NPI xenograft. A return to the diabetic state was noted, and spleen cells from these mice were isolated to characterize the adoptively transferred immune cells using flow cytometry. CFSE-labeled spleen cells from mouse recipients were also collected and analyzed by flow cytometry using PE-Cy5–conjugated anti-mouse TCRβ chain mAb. CFSE-specific fluorescence histograms were created using a BD FACS Calibur flow cytometry machine by gating on TCR$^+$ T-cells.

TABLE 1
Survival of NPI xenografts in B6 mice that were not treated or treated with short-term administrations of a combination of anti–LFA-1 and anti-CD154 mAbs

	n	Graft survival (days post-transplantation)	% Graft survival
Group and end point of the study (days post-transplantation)			
Untreated group			
100	10	0 (×10)	0
Treated group			
100	50	>100 (×50)	100
150	40	>150 (×39), 105	97.5
200	20	>200 (×18), 160, 185	90
250	10	>250 (×9), 216	90
300	8	>300 (×7), 266	85.7

Transplantation of a second-party NPI. Some B6 mice that maintained normoglycemia for >100 days post-transplantation were re-transplanted with a second-party NPI under the right kidney capsule. Blood glucose levels of these mice were monitored for another 100 days (200 days after the first NPI transplant) and, at this time, the left kidney bearing the first NPI xenograft was removed. Blood glucose levels of these mice were measured for an additional 100 days to monitor the function of the second-party NPI xenograft. After 100 days (300 days after transplantation of the first NPI), the remaining right kidney that contained the second NPI xenograft was removed to determine that maintenance of long-term normoglycemia was due to the presence of the second-party NPI xenograft.

Statistical analysis. Statistical differences between groups were sought using Mann-Whitney nonparametric unpaired Student's *t* test in SPSS statistical software, version 13.0 for Windows (Chicago, IL). A P value of <0.05 was considered to be statistically significant.

RESULTS

Short-term administrations of a combination of anti–LFA-1 and anti-CD154 mAbs resulted in indefinite NPI xenograft survival in B6 mice. To determine if short-term administrations of a combination of anti–LFA-1 and anti-CD154 mAbs can induce durable NPI xenograft protection, we lengthened the metabolic follow-up period of B6 mice up to 300 days post-transplantation. All 50 NPI-transplanted mice treated with the combination of mAbs achieved normoglycemia within 70–98 days post-transplantation, whereas none (n = 10) of the untreated NPI recipients achieved normoglycemia (Table 1). At 150 days post-transplantation, which defines our standard end point of the study, 39 of 40 treated mice maintained normoglycemia, and one mouse became diabetic at 105 days post-transplantation. At this time point, the ability of some treated recipients to respond to glucose challenge in vivo was performed (Fig. 1*A*). The blood glucose levels of these recipients at the beginning of the challenge (time 0 min) and at the end of the challenge (time 90 and 120 min) were not significantly different than those observed in B6

