Ethyleneurediamine-Fixed Donor Splenocyte Infusions Differentially Target Direct and Indirect Pathways of Allorecognition for Induction of Transplant Tolerance

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Ethlenecarbodiimide-Fixed Donor Splenocyte Infusions Differentially Target Direct and Indirect Pathways of Allorecognition for Induction of Transplant Tolerance


Strategic exposure to donor Ags prior to transplantation can be an effective way for inducing donor-specific tolerance in allogeneic recipients. We have recently shown that pretransplant infusion of donor splenocytes treated with the chemical cross-linker ethenecarbodiimide (ECDI-SPs) induces indefinite islet allograft survival in a full MHC-mismatched model without the need for any immunosuppression. Mechanisms of allograft protection by this strategy remain elusive. In this study, we show that the infused donor ECDI-SPs differentially target T cells with indirect versus direct allospecificities. To target indirect allospecific T cells, ECDI-SPs induce upregulation of negative, but not positive, costimulatory molecules on recipient splenic CD11c+ dendritic cells phagocytosing the injected ECDI-SPs. Indirect allospecific T cells activated by such CD11c+ dendritic cells undergo robust initial proliferation followed by rapid clonal depletion. The remaining T cells are sequestered in the spleen without homing to the graft site or the graft draining lymph node. In contrast, direct allospecific T cells interacting with intact donor ECDI-SPs not yet phagocytosed undergo limited proliferation and are subsequently anergized. Furthermore, CD4+CD25+Foxp3+ T cells are induced in lymphoid organs and at the graft site by ECDI-SPs. We conclude that donor ECDI-SP infusions target host allogeneic responses via a multitude of mechanisms, including clonal deletion, anergy, and immunoregulation, which act in a synergistic fashion to induce robust transplant tolerance. This simple form of negative vaccination has significant potential for clinical translation in human transplantation. The Journal of Immunology, 2012, 189: 000–000.

Current standard practice for controlling allogeneic transplant rejection is life-long immunosuppression. These pharmacological agents have significant immunologic and metabolic toxicities. Donor-specific tolerance is an attractive concept, but has not yet been reliably and consistently achieved in humans.

One approach for inducing donor-specific tolerance is by strategic exposures of the recipient to donor Ags prior to transplantation. A critically important aspect of such exposures is to identify conditions that will ensure host tolerance rather than sensitization. Such “planned preimmunization” was experimented in the 1980s in the form of donor-specific transusions (1–4). Despite initial promises, unacceptably high incidences of sensitization following donor-specific transusions alone precluded its clinical application (2, 3). Donor-specific transusions in combination with costimulation blockade through anti-CD154 resulted in tolerance in some animal models, including nonhuman primates. However, the prothrombotic effect of this mAb has limited its clinical application (5–8).

A robust strategy for induction of Ag-specific tolerance has been described in models of autoimmune diseases such as experimental autoimmune encephalomyelitis and autoimmune diabetes (9–12), in which peptides or proteins implicated in the specific autoimmune disease (e.g., insulin protein) are cross-linked to the cell surface of splenocytes (SPs) using a chemical cross-linker ethenecarbodiimide (ECDI). Infusion of such autoantigen-coupled SPs induces robust Ag-specific tolerance by preventing Th1- and Th17-mediated autoimmunity and restores self-tolerance (10–13).

Translating this strategy to transplantation tolerance, we have recently reported that infusions of ECDI-coupled donor splenocytes (ECDI-SPs) on day −7 and day +1 in recipients (with day 0 being the day of transplant) induce long-term allograft tolerance in a full MHC-mismatched mouse model of islet transplantation (14, 15). This tolerance strategy does not require generalized T and/or B cell depletion or costimulation blockade, therefore making it highly attractive for potential clinical translation for human allogeneic transplantations. Although cellular and humoral antidonor responses are significantly suppressed by the infusion of donor ECDI-SPs, the exact mechanisms leading to such effective control of alloimmunity remain elusive.
In this study, we report that donor ECDI-SPs are capable of targeting both direct and indirect pathways of allore cognition via distinct mechanisms. Most donor ECDI-SPs are rapidly internalized by recipient splenic APCs, particularly the CD11c<sup>+</sup> dendritic cells (DCs), which selectively upregulate negative, but not positive, costimulatory molecules. Upon encountering with such recipient APCs, T cells with indirect allospecificity undergo rapid expansion followed by profound clonal contraction, with the remaining T cells sequestered in the spleen without trafficking to the graft or graft draining lymph nodes (dLNs). Alternatively, residual donor ECDI-SPs not internalized by host phagocytes weakly stimulate T cells with direct allospecificity and render them resistant to subsequent stimulation (anergy). Additionally, the regulatory T cell (Treg) population is expanded by ECDI-SPs. Thus, donor ECDI-SP-based therapy employs several distinct yet synergistic mechanisms to achieve robust and durable transplant tolerance.

