Transient B-Cell Depletion Combined With Apoptotic Donor Splenocytes Induces Xeno-Specific T- and B-Cell Tolerance to Islet Xenografts

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Peritransplant infusion of apoptotic donor splenocytes cross-linked with ethylene carbodiimide (ECDI-SPs) has been demonstrated to effectively induce allogeneic donor-specific tolerance. The objective of the current study is to determine the effectiveness and additional requirements for tolerance induction for xenogeneic islet transplantation using donor ECDI-SPs. In a rat-to-mouse xenogeneic islet transplant model, we show that rat ECDI-SPs alone significantly prolonged islet xenograft survival but failed to induce tolerance. In contrast to allogeneic donor ECDI-SPs, xenogeneic donor ECDI-SPs induced production of xenodonor-specific antibodies partially responsible for the eventual islet xenograft rejection. Consequently, depletion of B cells prior to infusions of rat ECDI-SPs effectively prevented such antibody production and led to the indefinite survival of rat islet xenografts. In addition to controlling antibody responses, transient B-cell depletion combined with ECDI-SPs synergistically suppressed xenodonor-specific T-cell priming as well as memory T-cell generation. Reciprocally, after initial depletion, the recovered B cells in long-term tolerized mice exhibited xenodonor-specific hyporesponsiveness. We conclude that transient B-cell depletion combined with donor ECDI-SPs is a robust strategy for induction of xenodonor-specific T- and B-cell tolerance. This combinatorial therapy may be a promising strategy for tolerance induction for clinical xenogeneic islet transplantation. *Diabetes* 62:3143–3150, 2013

Pancreatic islet transplantation is a promising treatment option for type 1 diabetes (1). However, a major limitation to its widespread clinical application is the shortage of human donor pancreata (2,3). Xenogeneic sources of islets are an attractive alternative. Currently, porcine islets are considered the best suitable substitute for human transplantation because of the unlimited donor source and their functional compatibility in humans (4). Moreover, they may be resistant to recurrent autoimmunity that is potentially present in recipients of islet transplantation (5,6). Unfortunately, the need for aggressive immunosuppression to control xenogeneic rejection is currently prohibitive for its application as a standard therapy for β-cell replacement in humans (7,8). Therefore, effective tolerance strategies for xenogeneic transplantation are urgently needed.

Early studies in xenogeneic transplant models point to a critical role of T-cell–mediated processes in xenograft rejection (7–10). However, B cells are increasingly recognized for their role in xenogeneic immunity (11,12). In addition to mediating humoral responses by differentiating into antibody-producing plasma cells, B cells have also been shown to influence T-cell priming, expansion, and differentiation through a variety of mechanisms, including antigen presentation, costimulation, and cytokine production (13–16). Consequently, B-cell deficiency or depletion ameliorates autoimmune diseases, including type 1 diabetes, multiple sclerosis, and rheumatoid or collagen-induced arthritis (17–19). Likewise, B-cell depletion has been demonstrated to prolong allogeneic and xenogeneic graft survival in nonhuman primates (12,20).

We have previously shown that intravenous infusion of donor splenocytes cross-linked with ethylene carbodiimide (ECDI-SPs) induces donor-specific tolerance to allogeneic islet and heart grafts (21–23), and the mechanisms of graft protection in these models involve deletion, anergy, and regulation of T cells of direct and indirect allo-specificities (24).

In the current study, we tested donor ECDI-SPs in a concordant (rat-to-mouse) xenogeneic islet transplant model. We show that although ECDI-SPs alone significantly prolong islet xenograft survival, additional transient B-cell depletion is required to promote xenogeneic tolerance and indefinite islet xenograft survival. Furthermore, transient B-cell depletion significantly impairs xenogeneic T-cell priming and memory T-cell generation. Reciprocally, during B-cell reconstitution after transient B-cell depletion, the recovered B cells exhibit xenoantigen-specific unresponsiveness in the long-term tolerized hosts. Collectively, our findings establish a novel and effective tolerance therapy for xenogeneic islet transplantation and underscore the critical role of B-cell depletion in this process.

RESEARCH DESIGN AND METHODS

Animals and induction of diabetes. Male C57BL/6 (B6) mice (7–10 weeks old) were from The Jackson Laboratory (Bar Harbor, ME). Male Lewis rats and Wistar-Furth rats (7–10 weeks old) were from Harlan (Indianapolis, IN). B6 mice were rendered diabetic by an intraperitoneal injection of 200 mg/kg streptozotocin (Sigma). Diabetes was confirmed by a blood glucose concentration >250 mg/dL on 2 consecutive days. All studies were approved by Northwestern University Animal Care and Use Committee.

Islet isolation and transplantation. Lewis rat islets were isolated by a mechanically enhanced enzymatic digestion using collagenase (Roche). After filtration through a mesh screen, the filtrate was applied to a discontinuous Ficoll (Sigma) gradient. Islets were handpicked, washed, and counted. A total of 550 rat islets were transplanted under the kidney capsule of diabetic mice. Rejection was diagnosed when the blood glucose concentration was >250 mg/dL for at least 2 consecutive days.