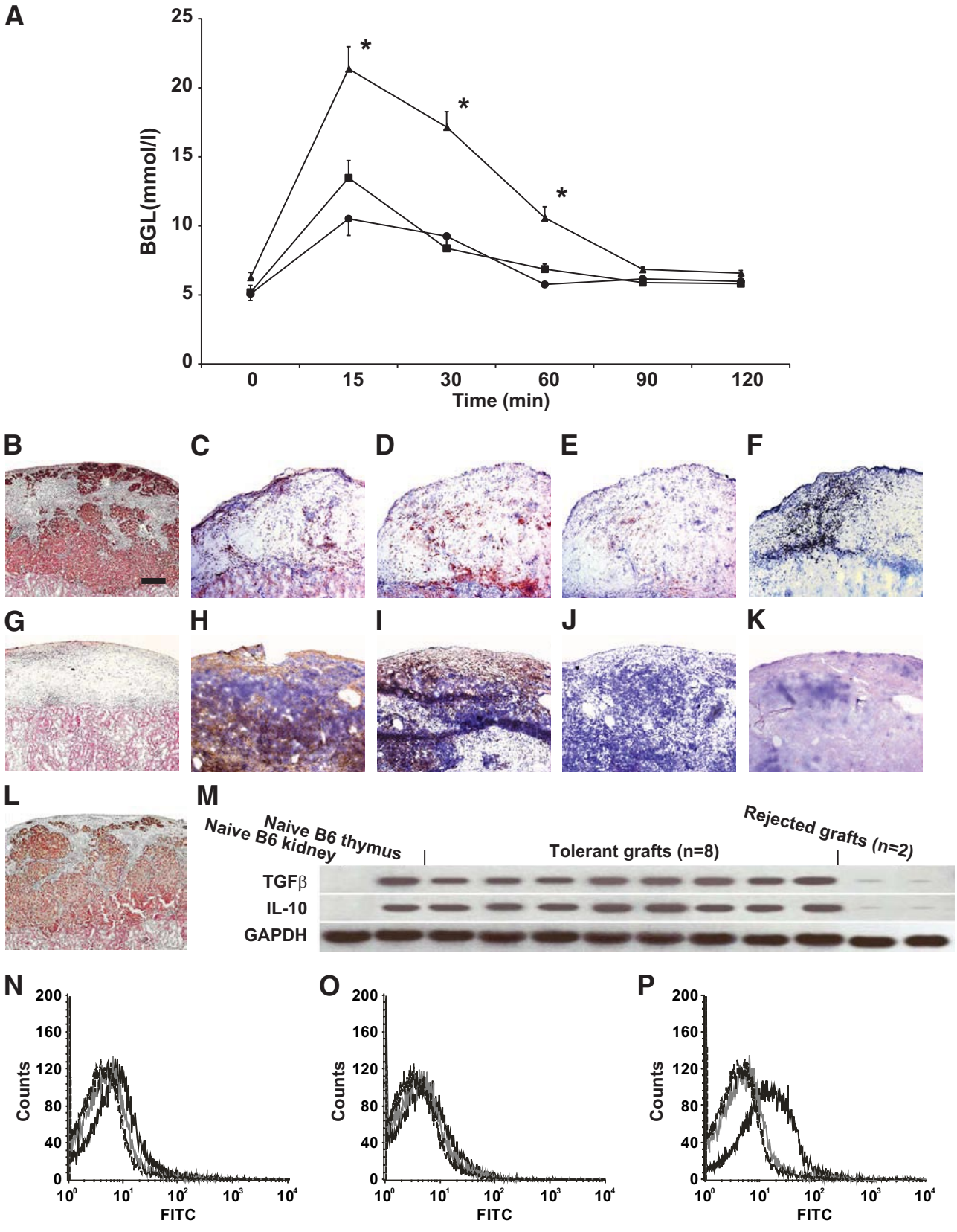

FIG. 1. Blood glucose levels (BGLs) of B6 mouse recipients of NPI (■) responding to oral glucose challenge at 150 days post-transplantation (*A*). Control groups are age-matched B6 *rag*$^{-/-}$ mouse recipients of the same NPI (●) and naïve nontransplanted B6 mice (▲). $n = 5$ in each group, *$P < 0.008$ versus B6 and B6 *rag*$^{-/-}$ mouse recipients of NPI. Representative islet grafts from B6 (*B*) and B6 *rag*$^{-/-}$ (*L*) mice that had long-term normoglycemia contained intact islets with abundant insulin-positive cells (brown stain). Characterization of immune cells surrounding the NPI xenografts of B6 mice with long-term graft function showed some CD3$^+$, CD4$^+$, CD8$^+$, and foxp3$^+$ cells (brown structures; *C–F*). Representative NPI xenografts from B6 mice that became diabetic had no intact islets and contained massive amounts of CD3$^+$ and CD4$^+$ but few CD8$^+$ and foxp3$^+$ cells (*G–K*). Scale bar represents 100 μm. Higher expression of TGFβ1 and IL-10 were detected in NPI xenografts from B6 mice with

TABLE 2
Phenotype of immune cells from B6 mouse recipients of NPIs treated with a combination of anti–LFA-1 and anti-CD154 mAbs compared with the phenotype of immune cells in naïve non-transplanted B6 mice and B6 mouse recipients of NPIs that rejected their islet xenografts

	n	Mean ± SE total number ($\times 10^6$)	Mean composition of isolated spleen cells (%) ± SE		
			$CD4^+$	$CD8^+$	$CD19^+$
Naïve B6	12	89.61 ± 2.15	20.98 ± 0.82	13.04 ± 0.40	55.18 ± 2.0
Tolerant B6	19	86.02 ± 2.34	20.21 ± 0.66	13.32 ± 0.47	56.65 ± 2.07
Non-tolerant B6	5	94.33 ± 4.46	17.91 ± 2.50	10.45 ± 2.21	61.99 ± 6.80

$rag^{-/-}$ mouse recipients of the same NPI and naïve B6 mice. However, the blood glucose levels of both B6 and B6 $rag^{-/-}$ mouse recipients of the same NPI were significantly lower at the 15-, 30-, and 60-min time points when compared with naïve B6 mice.

At 200 days post-transplantation, 18 of 20 treated mice remained normoglycemic while two mice returned to the diabetic state at 160 and 185 days post-transplantation. Nine out of 10 treated mice maintained normoglycemia at 250 days post-transplantation, and one mouse became diabetic at 216 days post-transplantation. Finally, at 300 days post-transplantation, seven of eight treated recipients maintained normoglycemia, and one recipient returned to the hyperglycemic state at 266 days post-transplantation. The removal of the NPI xenograft-bearing kidney from randomly selected mouse recipients that maintained long-term normoglycemia at various time points post-transplantation resulted in return to the diabetic state, confirming that maintenance of normoglycemia depends on the islet xenograft.

NPI xenografts from B6 mice with long-term normoglycemia (Fig. 1*B*) showed intact islets composed of abundant insulin-positive cells comparable to those observed in B6 $rag^{-/-}$ mice transplanted with the same NPI (Fig. 1*L*). We observed immune cells surrounding the islet xenografts in these mice, and further characterization of the cells revealed that they are $CD3^+$, $CD4^+$, $CD8^+$, and $foxp3^+$ cells (Fig. 1*C–F*). In contrast, NPI xenografts from B6 mice that rejected their NPI xenografts and eventually became diabetic had massive amounts of immune cell infiltrate with no intact islets remaining in the transplant site (Fig. 1*G*) and had few $CD8^+$ and $foxp3^+$ cells (Fig. 1*H–K*). Analysis of the NPI xenografts harvested from B6 mice with long-term normoglycemia showed higher expression of transforming growth factor (TGF)-β1 and interleukin (IL)-10 transcripts compared with the expression observed in B6 mice that eventually rejected their grafts (Fig. 1*M*), suggesting that local production of these regulatory cytokines may be partly responsible for the protection induced by the combined mAb therapy.

