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

Shusen Wang,1,2 James Tasch,3 Taba Kheradmand,3 Jodie Ulaszek,3 Sora Ely,3 Xiaomin Zhang,1 Bernhard J. Hering,4 Stephen D. Miller,5 and Xunrong Luo1,3,5

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 the 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 combinatory 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 B-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 xenograft-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. 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 rats 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 (Ab; clone 5D2; Genentech) were administered on days 2, 10, and 1 day 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 transplantation 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 degragation 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.

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 \( \times 10^8 \) 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.

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 10^6 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).
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. 3A, at 72 h postinjection, the CD19+B220+PKH-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+PKH-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. 3B). Similar B220+ graft-infiltrating B cells were also observed in control, untreated islet xenografts at week 2 prior to their rejection (Fig. 3B). 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. 4A, 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. 4B). Of note, anti-CD20 treatment alone also significantly prolonged rat islet xenograft survival (Fig. 4B), 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. 4C). 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-adptive transfer experiment.
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. 4D, 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. 5A, 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. 5B, 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. 5B).

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. 6A, 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<sup>+</sup>CD44<sup>high</sup>CD62L<sup>−</sup> effector memory population and the CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>+</sup> 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. 6B, 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. 6C, 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 xenodonorspecific 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 tolerated recipients were able to respond to rat ECDI-SPs by producing anti-rat antibodies as they do in naive mice (Fig. 2B). Recipients initially tolerized with anti-CD20 plus rat ECDI-SPs were rechallenged with intravenous injection of $1 \times 10^8$ rat ECDILSPs 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...
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. 7A, no anti-rat antibodies could be detected at 4 weeks postchallenge. However, these mice were fully capable of producing anti-pig antibodies (Fig. 7B) 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 underline 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. 2B) and that these antibodies are themselves an impairment to tolerance induction (Fig. 4D). 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. 3A) 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. 4D). 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 xenotoxic antigens such as the c-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...
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. 3B) 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. 5B), 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. 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⁺CD62L⁻) and central memory (CD44⁺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.
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. 6A–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 tolerated 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. 7A), whereas humoral responses to fresh pig SPs are intact (Fig. 7B). 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, auto-antigen-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 xenoinnunity 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.

ACKNOWLEDGMENTS
S.W., B.J.H., S.D.M., and X.L. have received JDRF and Development grant 17-2009-804, T.K. has received JDRF Postdoctoral Fellowship grant 9-2010-447, and X.L. has received National Institutes of Health Directors New Innovator Award DP2 DK083099.

No potential conflicts of interest relevant to this article were reported.
S.W., S.D.M., and X.L. designed the research. S.W., J.T., T.K., J.U., S.E., X.Z., and S.D.M. performed the experiments. S.W., B.J.H., S.D.M., and X.L. analyzed the data. S.W. and X.L. wrote the manuscript. B.J.H. and S.D.M. reviewed and edited the manuscript. X.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank the Northwestern University Interdepartmental Immunobiology Flow Cytometry Core Facility for its support of this work.