Tolerance therapies and serum adoptive transfer. Two hundred fifty micrograms of anti-mouse CD20 monoclonal antibody (mAb; clone 5D2; Genentech) were administered on days −10 and 1, with day 0 being the day of rat islet transplantation. ECDI-treated donor SPs were prepared as described (21). Briefly, spleens from Lewis rats were processed into single-cell suspensions. Erythrocytes were lysed, and SPs were incubated with ECDI (150 mg/mL for every 3.2×10^8 cells; Calbiochem) on ice for 1 h with agitation followed by washing. A total of 1×10^8 ECDI-treated SPs in 200 μL PBS were injected on days −7 and 1. For the serum transfer experiment, serum was obtained from donor mice 14 days after injection with rat ECDI-SPs, and the presence of anti-rat antibodies was confirmed by fluorescence-activated cell sorter (FACS). Three hundred microliters of serum per mouse were transferred on the day of transplantation of rat islets.

Antibodies and FACS analysis. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 and anti-CD4, anti-CD8, and anti-CD69; phycoerythrin (PE)-conjugated anti-mouse B220 and anti-rat B220; PerCP (Peridinin Chlorophyll Protein Complex)-conjugated anti-CD4 and anti-CD19; and allophycocyanin (APC)-conjugated anti-CD62L mAbs were from BD Biosciences. PE-conjugated anti–interferon-γ (IFN-γ), anti-CD44, and APC-conjugated anti-mouse CD86, CD80, CD40, and OX40L were from eBioscience.

Mouse anti-rat or anti-pig antibody measurement. Rat or pig SPs were incubated with recipient mouse serum on ice for 1 h and washed. For anti-rat antibodies, rat SPs were further stained with PE-conjugated anti-rat–B220 mAb and FITC-conjugated anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 mAbs and were analyzed by FACS gating on rat B220− cells. For anti-pig antibodies, pig SPs were further stained with FITC-conjugated anti-mouse IgG1, IgG2a, IgG2b, or IgG3 mAbs and were analyzed by FACS gating on live cells. Serum from naive C57L/B6 was used as negative control.

Mixed lymphocytic reactions and delayed-type hypersensitivity. Responder cells were whole SPs from B6 recipients labeled with 0.5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE). This will allow assessment of both direct and indirect anti-xeno T-cell reactivity. Stimulator cells were Lewis rat SPs. A total of 2×10^5 responder cells were cultured in a 96-well plate with stimulator cells at a responder cell: stimulator cell ratio of 1:2 for 3–6 days. Cells were harvested on indicated days and stained with APC-conjugated anti-CD4 or anti-CD8, and PE-conjugated IFN-γ. Cell proliferation was quantified by CFSE dilution. Delayed-type hypersensitivity (DTH) experiments were performed as previously described (21). Briefly, the ear thickness of mice in various treatment groups was measured with a Mitutoyo engineer's micrometer (Schlesinger's Tools, Brooklyn, NY) at baseline (on day 7) immediately prior to injection of a total of 10^7 rat SP lysate in 10 μL PBS into the dorsal surface of the ear. Twenty-four hours later (on day 8), the increase in ear thickness over prechallenge baseline was determined.

Histology. Frozen sections of islet grafts were blocked with 10% donkey serum (Sigma-Aldrich). Sections were stained with rabbit anti-mouse insulin polyclonal antibody (1:200, rabbit IgG, H-86; Santa Cruz Biotechnology), rat anti-mouse Foxp3 mAb (1:400, rat IgG2a, κ clone FJK-16s; eBioscience), rat anti-mouse C4d mAb (1:100, rat IgG2a, clone 16D2; Abcam), and rat anti-mouse B220 mAb (1:250, rat IgG2a κ, clone RA3–6B2; eBioscience). For visualization of insulin, donkey anti-rabbit DyLight 488 (1:400; Jackson ImmunoResearch) was used. For visualization of Foxp3 and C4d, donkey anti-rat DyLight 549 (1:400; Jackson ImmunoResearch) was used. For visualization of B220, donkey anti-rat DyLight 594 (1:500; Jackson ImmunoResearch) was used. DAPI staining was concurrently performed. Negative controls were performed by eliminating primary antibodies during staining processes. Images were visualized using Leica DM5000 B microscope, acquired with a QImaging Retiga 4000r camera, and analyzed with Image Pro 6.2 software.

Statistical analysis. Data are expressed as the mean ± SEM. Kaplan-Meier analysis was used for graft survival analysis, and a log-rank test was used to compare survival among groups. Column statistics were performed by Student t test. A P value <0.05 was considered to be statistically significant. All analyses were performed with GraphPad Prism 5 software.

RESULTS

Rat ECDI-SP infusions significantly prolong rat islet xenograft survival but fail to provide indefinite protection.

To test whether donor ECDI-SP infusions could protect xenogeneic islet grafts, we used a rat-to-mouse islet transplant model. Diabetic B6 mice were injected intravenously with 1×10^8 ECDI-fixed Lewis rat SPs 7 days before (day −7) and 1 day after (day 1) transplantation with a Lewis rat islet graft on day 0. As shown in Fig. 1, two doses of rat ECDI-SPs significantly prolonged rat islet xenograft survival (mean survival time [MST]: 48 days for treated mice versus 18 days for control mice, $P = 0.0026$). The protection is donor specific, as infusions of ECDI-fixed third-party (Wistar-Furth rat) SPs did not protect Lewis rat islet xenografts (Fig. 1). However, unlike the indefinite graft protection seen in allogeneic islet transplantation (21), protection of xenogeneic islet grafts by donor ECDI-SPs was transient, as all rat islet xenografts were rejected by day 61.

Rat ECDI-SP infusions control anti-rat DTH response but induce production of anti-rat antibodies and graft site C4d deposition.

