Indefinite Survival of Neonatal Porcine Islet Xenografts by Simultaneous Targeting of LFA-1 and CD154 or CD45RB

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A variety of transient therapies directed against molecules involved in T-cell activation and function result in long-term islet allograft survival. However, there are relatively few examples of durable islet xenograft survival using similar short-term approaches, especially regarding highly phylogenetically disparate xenograft donors. Previous studies demonstrate that combined anti–lymphocyte function–associated antigen-1 (LFA-1) plus anti-CD154 therapy results in a robust form of islet allograft tolerance not observed with either individual monotherapy. Thus, the aim of this study was to determine whether the perturbation of anti–LFA-1, either alone or in combination with targeting CD154 or CD45RB, would promote neonatal porcine islet (NPI) xenograft survival in mice. NPI xenografts are rapidly rejected in wild-type C57BL/6 mice but reproducibly mature and restore durable euglycemia in diabetic, immune-deficient C57BL/6 rag-1−/− recipients. A short course of individual anti–LFA-1, anti-CD154, or anti-CD45RB therapy resulted in long-term (>100 days) survival in a moderate proportion of C57BL/6 recipients. However, simultaneous treatment with anti–LFA-1 plus either anti-CD154 or anti-CD45RB therapy could achieve indefinite xenograft function in the majority of recipient animals. Importantly, prolongation of islet xenograft survival using combined anti–LFA-1/anti-CD154 therapy was associated with little mononuclear cell infiltration and greatly reduced anti-porcine antibody levels. Taken together, results indicate that therapies simultaneously targeting differing pathways impacting T-cell function can show marked efficacy for inducing long-term xenograft survival and produce a prolonged state of host hyporeactivity in vivo.

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Endocrine replacement therapy by islet transplantation is an attractive alternative treatment for patients with type 1 diabetes. However, the widespread clinical application of this treatment is currently limited by the shortage of human cadaveric organs available for transplantation. Transplantation of islets derived from pigs may be one approach that could solve the shortage in human islets, provided that formidable xenograft rejection can be prevented. Neonatal pigs in particular are an attractive inexpensive alternative source of insulin-producing tissue for clinical transplantation. Single neonatal pig donors yield ~50,000 islet cell aggregates that consist primarily of epithelial cells and pancreatic endocrine cells (1). Importantly, neonatal porcine islets (NPIs) are responsive to glucose challenge in vitro and are capable of maturing and reversing hyperglycemia in immune-deficient animals (1). Although neonatal pig shows promise in providing an abundant number of islets for clinical transplantation, we have recently reported that NPI xenografts are vigorously rejected when transplanted into untreated mice (2). Thus, the potential of neonatal pigs as a source of insulin-producing tissue for clinical transplantation is greatly hampered by a formidable cellular xenograft response. While a number of short-term approaches to blocking/perturbing a variety of cell surface molecules involved in T-cell function can result in long-term islet allograft survival, it is less clear whether T-cell–directed therapies can result in prolonged xenograft survival. To make neonatal pigs a viable source of islets for clinical transplantation, it is essential to develop effective therapies that prevent the rejection and possibly induce tolerance to NPI xenografts. Therefore, the aim of this study was to determine the efficacy of combining therapies that target distinct molecular pathways for promoting the survival of NPI xenografts in mice.

Numerous approaches for achieving either islet allograft or xenograft prolongation involve monoclonal antibody therapy directed at a variety of cell surface molecules involved in T-cell activation and function. Based on the concept that T-cell receptor ligation in the absence of costimulatory signals can lead to T-cell unresponsiveness (3–5), costimulatory molecules have been a major focus as therapeutic targets for inducing allograft prolongation and tolerance. The potential efficacy of costimulation blockade for xenograft survival was first indicated through the administration of CTLA4-Ig treatment to inhibit CD28:
CD80/CD86 interactions, resulting in human-to-mouse xenograft prolongation (6). Costimulation through CD40: CD154 interaction has also served as a major therapeutic target for facilitating allograft and xenograft survival. Anti-CD154 monoclonal antibody therapy has been shown to induce long-term survival of islet allografts in rodent models (7–11) and in nonhuman primates (12,13). Anti-CD154 plus donor-specific transfusion is also effective for promoting rat and porcine islet xenografts in mice (14,15).