Materials and Methods

Mice

Eight- to 20-wk-old male BALB/c (H<sup>2</sup>b), congenic Thy1.1, Thy1.2, CD45.2, and CD45.1 C57BL/6 (B6; H<sup>2</sup>), SJL (H<sup>2</sup>), Foxp<sub>3</sub><sup>g+</sup> knock-in mice, CD11c-diphtheria toxin receptor (DTR) mice, and IFN-γ<sup>−/−</sup> mice on a B6 background were purchased from the The Jackson Laboratory. 4C mice were provided by Dr. Qizhi Tang from the University of California, San Francisco. All mice were housed under specific pathogen-free conditions at Northwestern University. Protocols were approved by the Northwestern University Institutional Animal Care and Use Committee.

Abs and FACS analysis

PE-conjugated anti–IFN-γ (XMG1.2), allophycocyanin-conjugated anti-Thy1.2 (104), PerCP-conjugated anti-CD4 (L7T4; GK1.5), allophycocyanin-conjugated anti-CD11c (HL3), PerCP-conjugated anti-CD11b (Mac-1), PE-conjugated anti-CD8<sub>α</sub> (53.6.7), PE-conjugated anti-CD8<sub>δ</sub> (7-2), FITC-conjugated anti-CD40 (3/23), PE-conjugated anti-CD80 (16-10A1), PE-conjugated anti-programmed death ligand (PD-L), PE-conjugated anti–PD-L1 (MIH-5), and allophycocyanin-conjugated anti-CD25 (PC6) were from BD Biosciences. PE-conjugated anti-mouse Foxp3 (FK-16a) was from eBioscience.

Diabetes experiments

Mice were treated with streptozotocin (Sigma-Aldrich) at 170 mg/kg. Confirmation of diabetes and protocol for islet transplantation were described previously (16). Graft rejection was determined by two consecutive blood glucose readings >250 mg/dl.

ECDI cell coupling and tolerance induction

Tolerance was induced by i.v. injection of ECDI-treated donor SPs as described (15). For tracking of ECDI-treated SPs in vivo, the cells were further labeled with PKH-67 (Sigma-Aldrich) at a final concentration of 20 µg/mouse i.p. 24 h prior to each dose of ECDI-SP injection. For CD11c<sup>+</sup> DC depletion, DT (10 ng/kg) was injected i.p. 18 h before each dose of ECDI-SP injection in CD11c<sup>+</sup>DTR mice. Depletion of respective cell populations was collected for each by FACS analysis. For neutralizing IFN-γ, anti–IFN-γ (XMG1.2; BioXCell) was given at 500 µg/mouse i.p. 24 h prior to each dose of ECDI-SP injection.

Immunofluorescence

Frozen sections of spleens were blocked with 10% donkey serum (Sigma-Aldrich). Staining was performed with anti-CD11c mAb (Armenian hamster IgG clone AP-MAB0814; Novus Biologicals), anti-CD45R (B220) mAb (rat IgG<sub>2a</sub>, k clone RA3-6B2, eBioscience), and anti-F4/80 mAb (rat IgG<sub>2a</sub>, k clone BM8; eBioscience). Ab binding was visualized using secondary Abs (DyLight 594-conjugated AffiniPure goat anti-Armenian hamster IgG for CD11c and DyLight 594-conjugated AffiniPure donkey anti-rat IgG for all other markers; Jackson ImmunoResearch Laboratories). Mounting medium with DAPI was used (Vector Laboratories). Images were visualized using a Zeiss Axio Scope A1 microscope, acquired with a Jenoptik ProgRes MAC camera, and analyzed with ProgRes Capture Pro 2.7 software.