REFERENCES
1. Barton PB, Rickels MR, Alejandro R, et al. Improvement in outcomes of clinical islet transplantation: 1999-2010. Diabetes Care 2012;35:1436–1445
2. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230–238
3. Shapiro AM, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355:1318–1330
4. Korbutt GS. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes—chapter 3: pig islet product manufacturing and release testing. Xenotransplantation 2009;16:223–228
5. Koumanda M, Qipo A, Smith RN, Aucincloss H Jr. Pig islet xenografts are resistant to autoimmune destruction by non-obese diabetic recipients after anti-CD4 treatment. Xenotransplantation 2003;10:178–184
6. Guo Z, Wu T, Kirchhof N, et al. Immunotherapy with nondepleting anti-CD4 monoclonal antibodies but not CD28 antagonists protects islet graft in spontaneously diabetic nod mice from autoimmune destruction and allo- geneic and xenogeneic graft rejection. Transplantation 2001;71:1656–1665
7. Hering BJ, Wijkstra M, Graham MI, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat Med 2006;12:301–303
8. Cardona K, Korbutt GS, Milas Z, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat Med 2006;12:304–306
9. Friedman T, Smith RN, Colvin RB, Iaconini J. A critical role for human CD4+ T-cells in rejection of porcine islet cell xenografts. Diabetes 1999;48: 2340–2348
10. Mai G, Burcher P, Morel P, et al. Anti-CD154 mAb treatment but not recipient CD154 deficiency leads to long-term survival of xenogeneic islet grafts. Am J Transplant 2005;5:1021–1031
11. Wang H, DeVries ME, Deng S, et al. The axis of interleukin 12 and gamma interferon regulates acute vascular xenogeneic rejection. Nat Med 2000;6: 549–555
12. Muthukumaran MM, Corcoran PC, Singh AK, et al. B-cell depletion extends the survival of GTKO.bCD46Tg pig heart xenografts in baboons for up to 8 months. Am J Transplant 2012;12:763–771
13. Gavanescu I, Benoist C, Mathis D. B cells are required for Aire-deficient mice to develop multi-organ autoinflammation: a therapeutic approach for APECED patients. Proc Natl Acad Sci USA 2008;105:13000–13014
14. Ding Q, Yeung M, Camirand G, et al. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. J Clin Invest 2011;121:3645–3656
15. Crawford A, Macleod M, Schumacher T, Corlett L, Gray D. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. J Immunol 2006;176:3488–3506
16. Noorchashm H, Reed AJ, Rostami SY, et al. B cell-mediated antigen presentation is required for the pathogenesis of acute cardiac allograft rejection. J Immunol 2006;177:7715–7722
17. Hu CY, Rodriguez-Pinto D, Du W, et al. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest 2011;117:3857–3867
18. Bouaziz JD, Yanaba K, Venturi GM, et al. Therapeutic B cell depletion via rituximab in relapsing-remitting multiple sclerosis. N Engl J Med 2008;358:670–688
19. Liu C, Noorchashm H, Sutter JA, et al. B lymphocyte-directed immunotherapy promotes long-term islet allograft survival in nonhuman primates. Nat Med 2007;13:1295–1298
20. Lao X, Pothoven KL, McCarthy D, et al. ECDI-fixed allogeneic splenocytes induce donor-specific tolerance for long-term survival of islet transplants via two distinct mechanisms. Proc Natl Acad Sci USA 2008;105:14527–14532
21. Kheradmand T, Wang S, Gibby RF, et al. Permanent protection of PLG scaffold transplanted allogeneic islet grafts in diabetic mice treated with ECDI-fixed donor splenocyte infusions. Biomaterials 2011;32:4517–4524
22. Chen G, Kheradmand T, Bryant J, et al. Intragastric CD11b(+) IDO(+) cells mediate cardiac allograft tolerance by ECDI-fixed donor splenocyte infusions. Am J Transplant 2012;12:2920–2929
23. Kheradmand T, Wang S, Bryant J, et al. Ethylenecarbodiimide-fixed donor splenocyte infusions differentially target direct and indirect pathways of allore cognition for induction of transplant tolerance. J Immunol 2012;189:804–812
24. Ueki I, Abiru N, Kobayashi M, et al. B cell-targeted therapy with anti-CD20 monoclonal antibody in a mouse model of Graves’ hyperthyroidism. Clin Exp Immunol 2011;163:309–317
25. Hering BJ, Walawalkar N. Pig-to-nonhuman primate islet xenotransplantation. Transpl Immunol 2009;21:81–86
26. Arefanian H, Tredget EB, Rajotte RV, Gill RG, Korbutt GS, Rayat GR. Short-term administrations of a combination of anti-LFA1-1 and anti-CD154 monoclonal antibodies induce tolerance to neonatal porcine islet xenografts in mice. Diabetes 2010;59:958–966
27. Cui H, Tucker-Burden C, Caufield SM, et al. Long-term metabolic control of autoimmune diabetes in spontaneously diabetic nonobese diabetic mice by nonvascularized microencapsulated adult porcine islets. Transplantation 2009;88:160–169
28. Li S, Yan Y, Lin Y, et al. Rapidly induced, T-cell independent xenotransplantation in diabetic mice mediated by marginal zone B cells and requires help from NK cells. Blood 2007;110:3926–3935
29. Kanaan N, Bachman LA, McGregor CG, Griffin MD. Porcine antigen presenting cells produce soluble adjuvants that stimulate B cells within and across the species. Am J Transplant 2003;3:403–415
30. Chen G, Qian H, Starzl T, et al. Acute rejection is associated with antibodies to non-Gal antigens in baboons using Gal-knockout pig kidneys. Nat Med 2005;11:1295–1298
31. Chen G, Sun H, Yang H, et al. The role of anti-non-Gal antibodies in the development of acute humoral xenograft rejection of hDAF transgenic porcine kidneys in baboons receiving anti-Gal antibody neutralization therapy. Transplantation 2006;81:273–283
32. Carvello M, Petrelli A, Vergani A, et al. Inotuzumab ozogamicin murine analog-mediated B-cell depletion reduces anti-islet allo- and autoimmune responses. Diabetes 2012;61:155–165
33. Brosdie GM, Wallberg M, Santamaria P, Wong FS, Green EA. B-cells promote intrasplenic CD8+-cytotoxic T-cell survival to enhance type 1 diabetes. Diabetes 2008;57:909–917
34. Pierson RN 3rd, Winn HH, Russell PS, Achincloss H Jr. Xenogeneic skin graft rejection is especially dependent on CD4+ T cells. J Exp Med 1989;170:991–996
35. Gill RG, Wolf L, Daniel D, Coulombe M. CD4+ T cells are both necessary and sufficient for islet xenograft rejection. Transplant Proc 1994;26:1203
36. Ng YH, Oberbarnscheidt MH, Chandramoorthy HC, Hoffman R, Chalassani G. B cells help alloreactive T cells differentiate into memory T cells. Am J Transplant 2010;10:1970–1980
37. Tsai MK, Chien HF, Tseng MC, Lee PH. Effects of B cell depletion on T cell alloimmune immune responses: a strategy to reduce alloimmune sensitization. Transpl Immunol 2009;21:215–220
38. Lee KM, Kim JJ, Stott R, et al. Anti-CD45RB/anti-TIM-1-induced tolerance requires regulatory B cells. Am J Transplant 2012;12:2072–2078
39. Kouskoff V, Lacaud G, Pape K, Retter M, Nemazee D. B cell receptor expression level determines the fate of developing B lymphocytes: receptor editing versus selection. Proc Natl Acad Sci USA 2000;97:7435–7439
40. ECDI-SP