The levels of anti-porcine IgG antibodies in B6 mice that maintained long-term normoglycemia (Fig. 1*N*) were comparable to the anti-porcine IgG antibody levels detected in naïve B6 mice (Fig. 1*O*). In contrast, the anti-porcine IgG antibody levels of B6 mouse recipients that eventually rejected the NPI xenografts were significantly higher than the levels detected in B6 mice that maintained long-term normoglycemia (Fig. 1*P*). Taken together, these results indicate that short-term administration of combined anti–LFA-1 and anti-CD154 mAbs induce tolerance to NPI xenografts.

Combined anti–LFA-1 and anti-CD154 mAb therapy resulted in changes in $CD4^+$ T-cells expressing regulatory markers. The total number of immune cells and frequencies of $CD4^+$, $CD8^+$ T-cells, and $CD19^+$ B-cells from the spleen of B6 mice that maintained long-term normoglycemia (tolerant) were comparable to those detected in naïve B6 mice (Table 2). Although the total number of immune cells and $CD19^+$ B-cells in B6 mice that eventually rejected the graft (nontolerant) was higher compared with those detected in tolerant B6 mice and naïve B6 mice, the difference was not statistically significant. In addition, whereas the frequencies of $CD4^+$ and $CD8^+$ T-cells were lower in nontolerant B6 mice than in those observed in tolerant B6 and naive B6 mice, the difference was not statistically significant (Table 2). However, we found significant differences in the percentage of $CD4^+CD25^+$ and $CD8^+CD25^+$ T-cells between tolerant, nontolerant, and naïve B6 mice (Fig. 2*A*). Further analysis of $CD4^+CD25^+$ T-cells showed a significant increase in the frequencies of these cells expressing foxp3 and GITR compared with those found in nontolerant and naïve B6 mice (Fig. 2*B*). Similarly, the frequencies of $CD4^+$ T-cells expressing PD-1 and CTLA-4, but not BTLA co-inhibitory markers, were also significantly increased in tolerant B6 mice compared with those detected in nontolerant and naïve B6 mice (Fig. 2*C*). Collectively, these results indicate that combined anti–LFA-1 and anti-CD154 mAbs result in higher percentage of $CD4^+$ T-cells expressing regulatory markers, suggesting that tolerance to NPI xenografts may be mediated by T regulatory cells.

Combined anti–LFA-1 and anti-CD154 mAb therapy resulted in T regulatory cell–mediated tolerance to NPI xenografts. To better define the role of T regulatory cells in protection generated by combined anti–LFA-1 and anti-CD154 mAbs, a group of tolerant B6 mice was treated with depleting anti-CD25 mAb beginning at 150 days post-transplantation. All normoglycemic recipients became diabetic at 25.3 ± 2.5 days post-injection of anti-CD25 mAb (n = 7, Fig. 3*A*). NPI xenografts from these mice had infiltrating immune cells and no insulin-positive cells remaining in the grafts (Fig. 3*B*). The levels of mouse anti-porcine IgG antibody in these mice were significantly higher (Fig. 3*C*) than those detected in tolerant B6 mice

long-term normoglycemia at 150 days post-transplantation (n = 8, *M*) compared with B6 mice that rejected their NPI xenografts (n = 2). Positive control includes thymus cDNA from naïve nontransplanted B6 mice, while the negative controls include kidney from naïve nontransplanted B6 mice and water in place of experimental cDNA. GAPDH serves as a housekeeping gene. The levels of anti-porcine IgG antibodies (*solid black line*) in B6 mice that maintained long-term normoglycemia (1.69–5.07%, n = 25, *N*) were comparable to those detected in naïve nontransplanted B6 mice (1.73–4.57%, n = 10, *O*) but were significantly ($P < 0.001$) lower than those detected in B6 mice that eventually rejected the NPI xenografts (28.54–43.69%, n = 5, *P*). Representative histograms are shown and controls for this experiment consisted of unstained porcine spleen cells (*dashed black line*) and porcine spleen cells incubated with a secondary antibody in the absence of mouse serum (*solid gray line*). (A high-quality digital representation of this figure is available in the online issue.)