Facilitating islet allograft prolongation has also been achieved by targeting molecules involved in T-cell receptor signaling, such as CD3 (16,17) and CD45 (18). CD45 is a transmembrane protein tyrosine phosphatase essential for T-cell activation (19). CD45RB is a restricted isoform of CD45 that has been an effective target of monoclonal antibody therapy for facilitating transplant prolongation and induction of tolerance. Administration of antibody to CD45RB can result in prolonged allograft and xenograft survival in experimental models of islet transplantation (18–24). In addition, combination of anti-CD45RB antibody with anti-CD154 antibody has been shown to promote long-term islet allograft survival in both chemically induced mice (21) and spontaneously diabetic nonobese diabetic (NOD) mice (25). Thus, manipulation of T-cell receptor signaling (signal 1) alone or in combination with costimulation blockade (signal 2) is a promising target for achieving islet graft survival.

Another highly effective route for achieving allograft prolongation is through perturbation of cell adhesion/homing receptors, especially the β2 integrin lymphocyte function–associated antigen-1 (LFA-1; CD11a). Short-term blockade of LFA-1 with monoclonal antibody therapy is highly effective for promoting long-term islet allograft survival and tolerance (11,26–29). Furthermore, the combination of anti–LFA-1 plus anti-CD154 monoclonal antibody has been recently shown to be effective in preventing the rejection of islet allografts in either chemically induced (30) or spontaneously diabetic (31) mice and can induce a robust form of “dominant” allograft tolerance (30). Blockade of intracellular adhesion molecule-1 (ICAM-1), the major ligand of LFA-1, has proven effective for promoting islet xenograft survival in mice (32), providing evidence of a role for LFA-1/ICAM-1 in xenograft rejection. Given the tolerizing potential of these therapies, including anti–LFA-1 therapy, we determined whether a short-course administration of monoclonal antibodies directed against LFA-1, CD154, or CD45RB alone or in combination could promote NPI xenograft survival and function in diabetic B6 mice. Results show that anti–LFA-1 combined with either anti-CD154 or anti-CD45RB therapy could achieve striking prolongation of NPI xenografts in mice. Alternatively, we also determined whether rapamycin treatment either alone or with anti–LFA-1 can result in long-term acceptance of NPI xenografts in B6 mice. Interestingly, although rapamycin monotherapy can promote long-term islet allograft survival (33) and is used as a mainstay antirejection drug in the Edmonton protocol (34), we observed little benefit in using rapamycin in the islet xenograft protocols described.

**RESEARCH DESIGN AND METHODS**

Male C57BL/6ByJ (B6, H-2b) and immune-deficient C57BL/6-rag-1<sup>tm1/mom</sup> (B6 <sup>rag-1</sup>−<sup>−</sup>, H<sup>2</sup>b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as islet transplant recipients. One- to 3-day-old Landrace-Yorkshire (1.5–2 kg body wt) neonatal pigs of either sex were purchased from the University of Alberta farm. Recipient mice were rendered diabetic by a single intravenous injection of streptozotocin (160 mg/kg body wt; Calbiochem, La Jolla, CA) 4–5 days before transplantation. All diabetic mice had two consecutive nonfasting blood glucose levels ≥17 mmol/l. Blood samples were obtained from the tail vein to monitor glucose levels. Human intravenous insulin (Humulin R; Eli Lilly and Co., Indianapolis, Indiana, United States) was administered subcutaneously to maintain blood glucose levels <5 mmol/l. Graft rejection was defined as the first of 3 consecutive days of hyperglycemia (≥10 mmol/l glucose), and rejection was confirmed by histological analysis of the graft. Nephrectomy of the graft-bearing kidney was performed on recipients with long-term graft function (>100 days posttransplant) to confirm that hyperglycemia ensued, indicating that normal blood glucose was xenograft dependent.