Correlating with prolonged islet xenograft survival, recipient mice treated with rat ECDI-SPs showed diminished DTH response to rat antigen challenge (Fig. 2A) as previously seen in the allogeneic islet transplant model (21). To determine the potential causes of eventual graft rejection, we next examined whether the infusion of rat ECDI-SPs could induce anti-rat antibodies. Recipient serum was harvested 7 and 14 days after an infusion of rat ECDI-SPs and was tested for the development of anti-rat IgGs. As shown in Fig. 2B, low levels of anti-rat IgG2a, 2b were already detectable as early as 7 days after an infusion of rat ECDI-SPs, and by 14 days IgG1, 2a, 2b, and 3 were all detected at high levels (Fig. 2B), such that antibodies were still detectable in sera at 1:128 serial dilution (Supplementary Fig. 1). This is in sharp contrast to infusions of allogeneic ECDI-SPs, which could not induce allogeneic anti-donor antibodies themselves but in fact were able to suppress anti-donor antibody production induced by islet allografts (21). To determine whether the induced anti-rat antibodies contribute to the rejection of rat islet xenografts in recipients treated with rat ECDI-SPs, we next examined rat islet xenografts for the deposition of the complement degradation product C4d. As shown in Fig. 2C, robust C4d deposition was observed in day 14 and day 28 rat islet xenografts from recipients treated with rat ECDI-SPs, indicating that the induced anti-rat antibodies are likely pathogenic.
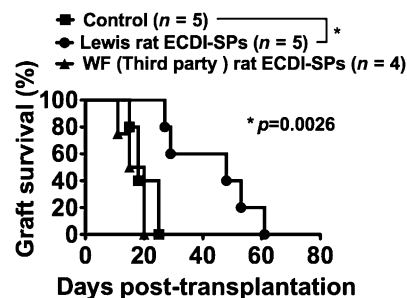

FIG. 1. Rat ECDI-SP infusions significantly prolong rat islet xenograft survival but fail to provide indefinite islet xenograft protection. Lewis rat SPs were treated with ECDI, as detailed in RESEARCH DESIGN AND METHODS, and 10^8 Lewis rat ECDI-SPs were injected into diabetic B6 recipients on days −7 and 1 relative to the transplantation of Lewis rat islets on day 0. Wistar-Furth (WF) rats were used as a third party to demonstrate donor specificity. The rejection of an islet xenograft was determined by a blood glucose level of >250 mg/dL on two consecutive days. The y-axis shows the percentage of graft survival, which was calculated as the number of surviving grafts (not yet rejected as defined above) divided by the number of total grafts (shown as the "n" for each group in the graph).
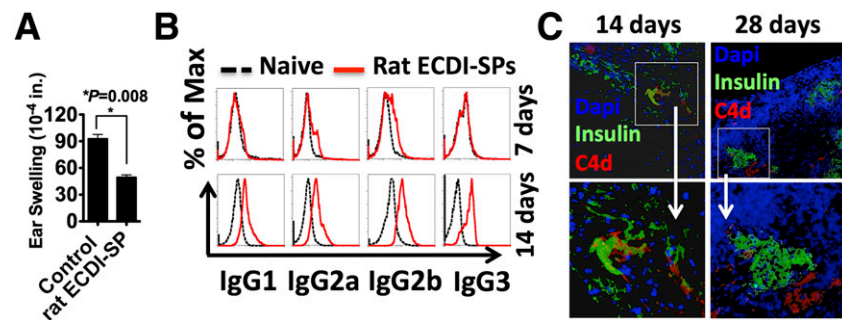
FIG. 2. Rat ECDI-SP infusions induce production of anti-rat antibodies and graft site C4d deposition. *A*: Mice treated with rat ECDI-SPs showed a diminished DTH response to rat antigen challenge compared with untreated controls. *B*: Anti-rat IgG1, IgG2a, IgG2b, and IgG3 levels were measured in the serum of mice 7 and 14 days after an infusion of rat ECDI-SPs. The data shown are representative of at least eight mouse serum samples from recipients treated with rat ECDI-SPs. *C*: Triple-immunofluorescent staining with insulin, C4d, and DAPI of a day 14 and a day 28 rat islet xenograft from recipients treated with rat ECDI-SPs. Original magnification ×10. Histology is representative of two rat islet xenografts obtained and sectioned from two recipients treated with rat ECDI-SPs at each time point. Max, maximum.

Rat ECDI-SPs induce B-cell activation and graft site B-cell infiltration. Our previous studies of donor ECDI-SPs in allogeneic transplant models indicate that the injected donor ECDI-SPs are rapidly internalized by recipient splenic marginal zone dendritic cells, macrophages, and B cells (24). Given the above result of anti-rat antibody production, we next examined the effect of rat ECDI-SPs on recipient splenic B cells internalizing the injected rat ECDI-SPs. Rat ECDI-SPs were labeled with PKH-67 prior to injection into B6 mice. Seventy-two hours later, spleens were harvested and splenic CD19$^+$B220$^+$ B cells were examined for markers of activation. As shown in Fig. 3*A*, at 72 h postinjection, the CD19$^+$B220$^+$PHK-67$^+$ B cells (B cells that had internalized the injected rat ECDI-SPs) showed significantly upregulated expression of costimulatory molecules CD80, CD86, CD40, and OX40L compared with the CD19$^+$B220$^+$PHK-67$^-$ B cells (B cells that had not internalized the injected rat ECDI-SPs). Further supporting an important role of B cells in the rejection of rat islet xenografts in recipients treated with rat ECDI-SPs, islet xenografts retrieved at 2 weeks post-transplantation from these recipients showed a significant number of B220$^+$ graft-infiltrating B cells, and this infiltration persisted at 4 weeks post-transplantation preceding the eventual rejection of these grafts (Fig. 3*B*). Similar B220$^+$ graft-infiltrating B cells were also observed in control, untreated islet xenografts at week 2 prior to their rejection (Fig. 3*B*). Collectively, these findings suggest that B cells can be activated by infusions of rat ECDI-SPs and may consequently play a prominent role in the rejection of rat islet xenografts in recipients treated with rat ECDI-SPs.