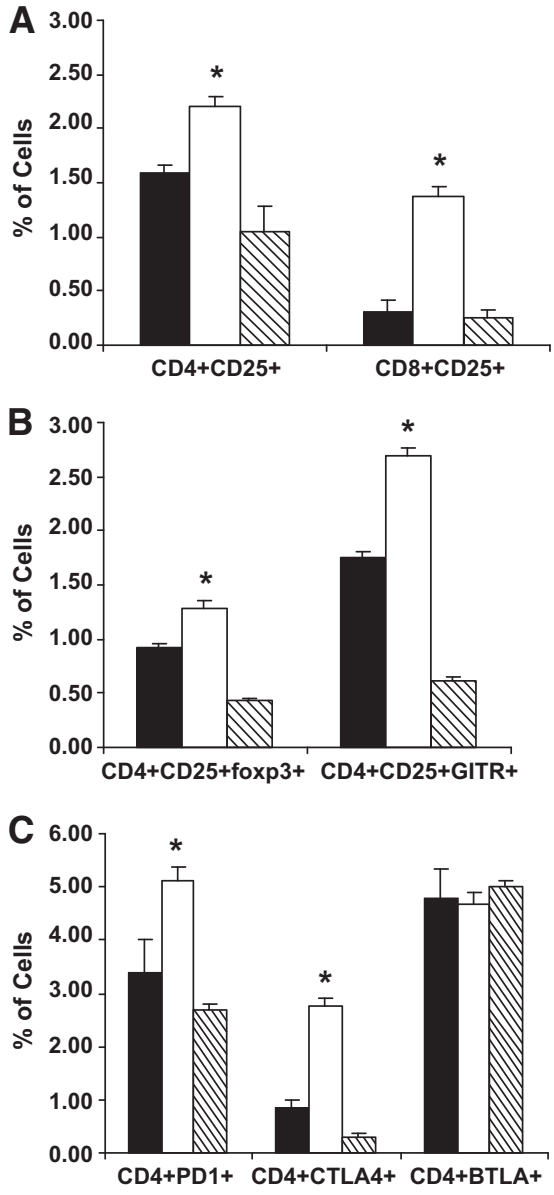

FIG. 2. High frequency of $CD4^+$ T-cells expressing regulatory markers were detected in B6 mice with long-term graft function. The frequency of $CD4^+CD25^+$ (2.20 ± 0.08%, n = 19, *A*) and $CD8^+CD25^+$ (1.38 ± 0.09%, n = 19) T-cells in B6 mice with long-term graft function (□) was significantly (P < 0.0001) higher than those observed in naïve nontransplanted B6 mice (■, 1.58 ± 0.08 and 0.30 ± 0.11%, respectively, n = 12, *A*) and in B6 mice that rejected their NPI xenografts (▨, 1.04 ± 0.25 and 0.26 ± 0.07%, respectively, n = 5, *A*). In this population, there were also significantly more cells expressing foxp3 (1.29 ± 0.08%, n = 19, P = 0.0013, *B*) and GITR (2.69 ± 0.08%, n = 19, P < 0.0001) compared with those found in naïve nontransplanted B6 mice (0.92 ± 0.03 and 1.76 ± 0.05%, respectively, n = 12) and in B6 mice that rejected their NPI xenografts (0.43 ± 0.02 and 0.62 ± 0.03%, respectively, n = 5). The frequencies of $CD4^+$ T-cells (*C*) expressing PD-1 (5.12 ± 0.24%, n = 19) and CTLA-4 (2.78 ± 0.12%, n = 19) but not BTLA (4.69 ± 0.23%, n = 19) co-inhibitory markers were also significantly (P < 0.0001) increased in B6 mice with long-term NPI xenograft survival compared with those detected in naïve nontransplanted B6 mice (3.38 ± 0.63, 0.83 ± 0.18, and 4.79 ± 0.55%, n = 12, respectively) and in B6 mice that became diabetic (2.68 ± 0.13, 0.31 ± 0.06, and 5.01 ± 0.11%, n = 5, respectively).

that were not treated with anti-CD25 mAbs and in naïve B6 mice (Fig. 1*N* and *O*, respectively). These results further indicate that T regulatory cells mediate long-term immune protection of NPI xenografts rendered by combined anti–LFA-1 and anti-CD154 mAb therapy.

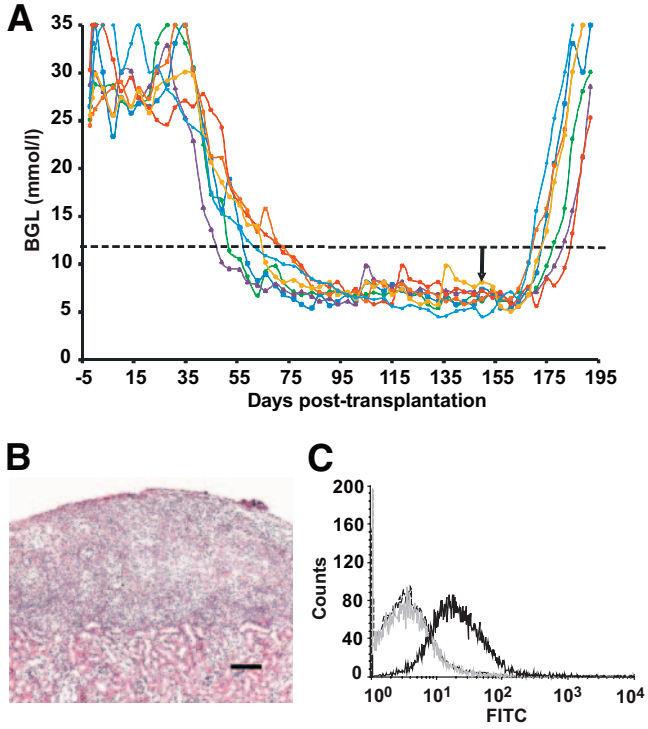

FIG. 3. T regulatory cells mediate long-term immune protection of NPI xenografts rendered by combined anti–LFA-1 and anti-CD154 mAb therapy. All normoglycemic recipients (n = 7) that received depleting anti-CD25 mAb on 0, 2, 4, and 6 days post-administration beginning at 150 days post-transplantation (*arrow*) became diabetic at 175.3 ± 2.5 days post-transplantation (25.3 ± 2.5 days post-injection of anti-CD25 mAb; *A*). NPI xenografts had infiltrating immune cells and absence of insulin-positive cells (*B*). Scale bar represents 100 μm. The levels of mouse anti-porcine IgG antibody (*solid black line*) in these mice were significantly (P < 0.0001) higher (37.28 ± 1.80%, n = 7, *C*) compared with the levels detected in tolerant B6 mice that were not treated with anti-CD25 mAb and in naïve nontransplanted B6 mice (Fig. 1*N* and *O*, respectively). Representative histograms are shown, and controls for this experiment consisted of unstained porcine spleen cells (*dashed black line*) and porcine spleen cells incubated with a secondary antibody without mouse serum (*solid gray line*). BGL, XXX; FITC, fluorescein isothiocyanate. (A high-quality color representation of this figure is available in the online issue.)