**Antirejection therapies.** Streptozotocin-induced diabetic B6 mice transplanted with NPIs were randomly designated to receive the following antirejection treatments intraperitoneally: 1) rapamycin alone (0.2 mg/kg on days 0–14 posttransplantation; generous gift from Dr. James Shapiro, Edmonton, Alberta), 2) anti–LFA-1 monoclonal antibody alone (KBA; rat IgG2a; hybridoma kindly provided by Dr. Ihara, Charlestown, MA; 200 μg on days 0, 1, 7, 14 posttransplantation), 3) anti-CD154 monoclonal antibody (MR-1; hamster IgG1; Bio Express, West Lebanon, NH; 250 μg on days –1 and 1 and 2 times a week for additional 4 weeks), 4) anti-CD45RB monoclonal antibody (MB236G2, rat IgG2a; hybridoma purchased from American Type Culture Collection, Manasas, VA; 250 μg on days 0–5 posttransplantation), 5) rapamycin plus anti–LFA-1 monoclonal antibody, 6) anti–LFA-1 plus anti-CD154 monoclonal antibodies, and 7) anti–LFA-1 plus anti-CD45RB monoclonal antibodies.

**Immunohistological analysis.** Graft-bearing kidneys were harvested at the end of the study and divided in two sections. One half of the kidney was fixed in 10% buffered formalin solution, cut into 5-mm-thick sections, and embedded in paraffin. Sections 5 μm thick were stained to determine the presence of insulin-containing β-cells. The other half of the kidney was embedded in OCT compound (Miles Scientific, Naperville, IL) and kept frozen at −70°C to determine the presence of T-cells and macrophages. Insulin-containing β-cells were detected by applying guinea pig anti-porcine primary antibody (1:200; DAKO, Carpinteria, CA) for 30 min, followed by the addition of biotinylated goat anti–guinea pig IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA). The avidin–biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories) and 3,3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA) were used to produce a brown color. All paraffin sections were counterstained with Harris’ hematoxylin and eosin.
Detection of mouse anti-porcine antibodies in mouse serum. To determine the effect of various antirejection therapies in the production of mouse anti-porcine antibodies, we determined the levels of mouse anti-porcine IgG antibodies from the blood serum of each transplant recipient using flow cytometry. Peripheral blood samples from B6 mice with long-term islet xenograft survival were collected, and sera were isolated. Porcine spleen cells \((1 \times 10^6)\) obtained from the same pig islet donors were incubated with mouse serum at 1/128 dilution for 1 h at 37°C (5% CO\(_2\), 95% air). Spleen cells were then washed with PBS and incubated with Cy2-conjugated donkey anti-mouse IgG antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at 4°C. The percentage of cells bound to antibody was detected from single parameter fluorescence histograms on an Elite flow cytometer (Coulter Electronics, Palo Alto, CA) after gating on viable lymphocytes. Controls for this experiment include sera from nontransplanted naïve B6 mice, unstained pig spleen cells, and pig spleen cells stained with secondary antibody alone without mouse serum.

**Statistical analysis.** Statistical differences in graft survival among treated groups were sought using the Kaplan-Meier log rank test. A \(P\) value < 0.05 was considered to be statistically significant.