B-cell depletion synergizes with rat ECDI-SP infusions to promote indefinite rat islet xenograft survival. Given the above findings, we reasoned that the depletion of B cells in combination with rat ECDI-SPs could further enhance graft protection. An anti-murine CD20 antibody was used to deplete circulating and splenic B cells (25). As shown in Fig. 4*A*, B-cell depletion prior to rat ECDI-SP infusion completely abolished the production of anti-rat antibodies of all Ig subclasses at 14 days after an infusion of rat ECDI-SPs. Furthermore, combination of B-cell depletion with rat ECDI-SPs allowed indefinite survival of the transplanted rat islet xenografts (100% graft survival >100 days, Fig. 4*B*). Of note, anti-CD20 treatment alone also significantly prolonged rat islet xenograft survival (Fig. 4*B*), suggesting a role of B cells in the process of xenogeneic rejection beyond that of mediating antibody production (see

below). Consistent with superior graft survival, protected islet xenografts (at 4 weeks post-transplant) in recipients treated with anti-CD20 plus rat ECDI-SPs showed robust insulin staining with minimal infiltration with B220$^+$ cells and negative staining for C4d (Fig. 4*C*). Of note, regulatory T cells expressing Foxp3 were abundantly detected in the protected islet xenografts at 4 weeks (Supplementary Fig. 2), indicating that this cell population may play a role in the long-term protection of islet xenografts.

To further confirm that the anti-rat antibodies induced by rat ECDI-SPs are detrimental to rat islet xenograft survival, we performed a serum-adoptive transfer experiment.
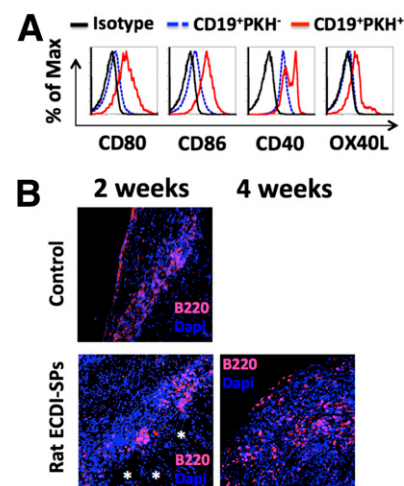

FIG. 3. Rat ECDI-SPs induce B-cell activation and graft site B-cell infiltration. *A*: Rat ECDI-SPs were labeled with PKH-67 prior to injection. At 72 h postinjection, spleens were harvested and prepared into single-cell suspensions. SPs were stained with CD19, B220, and respective antibodies shown and were analyzed by FACS. B cells that had internalized rat ECDI-SPs were identified as CD19$^+$B220$^+$PKH-67$^+$ cells, whereas B cells that had not were identified as CD19$^+$B220$^+$PKH-67$^-$ cells. Histograms show comparisons of the expression of selected markers between the CD19$^+$B220$^+$PKH-67$^-$ cells and the CD19$^+$B220$^+$PKH-67$^+$ cells. *B*: Rat islet xenografts were retrieved from recipients treated with rat ECDI-SPs at 2 and 4 weeks post-transplantation or control untreated recipients at 2 weeks post-transplantation, sectioned, and stained with B220 and DAPI. Asterisks (*) mark discernable islets in the 2-week islet graft from a recipient treated with rat ECDI-SPs. Original magnification ×10. Histology shown is representative of three rat islet xenografts obtained and sectioned from three recipients treated with rat ECDI-SPs at each time point. Max, maximum.
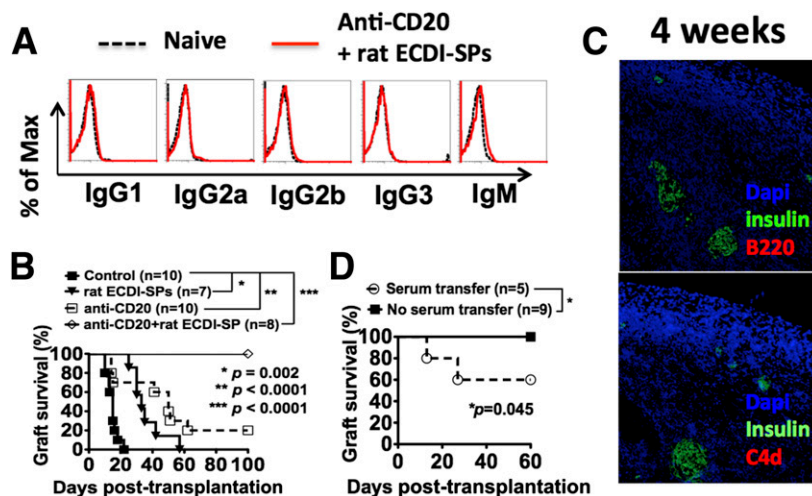
FIG. 4. B-cell depletion synergizes with rat ECDI-SP infusions to allow indefinite rat islet xenograft survival. *A*: B-cell depletion prior to rat ECDI-SP infusion completely abolished the production of anti-rat antibodies in all Ig subclasses previously seen induced by rat ECDI-SPs 14 days after an infusion of rat ECDI-SPs. *B*: Rat islet xenograft survival in recipients treated with rat ECDI-SPs alone, anti-CD20 alone, or anti-CD20 plus rat ECDI-SPs. Untreated mice were used as controls. *C*: Rat islet xenografts were retrieved from recipients treated with anti-CD20 plus rat ECDI-SPs at 4 weeks post-transplantation, sectioned, and stained with indicated antibodies. Original magnification ×10. Histology shown is representative of three rat islet xenografts obtained and sectioned from three recipients treated with anti-CD20 plus rat ECDI-SPs. *D*: 300 μL of serum (taken from mice 2 weeks after rat ECDI-SP infusion and with confirmed anti-rat antibodies) was injected intravenously on the day of rat islet transplantation in recipients treated with anti-CD20 plus rat ECDI-SPs. Graft outcome was compared with those in recipients treated with anti-CD20 plus rat ECDI-SPs without serum-adoptive transfers. For *B* and *D*, the *y*-axis shows the percentage of graft survival, which was calculated as the number of surviving grafts (not yet rejected) divided by the number of total grafts (shown as the "*n*" for each group in the graphs). Max, maximum.