Generation and adoptive transfer of activated DCs

B6 or BALB/c bone marrow-derived DCs (BMDCs) were generated using a published protocol (17). B6 BMDCs were pulsed with BALB/c or SJL (third-party) lysates (prepared with three cycles of freeze-thaw of splenocytes) for 8 h followed by LPS (100 ng/ml) activation overnight. BALB/c BMDCs were similarly activated with LPS overnight. CD11c<sup>+</sup> DCs were enriched by the day 0 DCs isolation kit (Miltenyi Biotec). CD11c<sup>+</sup> cells (2 × 10<sup>6</sup>) were injected i.p. into B6 recipients on indicated days.

Adaptive transfer of T cells

TCR transgenic T<sub>Ea</sub> (CD45.2<sup>+</sup>) and 4C (Thy1.1<sup>+</sup>) CD4 T cells were purified from spleens of respective TCR transgenic mice using a CD4<sup>+</sup> negative isolation kit (Miltenyi Biotec). T<sub>Ea</sub> and 4C CD4 T cells were labeled with 5 µM CFSE (Molecular Probes) and injected i.v. into CD45.1<sup>+</sup>T<sub>Ea</sub>. B6 congenic recipients on day -8 in the day to the day of islet transplantation (day 0), and analyzed on indicated days. In some experiments, CFSE-labeled T<sub>Ea</sub> (CD45.2<sup>+</sup>) CD4 T cells were injected into CD11c<sup>+</sup>DTR (CD45.2<sup>+</sup>) mice on day -8 and analyzed on indicated days using Vvo2 to gate on the injected T<sub>Ea</sub> cell population. For in vitro restimulation, harvested cells were further stimulated with PMA/ionomycin (50 and 200 ng/ml, respectively) for 4 h and further proliferation was examined by CFSE dilution.

Statistical analysis

Graft survival was calculated by Kaplan–Meier analysis. A log rank test was used to compare survival between groups. Column statistics was performed using a Student t test. A p value < 0.05 was considered to be statistically significant. All analyses were done with GraphPad Prism 5 software.

Results

Donor ECDI-SPs are rapidly internalized by recipient splenic APCs

To track the donor (BALB/c) ECDI-SPs in vivo after injection, the cells were labeled with a membrane fluorophore (PKH-67) prior to injection. The distribution of PKH-67<sup>+</sup> cells at 3 and 18 h postinjection was investigated. At 3 h postinjection, >85% of PKH-67<sup>+</sup> cells were also positive for recipient (B6) MHC class II I-A<sup>b</sup>, suggesting that most of the injected donor ECDI-SPs had been internalized by recipient class II<sup>+</sup> APCs (data not shown). This distribution of PKH-67<sup>+</sup> at 18 h postinjection (Fig. 1A) was not significantly different from that at 3 h, and therefore 18 h data are shown for subsequent analysis. As shown in Fig. 1B, most I-A<sup>b</sup>-<sub>k</sub> PKH-67<sup>+</sup> cells were found in the spleen, and to a lesser degree in the liver, of the injected mice. Few, if any, I-A<sup>b</sup>-<sub>k</sub>PKH-67<sup>+</sup> cells were found in peripheral LNs, the lungs (Fig. 1A, right panel), the bone marrow, or the thymus (data not shown). As shown in Fig. 1C, of the I-A<sup>b</sup>PKH-67<sup>+</sup> cells in the spleen, 4% were CD11b<sup>+</sup>CD11c<sup>+</sup> DCs, 4% were B220<sup>+</sup>CD11c<sup>+</sup>DTR, and 3.3% were CD8<sup>α</sup>CD11c<sup>+</sup> DCs. Additionally, 49% of I-A<sup>b</sup>PKH-67<sup>+</sup> cells in the spleen were B220<sup>+</sup>CD11c<sup>−</sup> B cells (confirmed by cell surface expression of CD19, data not shown), and the remaining I-A<sup>b</sup>PKH-67<sup>+</sup> cells were mainly F4/80<sup>+</sup>CD11b<sup>+</sup> (13%) and F4/80<sup>+</sup>CD11b<sup>+</sup> (8%) macrophages. Anatomically, at 18 h postinjection, PKH-67<sup>+</sup> cells were mainly distributed in the marginal zone of the spleen (Fig. 1D, left upper panel). Double fluorescent staining and confocal microscopic examination of the spleen tissue revealed PKH-67<sup>+</sup> fragments (green, intracellular) within CD11c<sup>+</sup>, B220<sup>+</sup>, and F4/80<sup>+</sup> cells (red, labeled with respective cell surface Abs), confirming that PKH-67<sup>+</sup> cells had indeed been internalized by host APCs (Fig. 1D).