**RESULTS**

**Survival and function of NPIs in chemically induced diabetic B6 \(\text{rag-}1^{-}\) mice.** It has previously been demonstrated that NPIs can survive and reverse hyperglycemia in alloxan-induced diabetic nude mice (1). In the present study, we confirmed that NPI xenografts could also survive and function in immune-deficient B6 \(\text{rag-}1^{-}\) mice. NPIs require a considerable time period to mature and function in vivo relative to adult islets (1). After transplantation of 2,000 NPIs, all streptozotocin-induced diabetic B6 \(\text{rag-}1^{-}\) recipients achieved normoglycemia, though the time to euglycemia was somewhat variable (7–16 weeks posttransplant; Fig. 1A). Immunohistological examination of these long-term functioning xenografts revealed highly vascularized tissues that consist predominantly of well-granulated insulin-containing \(\beta\)-cells with no cellular infiltration (Fig. 1B). In contrast, NPI xenografts obtained from untreated wild-type B6 mice demonstrated complete destruction of donor tissue architecture (Fig. 1C) and substantial mononuclear cell infiltration within 2 weeks posttransplantation. Thus, data show that NPI xenografts engrafted under the kidney capsule mature and restore euglycemia in immune-deficient B6 \(\text{rag-}1^{-}\) mice, similar to previous studies demonstrating that NPIs develop into mature insulin-producing \(\beta\)-cells in nude mice (1).

**Anti–LFA-1, anti-CD154, or anti-CD45RB, but not rapamycin, prevent the rejection of NPI xenografts.** We first tested the efficacy of individually blocking LFA-1, CD154, or CD45RB for promoting NPI xenograft survival and function in wild-type B6 mice. In addition, we determined whether addition of short-term rapamycin treatment would augment xenograft survival. All untreated B6 recipients acutely rejected NPI xenografts within 10 days posttransplantation. Since NPIs require several weeks to restore euglycemia in B6 \(\text{rag-}1^{-}\) mice, acute rejection in immune-competent B6 mice required histological assessment (Fig. 1C). Recipients that were treated with rapamycin remained diabetic throughout the study period, with none of the animals achieving euglycemia (Table 1; Fig. 2A). Although four of seven rapamycin-treated mice showed evidence of partial function (blood glucose levels of 13.3–15.2 mmol/l), these levels did not continue to decrease over time. Histological examination of such grafts at 100 days posttransplantation showed very few intact islets that remained in the graft (Fig. 2B). Thus, rapamycin demonstrated little efficacy for promoting NPI xenograft survival in B6 mice.

**FIG. 1.** Survival of NPI xenografts in B6 \(\text{rag-}1^{-}\) and B6 mice. Streptozotocin-induced diabetic (blood glucose level \(\geq 17.0\ \text{mmol/l}\) B6 \(\text{rag-}1^{-}\) (A,B) and B6 (C) mice were transplanted with NPIs under the kidney capsule and monitored for reversal of diabetes. Normal blood glucose level (\(<10\ \text{mmol/l}\) was achieved in B6 \(\text{rag-}1^{-}\) between 8 and 10 weeks posttransplantation (A). Histological examination of the xenografts at \(>100\) days posttransplantation showed numerous intact islets that contain large number of insulin-positive cells (brown stain) present in B6 \(\text{rag-}1^{-}\) recipient but absent in B6 mice. Scale bar represents 100 \(\mu\)m.
xenograft survival in this study. In contrast, antibody monotherapy using anti–LFA-1, anti-CD154, or anti-CD45RB monoclonal antibodies resulted in improved xenograft survival (Fig. 2A). Nearly half (7 of 15) of anti-LFA-1–treated mice achieved euglycemia within 4–14 weeks posttransplantation, with 6 of 7 of these mice achieving long-term xenograft survival (>100 days, \( P = 0.04 \) vs. rapamycin alone). Similarly, three of seven (42.9%) and three of eight (37.5%) mice that received anti-CD154 or anti-CD45RB monoclonal antibody therapy, respectively, achieved normoglycemia within 4–15 weeks posttransplantation, with a proportion of these animals demonstrating long-term xenograft function. Nephrectomy of the graft-bearing kidney in all animals with long-term function-