Three hundred microliters of serum containing anti-rat antibodies obtained from mice 14 days after the injection of rat ECDI-SPs were injected into recipient mice on the day of rat islet transplantation. As shown in Fig. 4*D*, adoptive transfer of serum containing anti-rat antibodies precipitated islet xenograft rejection in 40% of recipients treated with anti-CD20 plus rat ECDI-SPs in which indefinite islet xenograft survival was otherwise seen, demonstrating that these antibodies are themselves an impairment to tolerance induction.

B-cell depletion combined with rat ECDI-SPs impairs xeno-specific T-cell priming and memory generation. We next examined xenogeneic donor-specific T-cell responses in recipients treated with anti-CD20 plus rat ECDI-SPs. DTH responses to rat antigen challenge were first examined. As shown in Fig. 5*A*, the DTH response to rat antigen challenge was significantly diminished in mice treated with rat ECDI-SPs alone or rat ECDI-SPs plus anti-CD20. T-cell proliferation and effector cytokine production were next examined in in vitro 4-day restimulation assays that allowed the assessment of both direct and indirect anti-xeno T-cell reactivity. As shown in Fig. 5*B*, the proliferation of both CD4 and CD8 T cells to donor stimulation was markedly diminished in recipients treated with anti-CD20 or anti-CD20 combined with rat ECDI-SPs. Similarly, donor-stimulated production of effector cytokine IFN-γ was also decreased parallel to the decrease of proliferation (Fig. 5*B*).

To examine the effect of rat ECDI-SPs and/or anti-CD20 on donor-specific memory T-cell generation, we performed three lines of experiments. First, we quantified T cells with a memory phenotype at 90 days after the initial rat islet transplant. As shown in Fig. 6*A*, compared with control mice, mice treated with anti-CD20 alone, rat ECDI-SPs alone, or a combination of anti-CD20 plus rat ECDI-SPs showed a decrease in both the CD4$^+$CD44^{high}CD62L$^-$ effector memory population and the CD4$^+$CD44^{high}CD62L$^+$

central memory population. Second, we performed in vitro restimulation assays using T cells isolated from recipients 90 days after the initial rat islet transplants, and measured T-cell proliferation and IFN-γ production in a short 3-day stimulation. As shown in Fig. 6*B*, the proliferation of both CD4 and CD8 T cells to donor stimulation was markedly diminished in recipients treated with anti-CD20 alone, rat ECDI-SPs alone, or a combination of anti-CD20 plus rat ECDI-SPs compared with that seen in control mice. Similarly, donor-stimulated IFN-γ production was also decreased parallel to the decrease of proliferation. Third, we performed a second rat islet graft transplant on day 90 after the initial rat islet transplants in these four groups and monitored graft rejection kinetics. As shown in Fig. 6*C*, the second rat islet xenografts were rejected in an accelerated fashion in control mice (MST: 5 days). The second rat islet graft survival was significantly prolonged in recipients previously treated with anti-CD20 alone or rat ECDI-SPs alone (MST: 19.5 and 10 days, respectively). Remarkably, the second rat islet graft was not rejected in recipients previously treated with a combination of anti-CD20 plus rat ECDI-SPs, suggesting that tolerance was indeed induced in these recipients.