Recipient CD11c<sup>+</sup> DCs are obligatory for allograft tolerance induced by donor ECDI-SP infusions

To determine which APC population internalizing the injected donor ECDI-SPs might be responsible for the induction of toler-
PKH-67+ ECDI-SP fragments from intact PKH-67+ donor ECDI-SPs were rapidly internalized by recipient splenic APCs. ECDI-fixed, PKH-67-labeled BALB/c splenocytes (1 x 10^7) were injected into B6 mice. Eighteen hours later, organs were harvested, treated with collagenase, and stained with I-A^b (class II of B6), CD11c, CD11b, B220, and CD86. I-A^b was used to differentiate recipient APCs picking up PKH-67+ ECDI-SP fragments from intact PKH-67+ donor ECDI-SPs themselves. Portions of the spleen were processed for immunofluorescent staining. (A) Distribution of free PKH-67+ donor ECDI-SPs (I-A^b-PKH-67+) versus internalized PKH-67+ ECDI-SP fragments by recipient I-Ab+ APcs (I-A^b-PKH-67+) in the spleen. (B) Distribution of I-A^b-PKH-67+ in the spleen, the liver, peripheral LNs, and the lungs. The PKH-67+ gate was set by using splenocytes from B6 mice injected with unlabeled ECDI-fixed BALB/c splenocytes (No PKH [control]). (C) I-A^b-PKH-67+ cells were further characterized by cell surface markers of APC subtypes. (D) Confocal immunofluorescent images of B6 spleens 18 h after injection of ECDI-fixed, PKH-67–labeled BALB/c splenocytes. Sections were stained with respective Abs as shown. Panel 1. Overall distribution of PKH-67+ cells (green) within the spleen. Panels 2–4. Subcellular localization of PKH-67 fragments (green, intracellular) was seen in CD11c^+ B220^+ and F4/80^+ cells (red, cell surface). Data shown in (A)–(C) are representative of at least three independent experiments. Data shown in (D) are representative of sections from at least three identically treated individual spleens. Original magnification x10.

ance, an individual APC population was depleted at the time of donor ECDI-SP infusion and subsequent islet allograft survival was examined. Macrophages were depleted by injecting the mice with liposomal clodronate 18 h prior to ECDI-SP infusions. Recipient treatment with liposomal clodronate globally depleted macrophages (data not shown), including those that had taken up ECDI-SPs (Supplemental Fig. 1A). As shown in Fig. 2A, depletion of macrophages at the time of ECDI-SP infusions did not affect islet allograft survival. We next depleted B cells with antiserum CD20 mAb. The depletion Ab was given 3 d prior to donor ECDI-SP infusions and indeed it depleted B cells that had internalized ECDI-SPs (Supplemental Fig. 1B). The effect of B cell depletion using this Ab was long-lasting with slow gradual return between 13 and 23 d. Finally, the role of CD11c^+ DCs in tolerance induction was studied using CD11c-DTR mice. Administration of DT efficiently depleted CD11c^+ cells in these mice, including those CD11c^+ cells that had taken up ECDI-SPs (Supplemental Fig. 1C). Mice were injected with DT 18 h prior to donor ECDI-SP infusions, and islet allograft survival was examined. As shown in Fig. 2C, whereas depletion of CD11c^+ cells itself actually had a moderate beneficial effect on islet allograft survival, it completely abolished the graft tolerance effect by donor ECDI-SP infusions such that most of the mice rejected their islet allograft by day 30. These data indicate that the CD11c^+ cell population plays an obligatory role in allograft tolerance induced by donor ECDI-SP infusions.

Splenic CD11c^+ DCs from recipients treated with donor ECDI-SPs selectively upregulate negative costimulatory molecules