Lymphocytes from tolerant mice suppressed the in vitro proliferation of porcine-primed lymphocytes in a dose-dependent manner. Lymphocytes from spleen of tolerant B6 mice responded robustly after non–antigen-specific stimulation with Con A and anti-CD3ε mAb (Fig. 4*A*). The responses were comparable to the proliferative responses of lymphocytes from the spleen of naïve B6 mice after adding the same stimulator molecules (Fig. 4*B*). Similar to what we have previously reported (12), we observed no measurable proliferation of lymphocytes from naïve B6 mice several days after in vitro stimulation with porcine spleen cells (Fig. 4*C*). We also found that lymphocytes from the spleen of tolerant B6 mice did not proliferate after stimulation with porcine spleen cells from the islet donor (Fig. 4*C*). However, we found that initial priming of naïve B6 mice with porcine spleen cells resulted in strong proliferation of lymphocytes from these mice when re-stimulated with porcine spleen cells in vitro (Fig. 4*C*).

To determine whether lymphocytes from tolerant mice were capable of suppressing the proliferation of lymphocytes from porcine-primed B6 mice, we mixed different ratios of the two cell populations. Addition of lymphocytes from tolerant B6 mice resulted in a dose-dependent inhi-

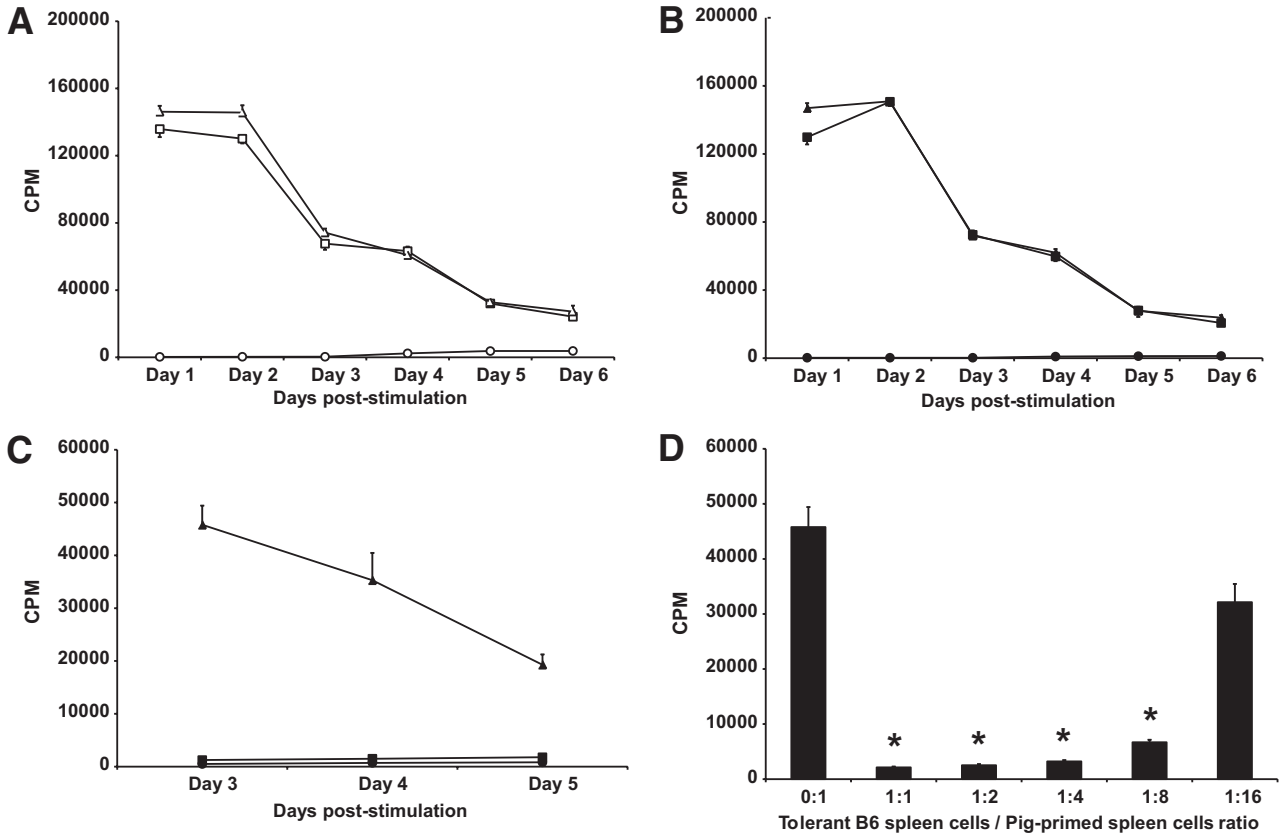

FIG. 4. Lymphocytes from tolerant mice suppressed the in vitro proliferation of porcine-primed lymphocytes in a dose-dependent manner. Lymphocytes from tolerant B6 mice ($n = 9$, *A*) responded robustly after stimulation with Con A (□) or anti-CD3ε mAb (△) comparable to what was observed in lymphocytes from naïve nontransplanted B6 mice (■ for Con A and ▲ for anti-CD3ε mAb, $n = 9$, *B*). Lack of proliferation was observed when these cells were not stimulated (○ for lymphocytes from tolerant mice [*A*] and ● for naïve mice [*B*]). Lymphocytes from porcine-primed B6 mice ($n = 6$) responded robustly when stimulated with porcine spleen cells (▲, *C*). Lymphocytes from tolerant B6 mice (□, $n = 9$) and naïve nontransplanted B6 mice (●, $n = 9$) did not proliferate after stimulation with porcine spleen cells from the islet donor (*C*). Lymphocytes from tolerant B6 mice significantly (*$P < 0.0005$, $n = 3$) inhibited the proliferation of porcine-primed lymphocytes in a dose-dependent manner on day 3 post-culture (*D*).