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Graft survival (days posttransplantation)( ^{a} )</th>
<th>Number of mice that achieved normoglycemia</th>
</tr>
</thead>
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<tr>
<td>Rapamycin</td>
<td>7</td>
<td>0 (×7)</td>
<td>0/7</td>
</tr>
<tr>
<td>Anti-LFA-1( ^{†} )</td>
<td>15</td>
<td>0 (×8), 86, &gt;100 (×6)</td>
<td>7/15</td>
</tr>
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<td>Anti-CD45RB</td>
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<td>0 (×4), 67, &gt;100, &gt;100</td>
<td>3/8</td>
</tr>
<tr>
<td>Anti-CD154</td>
<td>7</td>
<td>0, 0, 0, 83, &gt;100 × 2</td>
<td>3/7</td>
</tr>
<tr>
<td>Rapamycin plus anti-LFA-1</td>
<td>10</td>
<td>0 (×7), 99, &gt;100, &gt;100</td>
<td>3/10</td>
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<td>0, 0, &gt;100 (×12)</td>
<td>12/14</td>
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<tr>
<td>Anti-LFA-1 plus anti-CD45RB</td>
<td>12</td>
<td>0 (×4), 82, &gt;100 × 7</td>
<td>8/14</td>
</tr>
</tbody>
</table>

\( ^{a} \)Graft survival of 0 indicates that mice did not achieve normal blood glucose levels posttransplantation. Rapamycin (0.2 mg/kg) was administered intraperitoneally on days 0–14 posttransplantation; anti–LFA-1 (KBA; rat IgG2a; 200 μg) was given on days 0, 1, 7, and 14 posttransplantation; anti-CD154 (MR-1; hamster IgG1; 250 μg) was administered on days −1 and once and twice a week for an additional 4 weeks posttransplantation; and anti-CD45RB (MB23G2, rat IgG2a) was administered at 300 μg on day −1 and 100 μg on days 0–5 posttransplantation. \( ^{†} P < 0.04 \) vs. rapamycin, \( ^{‡} P = 0.01 \) vs. rapamycin plus anti–LFA-1.

In contrast, antibody monotherapy using anti–LFA-1, anti-CD154, or anti-CD45RB monoclonal antibodies resulted in improved xenograft survival (Fig. 2A). Nearly half (7 of 15) of anti-LFA-1–treated mice achieved euglycemia within 4–14 weeks posttransplantation, with 6 of 7 of these mice achieving long-term xenograft survival (>100 days, \( P = 0.04 \) vs. rapamycin alone). Similarly, three of seven (42.9%) and three of eight (37.5%) mice that received anti-CD154 or anti-CD45RB monoclonal antibody therapy, respectively, achieved normoglycemia within 4–15 weeks posttransplantation, with a proportion of these animals demonstrating long-term xenograft function. Nephrectomy of the graft-bearing kidney in all animals with long-term function-

**FIG. 2.** Effects of rapamycin and monoclonal antibody monotherapy on the survival of NPI xenografts. **A:** B6 mice treated with a monotherapy of rapamycin did not show long-term survival of NPI xenografts. None of the recipients in this group achieved normal blood glucose level. In contrast, mice treated with anti–LFA-1, anti-CD154, or anti-CD45RB antibody showed modest efficacy in prolonging islet xenograft survival. **B:** Islet xenografts from mice treated with monoclonal antibodies showed the presence of mononuclear cells around and inside the islet grafts. Some intact islets with insulin-positive cells (brown stain) are also detected. In contrast, grafts obtained from mice treated with rapamycin contain very few intact islets. Scale bar represents 100 μm.
ing xenografts resulted in rapid return to hyperglycemia, indicating that the maintenance of euglycemia in NPI recipients was xenograft dependent and not due to recovery of the endogenous pancreatic endocrine function. Immunohistochemical staining of these grafts showed intact islets stained positive for insulin (Fig. 2B). Some islets were infiltrated with mononuclear cells consisting of CD4, CD8 T-cells, and macrophages (data not shown), and some islets were surrounded but not infiltrated with these cells. Taken together, these data show that single therapy of anti–LFA-1, anti-CD154, or anti-CD45RB monoclonal antibody can induce long-term xenograft survival in at least a proportion of wild-type B6 recipients. **Combination of anti–LFA-1 plus anti-CD154 or anti-CD45RB monoclonal antibodies induce long-term islet xenograft acceptance in B6 mice.** A: Mice treated with a combination of anti–LFA-1 with either anti-CD154 or anti-CD45RB monoclonal antibodies showed normalization of the blood glucose levels within 8 weeks posttransplantation. B: Twelve of 14 mice treated with a combination of anti–LFA-1 and anti-CD154 monoclonal antibodies and 8 of 12 mice treated with a combination of anti–LFA-1 and anti-CD45RB monoclonal antibodies maintained normal blood glucose levels until the end of the study (>100 days posttransplantation). Histological examination of the islet grafts showed numerous intact islets that contain insulin granules (brown stain) and surrounded but not infiltrated with mononuclear cells. In contrast, only 3 of 10 mice that received a combination of anti–LFA-1 and rapamycin achieved normoglycemia, and these mice rejected their grafts before 100 days posttransplantation. Grafts obtained from mice treated with a combination of rapamycin and anti–LFA-1 contained few intact islets that stained positive for insulin, and a majority of the islets have been destroyed by infiltrating mononuclear cells. Scale bar represents 100 μm.