Long-term tolerized recipients exhibit xenodonor-specific B-cell unresponsiveness. After the initial anti-CD20 treatment, B cells gradually repopulated the host, and by day 50 post-treatment the number of peripheral B cells returned to baseline levels (data not shown). We next examined whether the B cells repopulating the tolerized recipients were able to respond to rat ECDI-SPs by producing anti-rat antibodies as they do in naive mice (Fig. 2*B*). Recipients initially tolerized with anti-CD20 plus rat ECDI-SPs were rechallenged with intravenous injection of 1×10^8 rat ECDI-SPs >300 days after the initial rat islet transplant, and anti-rat antibodies were measured 8 weeks later. In contrast to naive mice, long-term tolerized recipients were not able to produce anti-rat antibodies when
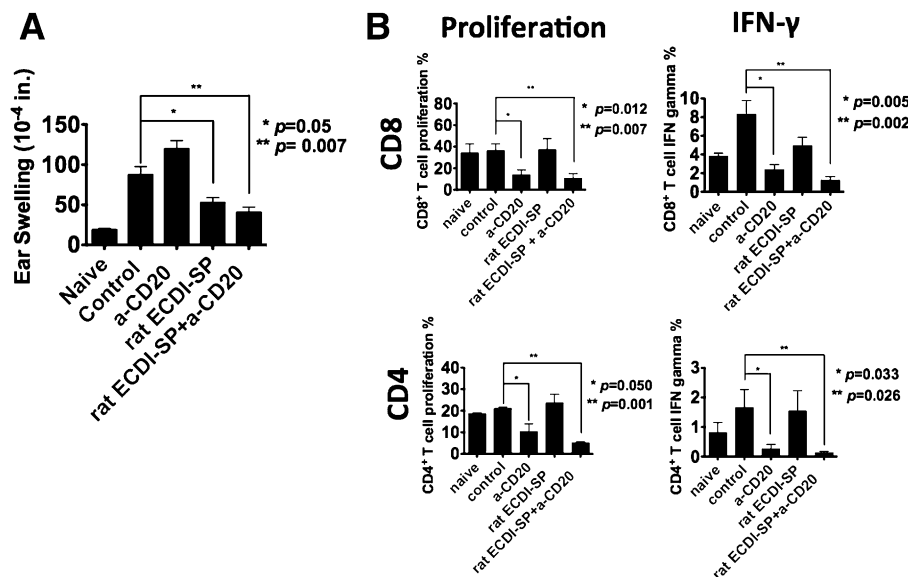
FIG. 5. B-cell depletion combined with rat ECDI-SPs impairs xeno-specific T-cell priming. *A*: DTH responses were measured in mice of various treatment groups as indicated and compared with that of naive unmanipulated mice as described in RESEARCH DESIGN AND METHODS. "Control" was the group of mice receiving rat islet grafts without any pretreatment. *B*: Mixed lymphocyte reactions were set up as described in RESEARCH DESIGN AND METHODS. Responder cells were obtained from the spleens of mice of various treatment groups at 2 weeks post-transplantation. Naive unmanipulated mice were used as control. Control was the group of mice receiving rat islet grafts without any pretreatment. Cultures were harvested on day 4; stained for CD4, CD8, and IFN-γ; and analyzed by FACS. The percentage of proliferated CD4 or CD8 cells was calculated as follows: $1 - [CFSE^{hi} CD4^+ (\text{or } CD8^+) \text{ cells/total } CD4^+ (\text{or } CD8^+) \text{ cells}]$. The percentage of proliferated IFN-γ+ cells was calculated as follows: IFN-γ+CD4+(or CD8+)/total CD4+(or CD8+) cells. The data shown are the average of two separate experiments with two to three mice in each group for each experiment. *P* values are listed to indicate the statistical significance of differences among the groups compared.

challenged with rat ECDI-SPs (data not shown). These mice were then further challenged with injections of highly immunogenic fresh rat SPs (without ECDI fixation), and, as shown in Fig. 7*A*, no anti-rat antibodies could be detected at 4 weeks postchallenge. However, these mice were fully capable of producing anti-pig antibodies (Fig. 7*B*) when challenged with fresh porcine SPs. Serial serum dilutions showed high titers of such anti-pig antibodies (Supplementary Fig. 3 and data not shown). These findings indicate that the recovered B cells exhibit xenodonor-specific unresponsiveness.

DISCUSSION

Compelling evidence in the literature indicates that T-cell–mediated processes play a critical role in the rejection of islet xenografts (9,26). Consequently, therapies targeting these processes such as costimulation blockade have been shown to provide long-term protection to islet xenografts in mice (27,28). Previous work in our laboratory has shown that donor ECDI-SPs effectively control donor-specific T-cell responses by parallel mechanisms including deletion, anergy, and induction of regulation (24). We therefore postulated that donor ECDI-SPs would be highly efficacious in tolerance induction to islet xenografts. However, our surprising findings of *1*) induction of anti-xenogeneic antibodies by donor ECDI-SPs themselves and *2*) the need for B-cell depletion for synergy with donor ECDI-SPs for tolerance induction to islet xenografts underlie the important and unique roles of B cells in xenogeneic responses, particularly in the setting of using donor-based cell therapy as a negative vaccine for tolerance induction.

Using a rat-to-mouse model, we showed that mouse anti-rat antibodies are induced by the infusion of xenogeneic