bition of proliferation of lymphocytes from porcine-primed B6 mice (Fig. 4*D*). At 1:1, 1:2, 1:4, and 1:8, but not 1:16, ratios of lymphocytes from tolerant B6 mice to lymphocytes from porcine-primed B6 mice, we observed significant inhibition of proliferation of the latter cell population at 3 days postculture. In contrast, the addition of lymphocytes from naïve B6 mice to lymphocytes from porcine-primed B6 mice at similar ratios did not result in the inhibition of proliferation but resulted in proliferation of lymphocytes from porcine-primed B6 mice (data not shown).

Tolerance rendered by combined anti–LFA-1 and anti-CD154 mAb therapy could be extended to second-party NPI xenografts. All B6 *rag*$^{-/-}$ mice transplanted with first- and second-party NPI remained normoglycemic for >60 days after the transfer of spleen cells from tolerant B6 mice (Table 3). However, B6 *rag*$^{-/-}$ mice with established NPI xenografts reconstituted with spleen cells from naïve B6 mice rejected their grafts by 15 days post-cell transfer (Table 3). CFSE labeling of T-cells from the spleen of these mice revealed that they exhibited robust proliferation (Fig. 5*A*). In contrast, CFSE labeling of T-cells from B6 mice tolerant to first-party NPI xenografts showed that a large proportion of these cells did not proliferate strongly after injection into B6 *rag*$^{-/-}$ mice with established first-party (Fig. 5*B*) and second-party (Fig. 5*C*) NPI xenografts. However, in the absence of NPI xenografts, these cells proliferated when injected into naïve B6 *rag*$^{-/-}$ mice (Fig. 5*D*).

The first-party (Fig. 5*E–I*) and second-party (Fig. 5*J–N*) NPI xenografts from B6 *rag*$^{-/-}$ mice reconstituted with spleen cells from tolerant B6 mice had intact islets with

TABLE 3
Survival of first- and second-party NPI xenografts in B6 *rag*$^{-/-}$ mice reconstituted with spleen cells from naïve or tolerant B6 mice

	Source of spleen cells	*n*	Graft survival (days post-cell transfer)
Source of islets			
First- or second-party newborn pig	Naïve non-transplanted B6 mice	5	10, 11, 13, 15 (×2)
First-party newborn pig	B6 mouse recipients with long-term normoglycemia	6	>60 (×6)
Second-party newborn pig	B6 mouse recipients with long-term normoglycemia	7	>60 (×7)