**FIG. 3.** Combination of anti–LFA-1 with anti-CD154 or anti-CD45RB monoclonal antibodies induce long-term islet xenograft acceptance in B6 mice. A: Mice treated with a combination of anti–LFA-1 with either anti-CD154 or anti-CD45RB monoclonal antibodies showed normalization of the blood glucose levels within 8 weeks posttransplantation. B: Twelve of 14 mice treated with a combination of anti–LFA-1 and anti-CD154 monoclonal antibodies and 8 of 12 mice treated with a combination of anti–LFA-1 and anti-CD45RB monoclonal antibodies maintained normal blood glucose levels until the end of the study (>100 days posttransplantation). Histological examination of the islet grafts showed numerous intact islets that contain insulin granules (brown stain) and surrounded but not infiltrated with mononuclear cells. In contrast, only 3 of 10 mice that received a combination of anti–LFA-1 and rapamycin achieved normoglycemia, and these mice rejected their grafts before 100 days posttransplantation. Grafts obtained from mice treated with a combination of rapamycin and anti–LFA-1 contained few intact islets that stained positive for insulin, and a majority of the islets have been destroyed by infiltrating mononuclear cells. Scale bar represents 100 μm.
porcine xenograft survival that was comparable to that found previously with allograft studies (30). Combined anti–LFA-1 plus anti-CD45RB therapy was somewhat less effective but nevertheless resulted in two-thirds (8 of 12) of the NPI xenografts achieving long-term survival (Table 1; Fig. 3A). Histological examination of porcine xenografts from recipients treated with anti–LFA-1/anti-CD154 combined therapy revealed extensive insulin-staining, intact islets that were largely devoid of detectable mononuclear cell (Fig. 4). A few islets from long-term functioning xenografts demonstrated noninvasive, peri-islet mononuclear cell accumulations (Fig. 3B and Fig. 4). In contrast, xenografts from mice treated with a combination of rapamycin and anti–LFA-1 showed fewer intact islets, and the majority of these islets demonstrated pronounced infiltration with mononuclear cells. Taken together, our data demonstrate that transient administration of a combination of anti–LFA-1 with anti-CD154 or anti-CD45RB monoclonal antibodies can be highly efficacious in preventing the rejection of NPI xenografts in B6 mice.