Because CD11c^+ DCs appeared to be the pivotal APC population mediating the tolerogenic effect of ECDI-SPs, we next examined the phenotype of splenic CD11c^+ DCs from recipients treated with donor ECDI-SPs. As shown in Fig. 3A, compared with CD11c^+ DCs from untreated recipients, DCs from recipients treated with ECDI-SPs upregulated the expression of the negative costimulatory molecule PD-L2, and to a lesser degree PD-L1. The mean fluorescent intensities for PD-L2 and PD-L1 are shown in the bar graph below the histograms. In contrast, expression of positive costimulatory molecules CD40, CD80, and CD86 was not upregulated. We therefore hypothesized that this altered pattern of negative versus positive costimulatory upregulation was critical in mediating the tolerogenic effect of ECDI-SPs. If so, providing activated DCs carrying the alloantigens would provide the missing positive costimulation signals in trans and effectively abrogate tolerance induced by donor ECDI-SP infusions. To test this hypothesis, B6 BMDCs were pulsed with BALB/c splenocyte lysate, activated with LPS overnight, and injected to B6 recipients (2 x 10^6 cells/mouse) on the same day of the first ECDI-fixed donor cell infusion, and islet allograft survival was examined. The uptake of BALB/c lysate by B6 BMDCs and the upregulation of CD80, CD86, and CD40 after LPS treatment of B6 BMDCs were both confirmed by FACS analysis (data not shown). As shown in Fig. 3B, transferring of activated B6 BMDCs pulsed with BALB/c lysate (activating the indirect pathway of allorecognition) effectively abolished tolerance induction. This process was exquisitely donor-specific, and not due to nonspecific production of inflammatory cytokines by the activated DCs, because transferring activated but unpulsed DCs or DCs pulsed with third-party (SJL) splenocyte lysate was unable to affect tolerance induced by donor (BALB/c) ECDI-SP infusions. Interestingly, transferring LPS-activated donor (BALB/c) BMDCs (activating the direct pathway of allorecognition) was also able to abolish tolerance induced by donor ECDI-SPs (Fig. 3B). These findings indicate that effective control of both the indirect and direct alloantigen presentation pathways is one of the critical mechanisms by which ECDI-SP infusions induce transplant tolerance and mediate allograft protection.

**Donor ECDI-SP infusions deplete T cells with indirect alloantigen specificity**

We next examined the effect of ECDI-SP infusions on CD4 T cells with indirect alloantigen specificity. To do so, T6a TCR transgenic mice were used. CD4^+ T cells from T6a mice carry a transgenic TCR specific for the I-E^k (BALB/c) allopeptide 52-68 cross-presented by B6 MHC class II I-A^b, therefore carrying indirect donor Ag specificity (18). As illustrated in Fig. 4A, 4 x 10^6
CD45.2<sup>+</sup>CD4<sup>+</sup> T cells were labeled with CFSE and adoptively transferred to CD45.1<sup>+</sup> B6 recipients 1 d prior to the first dose of donor ECDI-SP infusion on day −7. Mice were subsequently transplanted with islet grafts on day 0, tolerized again on day +1 according to our standard protocol, and sacrificed on days −4, 0, and +7 for examination of the injected TEa cells. Comparisons were made to untreated control mice. As shown in representative dot plots in Fig. 4B, in tolerized mice (+tol), within 72 h donor ECDI-SP infusion (day −4), TEa cells underwent six to seven cycles of cell divisions and expanded significantly (12.6%) in the spleen (Fig. 4B, left panels). Most expanded TEa cells remained in the spleen rather than traveling to peripheral LNs (Fig. 4B, middle panels). However, by day 0 (i.e., the day of islet transplantation and 7 d after the first donor ECDI-SP infusion), these cells underwent ∼10-fold contraction (from 12.6 to 1.2%). Furthermore, following the second donor ECDI-SP infusion on day +1, by day +7 their number in the spleen further diminished (0.6%). Moreover, only a small fraction of total TEa cells were able to be detected in the graft dLNs (0.3%; Fig. 4B, middle panel) or the islet graft (0.4%; Fig. 4B, right panel) at this time point. In contrast, in untreated naïve mice (−tol), TEa cells remained quiescent prior to islet graft transplantation and were distributed equally in the spleen and peripheral LNs (0.9 and 1%, respectively). After islet graft transplantation at day +7, a significantly higher percentage of TEa cells homed to the graft dLN (1.3%) and the islet graft (2.2%), and they proliferated rigorously there compared with those remaining in the spleen (0.7%). A summary of data from three to five independent experiments is presented in Fig. 4C with <em>p</em> values listed to indicate statistical significance. These data indicate that donor ECDI-SP infusions led to an initial expansion followed by profound depletion of CD4 T cells with indirect alloantigen specificities in the spleen. More importantly, the remaining T cells are preferentially retained in the spleen rather than trafficking to the graft dLNs or the islet graft to mediate anti-donor responses and graft destruction. One possible explanation for such splenic sequestration is the large burden of cognate Ags in the spleen provided by the infused donor ECDI-SPs.