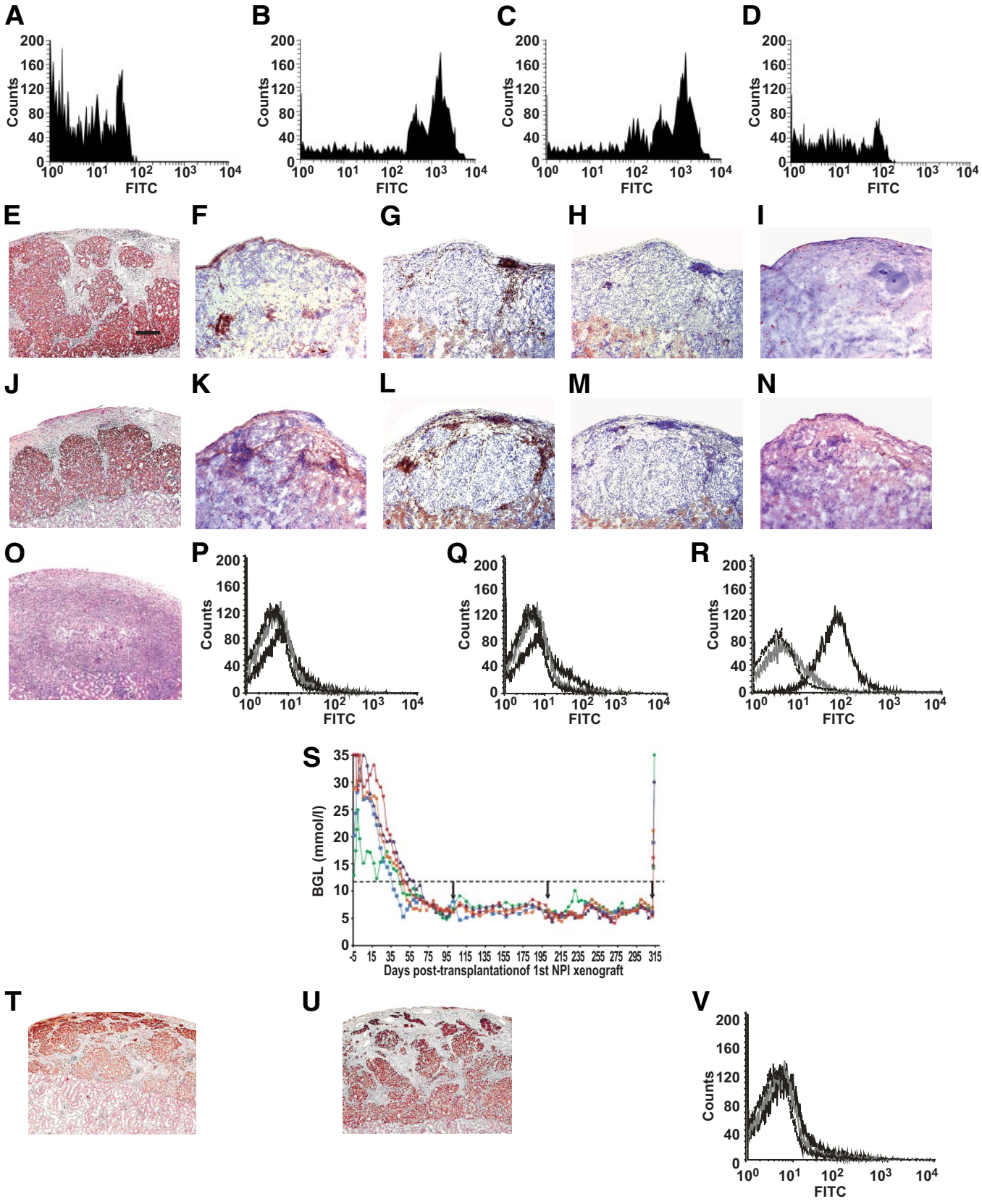

FIG. 5. Tolerance provided by combined anti–LFA-1 and anti-CD154 mAb therapy could be extended to second-party NPI xenografts. CFSE labeling of T-cells from B6 *rag*$^{-/-}$ mice that rejected the NPI xenografts after reconstitution of spleen cells from naïve B6 mice showed robust T-cell proliferation (*A*). In contrast, CFSE labeling of T-cells from B6 mice tolerant to first-party NPI xenografts showed that a large proportion of these cells did not proliferate robustly after injection into B6 *rag*$^{-/-}$ mice with established first-party (*B*) and second-party (*C*) NPI xenografts. However, in the absence of NPI xenografts, these cells were able to proliferate when injected into naïve B6 *rag*$^{-/-}$ mice (Fig. 5*D*). Representative histograms are shown (n = 3). Representative first-party (*E–I*) and second-party (*J–N*) NPI xenografts from B6 *rag*$^{-/-}$ mice injected with lymphocytes from tolerant B6 mice had intact islets composed of abundant insulin-positive cells and immune cells such as CD3^{+},

abundant insulin-positive cells and immune cells such as $CD3^+$, $CD4^+$, $CD8^+$, and $foxp3^+$ cells. NPI grafts from B6 $rag^{-/-}$ mouse recipients, injected with spleen cells from naïve B6 mice had no insulin-positive cells and contained massive immune cellular infiltrate (Fig. 5*O*). The humoral immune responses in B6 $rag^{-/-}$ mice with first-party (Fig. 5*P*) and second-party (Fig. 5*Q*) NPI xenografts that maintained normoglycemia for >60 days after injection of spleen cells from tolerant B6 mice were comparable. The IgG antibody levels of these mice, however, were significantly lower than those detected in B6 $rag^{-/-}$ mice that rejected the NPI xenografts after injection of spleen cells from naïve B6 mice (Fig. 5*R*).

To confirm that tolerance to first-party NPI xenografts could be extended to the second-party NPI xenografts, tolerant B6 mice were re-transplanted with a second-party NPI. All of the mice transplanted with a second-party NPI xenograft maintained normoglycemia for >100 days post-harvest of the first-party NPI xenograft. However, removal of the right kidney bearing the second-party NPI xenograft resulted in the recurrence of diabetes in all B6 mouse recipients (Fig. 5*S*), indicating that tolerance induced by combined anti–LFA-1 and anti-CD154 mAbs to first-party NPI xenografts could be extended to second-party NPI xenografts. The first-party (Fig. 5*T*) and second-party (Fig. 5*U*) NPI xenografts from tolerant B6 mice had intact islets composed of abundant insulin-positive cells. Low levels of anti-porcine IgG antibodies (Fig. 5*V*) were detected in these mice at the end of the study (>300 and >200 days post-transplantation of the first- and second-party NPIs, respectively).

DISCUSSION

The importance of T regulatory cells in tolerance to NPI xenografts induced by the combined mAb therapy was demonstrated when tolerant B6 mice became diabetic after depletion of $CD25^+$ cells using mAb. We found no significant difference in the total number of spleen cells and percentage of $CD4^+$, $CD8^+$ T-cells as well as B-cells from tolerant B6 mice compared with naïve B6 mice, suggesting that tolerance due to clonal deletion is unlikely. However, further analysis of the phenotype of immune cells from tolerant B6 mice using flow cytometry showed a higher percentage of $CD4^+$ T-cells, co-expressing cell surface markers for T regulatory cells (13–18) compared with those observed in naïve B6 mice.