**Combined monoclonal antibody therapy inhibits production of mouse anti-porcine antibodies.** Since significant prolongation of porcine xenograft survival was observed using a combined therapy of anti–LFA-1 with anti-CD154 or anti-CD45RB monoclonal antibodies, we examined the effect of these therapies on the production of mouse anti-porcine antibodies to porcine cells in vitro. We had previously found that anti–LFA-1 therapy profoundly inhibited both CD4 and CD8 alloreactivity in vitro, while anti-CD154 treatment led to modest inhibition of CD4 T-cells but not CD8 T-cells (30). Anti-CD45RB treatment did not inhibit T-cell reactivity to allogeneic antigen-presenting cells (APCs) (2). Thus, studying the impact of monoclonal antibody treatment on mouse anti-porcine T-cell reactivity was not considered informative. Alternatively, as an assessment of anti-donor reactivity, we chose to study anti-porcine antibody production rather than direct T-cells, since T-cell–dependent antibody responses are a consequence of “indirect” (host APC dependent) reactivity. When porcine spleen cells were incubated with sera from untreated mice with rejected islet xenografts, >90% of porcine cells were bound with xenoreactive mouse IgG antibodies (Fig. 5B). Similarly, sera from treated B6 mice, bearing long-term (>100 days) islet xenografts but eventually rejecting the NPI xenografts, demonstrated similar levels of anti-porcine antibody production (data not shown). In contrast, mice that were treated with combined monoclonal antibody therapies and having long-term islet xenograft survival (>100 days) displayed greatly reduced levels of anti-porcine IgG, comparable to levels found in naïve control (nontransplanted) B6 mice (Fig. 5A,C,D). These data indicate that combination of anti–LFA-1 with either anti-CD154 or anti-CD45RB monoclonal antibodies can result in long-term anti-porcine antibody hyporesponsiveness.

**DISCUSSION**

The importance of LFA-1 for islet xenograft rejection has been implicated by previous studies. For instance, pre-treatment of human islets with anti-ICAM monoclonal antibody resulted in significant prolongation of human islet xenografts in diabetic B6 mice (32). Our pilot study with anti–LFA-1 monoclonal antibody therapy also showed long-term acceptance of rat islet xenografts in BALB/c mice, and long-term survival of these xenografts required the presence of CD4 but not CD8 T-cells during the peritransplant period (35). Furthermore, the efficacy of monoclonal antibodies specific for certain molecules on the surface of T-cells can be greatly enhanced when combined with other monoclonal antibodies that act on distinct pathways of T-cell activation and function (21,30). In the present study, we demonstrate that a combination of anti–LFA-1 monoclonal antibody with either anti-CD154
or anti-CD45RB monoclonal antibody promotes robust, long-term survival of NPI xenografts in immune-competent B6 mice relative to individual single therapies. As such, combined anti–LFA-1/anti-CD154 treatment, which was highly effective for promoting islet allograft survival and tolerance in our previous studies (30), is nearly as effective for promoting NPI xenograft as well. We do not yet know if this approach results in a similar form of transplantation tolerance found in an islet allograft model (30). However, this short-term therapy results in long-term reduction in anti-donor antibody production, suggesting that an altered response to the NPI xenograft has occurred.

It is important to note that treatment with anti-CD154 alone showed at least partial efficacy for promoting long-term porcine islet xenograft survival, as recently shown by others (14). The interaction of CD154 with CD40 has been demonstrated to be critical in upregulating the antigen-presenting capacity of APCs, which in turn enhance the ability of these molecules to provide costimulatory signals required for CD4-dependent immune responses (36). Assuming that the CD4 response to xenografts is destructive and since we (2,37,38) and others (39,40) have previously demonstrated that islet xenograft rejection was mainly dependent on CD4 T-cells, it is probable that treatment of recipients with anti-CD154 monoclonal antibody may interfere with the interaction between CD154 expressed on xenoreactive CD4 T-cells and CD154 on APCs, resulting in long-term acceptance of porcine islet xenografts. Moreover, it has been shown that interaction between CD154 and anti-CD154 monoclonal antibody results in the downregulation of class II major histocompatibility complex (MHC) expression, B7-costimulatory receptors, adhesion molecules, and cytokine production (41) that may also contribute to the protective effect of this therapy. It remains unclear why anti–LFA-1/anti-CD154 combined treatment shows such striking efficacy for both islet allograft and xenograft survival.