rat ECDI-SPs (Fig. 2*B*) and that these antibodies are themselves an impairment to tolerance induction (Fig. 4*D*). This is in stark contrast to allogeneic ECDI-SPs, which are not able to induce allo-specific antibodies and can in fact suppress such antibody production (21). One possibility to account for such a difference may be the T-cell–independent (TI) nature of certain xenogeneic antigens such as species-specific oligosaccharides (29). It is possible that donor ECDI-SP infusions are only able to directly tolerize T-cell responses and subsequent T-cell–dependent B-cell responses. Consequently, TI B-cell responses may escape the tolerogenic effects of donor ECDI-SPs. Another possibility not mutually exclusive of the above is that signaling via B-cell receptors recognizing xenogeneic antigens might be particularly robust (30), which in the absence of effective T-cell tolerance early on leads to B-cell activation and differentiation into antibody-secreting plasma cells. Our data showing rapid B-cell activation after rat ECDI-SP infusion (Fig. 3*A*) support this possibility. However, although xenogeneic anti-donor antibodies in all Ig subclasses are seen to be induced by donor ECDI-SPs, it remains possible that the functionality of such antibodies are different from bona fide preexisting donor-specific antibodies induced by sensitizing events responsible for acute humoral rejection (31,32). This possibility is supported by the slow rejection kinetics seen in animals adoptively transferred with serum containing anti-rat antibodies induced by rat ECDI-SPs (Fig. 4*D*). Definitive elucidation of the ability of donor ECDI-SPs inducing tolerance toward T-cell–dependent versus TI xenogeneic antigens awaits future studies using T-cell–deficient athymic mice as well as model systems for specific xenoantigens such as the α-gal epitope. In addition to the concordant rat-to-mouse model demonstrated here, our preliminary studies in a discordant pig-to-mouse islet transplant model also suggest
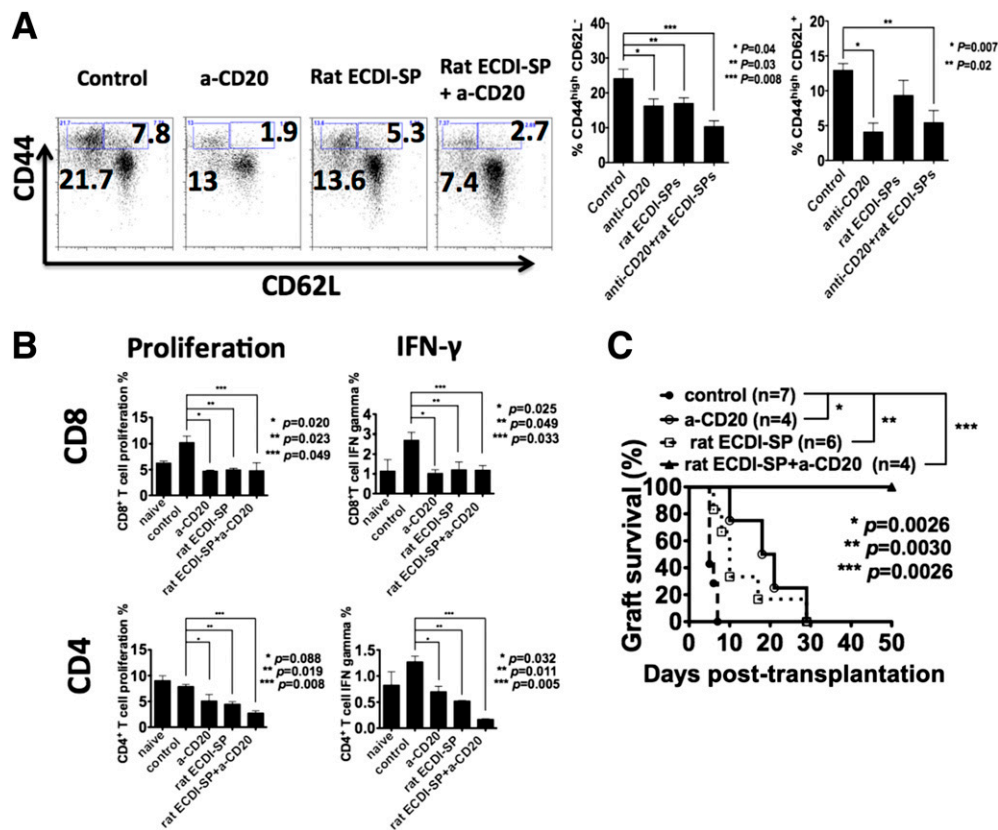
FIG. 6. B-cell depletion combined with rat ECDI-SPs impairs xeno-specific T-cell memory generation. *A*: SPs were obtained from the spleens of mice of various treatment groups at 90 days after rat islet transplantation and were stained for CD4, CD8, CD44, and CD62L. Plots are gated on CD4$^+$ cells. The plots shown are representative of data obtained from at least three mice from each treatment group. Bar graphs on right show the average (with error bars and *P* values) of the effector memory (CD44^{high}CD62L$^-$) and central memory (CD44^{high}CD62L$^+$) T-cell populations for the various treatment groups. *B*: Mixed lymphocyte reactions were set up as described in RESEARCH DESIGN AND METHODS. Responder cells were obtained from the spleens of mice of various treatment groups at 90 days post-transplantation. Naive unmanipulated mice were used as controls. Cultures were harvested on day 3; stained for CD4, CD8, and IFN-γ; and analyzed by FACS. The percentage of proliferated CD4 or CD8 cells and the percentage of IFN-γ$^+$ cells were calculated in a manner similar to that shown in Fig. 5. The data shown are the average of two separate experiments with two to three mice in each group for each experiment. *P* values are listed to indicate the statistical significance of the differences among the groups compared. *C*: Recipients from various treatment groups were transplanted with a second Lewis rat islet xenograft 90 days after the first Lewis rat islet transplantation. For recipients previously treated with anti-CD20 plus rat ECDI-SPs, the first rat islet xenograft was removed by graft nephrectomy on the same day of the second rat islet xenograft transplantation to ensure that subsequent glycemic control in these mice was a manifestation of the second islet xenograft function. For all other groups, mice were maintained on insulin therapy for the time period between the rejection of the first rat islet xenograft and the transplantation of the second rat islet xenograft in order to maintain their metabolic health. Rejection of the second islet xenograft was determined by a blood glucose level of >250 mg/dL on 2 consecutive days.

protective effects of pig ECDI-SPs to the transplanted pig islet xenografts (S. Wang and X. Luo, unpublished observations). However, although mouse models as such provide important insights for designing effective, clinically relevant tolerance strategies, they are nevertheless limited in their suitability for studying the role of natural xeno-reactive antibodies such as those existing in humans. Therefore, our ongoing effort now focuses on using nonhuman primates for testing the efficacy of this tolerance strategy for islet xenografts.