We also showed that T-cells from tolerant B6 mice did not proliferate after stimulation with porcine spleen cells. However, they proliferated robustly after non–antigen-specific stimulation with Con A or anti-CD3ε mAb, indicating that the in vitro unresponsiveness observed in T-cells from tolerant B6 mice is specific to porcine antigens. Furthermore, cells from tolerant B6 mice were able to suppress the proliferative response of lymphocytes from B6 mice immunized with the same porcine spleen cells that were used as stimulator cells in the mixed lymphocyte reaction assays. These results indicate that unresponsiveness of T-cells from tolerant B6 mice is not due to their anergic condition, but could be due to their regulatory function (19,20). Our results also showed that tolerance to first-party NPI xenografts could be extended to second-party NPI xenografts, as demonstrated by transfer of protection to NPI xenografts and survival of second-party NPI xenografts in tolerant B6 mice, suggesting that the state of unresponsiveness to islet xenografts may be species specific.

Similar to what was recently reported in islet allografts (21), our study demonstrates that T regulatory cells could be found in both the graft site and in the secondary lymphoid organ such as the spleen. These results implicate that in the case of maintaining tolerance to islet allografts and xenografts T regulatory cells could control the effector T-cells in both the sites of ongoing immune response and in the secondary lymphoid organs. It remains to be determined, however, whether T regulatory cells could also be found in the draining lymph nodes as what was shown in recipients of islet allografts or they are restricted to the spleen of recipients tolerant to islet xenografts. This will be an important study in the future to gain a better understanding of how T regulatory cells exhibit their suppressive effector function in porcine islet xenograft recipients.

Another important study in the future is to transplant porcine islets into nonhuman primates and treat them with an immunosuppressive protocol that includes simultaneous targeting of both adhesion and co-stimulatory pathways for T-cell activation. This would test whether our strategy could also result in tolerance to NPI xenografts in the presence of preexisting anti-Gal antibodies. Three recent studies (8,22,23) have demonstrated the feasibility of porcine islet xenotransplantation in preclinical nonhuman primate models, and these studies indicate that porcine islet xenografts do not undergo Gal-specific antibody-mediated hyperacute rejection, as seen in solid organ xenografts. However, these studies do not rule out the possibility that in the absence of Gal antigen, particularly on NPI, which we have previously demonstrated to highly expressed αGal (24), fewer islets may be required to reverse hyperglycemia, and less immunosuppression may be sufficient to demonstrate long-term survival of porcine islet xenografts. A study comparing the survival of Gal knockout with wild-type porcine islets in nonhuman primates using the same immunosuppressive protocol in the above-mentioned studies or immunosuppressive protocol that includes our strategy is warranted.

Although we have demonstrated that simultaneous per-

$CD4^+$,$CD8^+$, and $foxp3^+$ cells. NPI xenografts from B6 $rag^{-/-}$ mouse recipients reconstituted with spleen cells from naïve nontransplanted B6 mice had no intact islets (*O*). The scale bar represents 100 μm. The humoral immune responses of B6 $rag^{-/-}$ mouse recipients of first-party (*P*, $n = 9$) and second-party (*Q*, $n = 10$) NPI xenografts 60 days after reconstitution with lymphocytes from tolerant B6 mice were comparable (6.31 ± 0.59 and 7.55 ± 1.02%, respectively). The anti-porcine IgG antibody levels from these mice were significantly ($P < 0.0001$) lower than the IgG antibody levels detected in B6 $rag^{-/-}$ mice (67.42 ± 5.38%, $n = 8$) reconstituted with lymphocytes from naïve nontransplanted B6 mice, which rejected the NPI xenografts by 15 days post-cell injection (*R*). Tolerant B6 mice ($n = 5$) maintained normoglycemia after re-transplantation of a second-party NPI xenograft (*first arrow*, *S*) and remained normoglycemic after removal of the first-party NPI xenograft (*second arrow*). These mice became diabetic only after removal of the right kidney bearing the second-party NPI xenograft (*third arrow*). Representative first-party (*T*) and second-party (*U*) NPI xenografts from tolerant B6 mice had intact islets composed of abundant insulin-positive cells. Scale bar represents 100 μm. Low levels of anti-porcine IgG antibodies (3.95 ± 0.52%, $n = 5$) were detected in these mice at the end of the study (>300 and >200 days post-transplantation of the first- and second-party NPI, respectively; *V*). Representative histograms of porcine spleen cells bound to mouse IgG antibodies are shown, and controls for this experiment consisted of unstained porcine spleen cells (*dashed black line*) and porcine spleen cells incubated with a secondary antibody without mouse serum (*solid gray line*). BGL, blood glucose level; FITC, fluorescein isothiocyanate. (A high-quality digital representation of this figure is available in the online issue.)

turbation of both adhesion and co-stimulatory pathways for T-cell activation are highly effective at inducing tolerance to NPI xenografts in immune-competent mice, it is important to demonstrate that this strategy could be equally effective in an animal model of type 1 diabetes. We previously reported that combined anti–LFA-1 and anti-CD154 mAbs could also be effective at prolonging the survival and function of NPI xenografts in spontaneously diabetic NOD mice if $CD4^+$ T-cells were initially depleted (9). This suggests that in individuals with type 1 diabetes, targeting the autoreactive memory T-cell population may be required in addition to interfering with the adhesion and co-stimulatory pathways of T-cell activation to achieve long-term survival of porcine islet xenografts.

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