Alternatively, results show that a combination of anti–LFA-1 and anti-CD45RB monoclonal antibody is also highly efficacious in preventing the rejection of NPI xenografts. The mechanism of protection by this therapy is not known; however, it has been reported that the antibody to CD45 causes a change in CD45 isoforms. The change in CD45 isoforms differentially regulates tyrosine phosphorylation of particular signaling intermediates, including VAV and SLP-76 as well as the secretion of interleukin-2 (42–44). Treatment with therapeutic anti-CD45RB antibody results in downregulation of high molecular weight isoforms and upregulation of the low molecular weight CD45RO isoform in vivo (20). It has also been shown that this change causes a shift in the functional repertoire of responding T-cells, which skews the immune response toward tolerance (20). How anti-CD45RB therapy specifically results in islet xenograft prolongation and why this approach is highly effective when combined with anti–LFA-1 therapy will be essential to determine in future studies.

A potential common feature of therapies effective for promoting xenograft prolongation, such as those described in the present study, is the ability to inhibit “indirect” (host APC dependent) T-cell reactivity. Our previous studies indicate that NPI xenograft rejection is especially dependent on CD4 T-cell–dependent antigen recognition in association with host MHC class II molecules (2). Unlike allograft rejection that is independent of

FIG. 5. Combination of anti–LFA-1 with anti-CD154 or anti-CD45RB inhibits the production of anti-porcine antibodies in B6 mice. Neonatal porcine spleen cells (1 × 10⁶) from islet donor pigs were incubated with serum (1/128 dilution) collected from nontransplanted (naïve) mice (A), transplanted but untreated mice (B), or mice treated with combined monoclonal antibody therapy that showed long-term graft acceptance (C and D). Binding of mouse anti-porcine IgG (--) was measured and analyzed by flow cytometry. Controls for this experiment consisted of unstained (---) and secondary antibody without serum (...).
host MHC class II expression, porcine xenograft rejection was found to be entirely dependent on this indirect pathway (2). Importantly, T-cell–dependent antibody responses to allografts or xenografts are presumptive evidence of this “indirect” antigen recognition since T-B–cell collaboration requires interaction of helper T-cells, with antigens acquired and presented by the specific B-cell (45). We have frequently found that islet xenografts demonstrate an exaggerated antibody response relative to allografted animals, suggestive of strong indirect T-cell reactivity in vivo (R.G.G. et al., unpublished observations). A significant result from this study is that combined monoclonal antibody therapy can result in long-term inhibition of the humoral immune response to NPI xenografts. The levels of mouse anti-porcine IgG detected in recipients that had long-term graft survival was markedly reduced compared with the levels detected in recipients that rejected the islet xenografts. Thus, the relative anti-donor antibody hyporeactivity suggests that the therapies used are effective for preventing indirect antigen responses involved in xenograft immunity. It is unclear whether the inhibition of B-cell reactivity observed is due to a proximal effect on T-B–cells collaboration and/or is due to a perturbation of other initial CD4-APC interactions. The precise nature of altered xenograft recognition observed in these studies will require further study.

In conclusion, our data indicate that a short-course treatment with a combination of anti-LFA-1 monoclonal antibody with anti-CD154 or anti-CD45RB monoclonal antibody is quite effective in promoting the long-term survival of NPI xenografts in mice. The protection induced by the combination of monoclonal antibody therapy is associated with reduced cellular infiltration of the islet grafts as well as decreased levels of mouse anti-porcine IgG antibodies. This study also supports the importance of the interaction between LFA-1, CD154, and CD45RB and their ligands in the rejection of NPI xenografts. Importantly, therapies that are effective in promoting islet allograft survival and tolerance can show similar efficacy in promoting xenograft survival. It is unclear whether similar results can be achieved in large animal models in which vigorous innate immune responses and/or preexisting xenoreactive natural antibodies are likely to comprise a more stringent barrier to xenograft survival (45). However, our results indicate that strategic simultaneous targeting of differing immune pathways can be highly efficacious in preventing the rejection of NPI xenografts and that such approaches may form an important future component of therapeutic regimens applied in clinical islet xenotransplantation.

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REFERENCES