Anatomically, the large number of graft-infiltrating B cells present in the rat islet xenograft prior to and at the time of their rejection (Fig. 3*B*) suggests that these cells may play an important role in the eventual rejection of islet xenografts—more so than their role in the rejection of islet allografts (24,33). We speculate that in close proximity to their cognate antigens, these graft-infiltrating B cells may clonally expand, interact with follicular T cells, undergo isotype switching, and differentiate to antibody-producing plasma cells in situ. In addition, they may further act as

APCs and promote priming and/or survival of intragraft helper T cells and cytotoxic T cells (34). Ongoing research is attempting to elucidate the potential functions of graft-infiltrating B cells in comparison with splenic B cells.

CD4 T cells are thought to be the predominant T-cell subset driving acute xenograft rejection (35,36). Therefore, recipient APCs, including recipient B cells, likely play a critical role in activating xeno-specific CD4 T cells via indirect antigen presentation. In our model, the depletion of B cells significantly diminished CD4 and CD8 T-cell clonal expansion and effector cytokine production to xenogeneic stimulation (Fig. 5*B*), demonstrating the essential role of B cells for optimal xenogeneic T-cell priming in vivo. A similar role of B cells in pathogenic T-cell priming has been demonstrated in several models of autoimmunity (13,15,18). However, their role in the priming of alloreactive T cells appears to be more controversial (16,37,38) and in some cases has been shown to be obligatory for T-cell tolerance via an interleukin-10-dependent pathway (14,39),
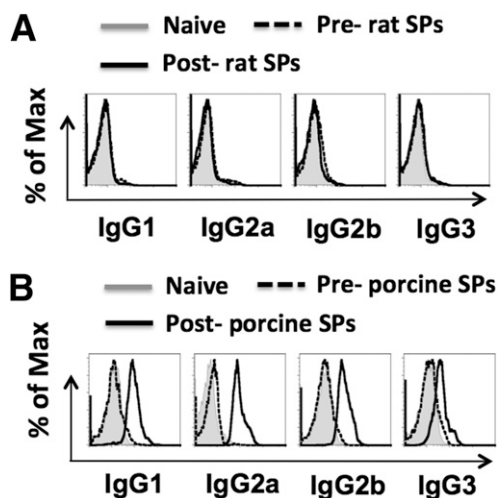
FIG. 7. Long-term tolerized recipients exhibit donor-specific B-cell unresponsiveness. Long-term tolerized recipients previously treated with anti-CD20 plus rat ECDI-SPs were rechallenged with intravenous infusions of 10^7 fresh rat (*A*) or pig (*B*) SPs, and 4 weeks later anti-rat or anti-pig Abs were measured. Max, maximum.

pointing to an important difference between alloreactive and xenoreactive T-cell activation. In addition to the initial priming, our data further demonstrate that T-cell differentiation to memory cells is also impaired in the absence of B cells (Fig. 6*A*–*C*), an effect that synergizes with that of donor ECDI-SP infusions and ultimately leads to tolerance induction to islet xenografts. These data are consistent with previous literature demonstrating the role of B cells in memory T-cell differentiation (37,38).

In long-term tolerized recipients of rat islet xenografts with fully recovered peripheral B cells, not only are rat ECDI-SPs not able to elicit anti-rat antibody responses, but fresh rat SPs, which are otherwise highly sensitizing, are also incapable of eliciting anti-rat antibody responses (Fig. 7*A*), whereas humoral responses to fresh pig SPs are intact (Fig. 7*B*). These findings indicate that initial B-cell depletion plus rat ECDI-SPs can induce xenodonor-specific B-cell tolerance. The mechanism underlying such B-cell tolerance remains to be elucidated. It is possible that after initial anti-CD20 treatment, xenodonor-specific B cells are deleted at the immature B-cell stage during B-cell recovery in the presence of a protected islet xenograft (40). Since ECDI-SPs are highly efficient in tolerizing T-helper cells (21,24), it is also possible that the returning B cells are hyporesponsive because of the lack of T-cell help. It has also been shown that reconstituted B cells after B-cell depletion are functionally less mature (41,42) and that B cells with regulatory capacity are preferentially enriched (43). Other potential mechanisms, including receptor editing, altered levels of coreceptors, and germinal center censoring, may also play a role in mediating the observed xenodonor-specific B-cell tolerance (44–46). Given the role of memory B cells as well as anti-donor antibodies in the impairment of tolerance induction and maintenance (47,48), the observed xenodonor-specific B-cell tolerance in our model is highly encouraging and promising for this strategy to develop into potential clinical applications.

In summary, our studies demonstrate that initial B-cell depletion combined with xenogeneic donor ECDI-SPs is a highly effective strategy for inducing durable tolerance to islet xenografts. To our knowledge, this is the first successful tolerance strategy for xenogeneic islet transplantation that does not require generalized T-cell depletion or costimulation blockade, or bone marrow conditioning regimens for the establishment of bone marrow chimerism, and is therefore safe. In addition, autoantigen-coupled ECDI-SPs have been shown to be highly effective at controlling autoimmunity in models of type 1 diabetes (49,50). Therefore, combining tolerance to both xenoimmunity and recurrent autoimmunity using ECDI-SPs would provide a promising therapeutic option for tolerance induction to islet xenografts in patients with autoimmune type 1 diabetes.

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