Given the critical importance of CD11c<sup>+</sup> cells in the graft protection induced by ECDI-SPs as shown in Fig. 2C, we next examined the effect of CD11c<sup>+</sup> cell depletion on the behavior of the injected TEa cells. As shown in Fig. 4D, when CD11c<sup>+</sup> cells were depleted from the ECDI-SP-treated recipients, the initial (day −4) TEa cell division and expansion seen in the spleen were much diminished compared with those seen in recipients not depleted of CD11c<sup>+</sup> cells (2.04% in depleted versus 6.78% in undepleted recipients). Consequently, on day 0, a much less profound contraction was seen in the depleted recipients (∼2-fold, from 2.04 to 0.92%) compared with that seen in the undepleted recipients (∼13-fold, from 6.78 to 0.54%). We speculate that the remaining TEa cells in the CD11c<sup>+</sup> cell-depleted recipients are fully capable of responding to further antigenic stimuli of an islet transplantation, and trafficking to the graft and the graft dLN to mediate effector function.

Consistent with the notion that T cells with indirect allospecificities are by and large depleted by our strategy, in long-term tolerized mice by donor ECDI-SP infusions (>100 d after the initial transplant and with functioning islet grafts), adoptive transfer of LPS-activated recipient CD11c<sup>+</sup> DCs pulsed with donor lysate (therefore presenting to and activating T cells with indirect allospecificities) could not break established graft tolerance to precipitate islet graft rejection (Fig. 4E). This is in sharp contrast to the ability of such activated DCs to prevent tolerance at the induction stage (Fig 3B) when T cells with indirect allospecificities are still abundantly present.

**IFN-γ contributes to the depletion of allo-specific T cells and is obligatory for tolerance induced by donor ECDI-SP infusions**

Examination of cytokine expression in the spleen after donor ECDI-SP infusion revealed that IFN-γ was expressed by a significant percentage (12.6%) of the proliferating TEa cells (Fig. 5A). IFN-γ has been previously implicated in activation-induced cell death of T cells through caspase pathway activation (19). We therefore tested whether production of this cytokine by proliferating TEa T cells contributed to the ultimate decrease in numbers of these cells. To do so, mice were treated with a neutralizing anti–IFN-γ mAb concomitant with donor ECDI-SP infusion, and TEa cells were examined 7 d later. As shown in Fig. 5B, representative contour plots on left, and summary bar graph on right, with <em>p</em> values listed to indicate statistical significance, treatment with anti–IFN-γ mAb increased the number of TEa cells in the spleen and LNs by ∼2- to 3-fold. To further ascertain the role of IFN-γ in the induction of tolerance by donor ECDI-SP infusions, we attempted to tolerate IFN-γ<sup>−/−</sup> recipients using the same regimen. As shown in Fig. 5C, IFN-γ<sup>−/−</sup> recipients were resistant to tolerance by this regimen and rejected their islet grafts 15–20 d after transplantation in a manner similar to untreated IFN-γ<sup>−/−</sup> recipients. These data suggest that IFN-γ plays an obligatory role in the tolerance induced by donor ECDI-SP infusions, potentially through depletion of donor-specific T cells during initial activation.

**Donor ECDI-SP infusions induce anergy in T cells with direct alloantigen specificity**

After injection, a small percentage of the donor ECDI-SPs remained free without being internalized by recipient APCs (1.1%; Fig. 1A).
These donor cells are likely able to transiently activate host T cells with direct alloantigen specificities. To examine this, we took advantage of the 4C transgenic mice. CD4+ cells from these mice carry a transgenic TCR specific for a BALB/c allopeptide presented by BALB/c class II MHC I-A\(^d\), and they therefore display direct donor Ag specificity (20). Similar to what is shown schematically in Fig. 4A, 4 \( \times 10^5 \) Thy.1.1^CD4^+ 4C T cells were labeled with CFSE and adoptively transferred to Th1.2^+ B6 recipients 1 d prior to the first dose of donor ECDI-SP infusion. Mice were subsequently transplanted with islet grafts on day 0 and tolerized again on day +1 according to our standard protocol. Comparisons were made to untreated control mice and mice injected with BALB/c SPs without ECDI treatment. As shown in Fig. 6A, 72 h after donor ECDI-SP infusion, a small fraction of the undivided 4C T cells recovered from untreated mice, which proliferated vigorously in response to PMA and ionomycin stimulation (Fig. 6B, top panels). Furthermore, after receiving an islet transplant and following the second donor ECDI-SP infusion on day +1, the previous undivided 4C cells in the ECDI-SP–treated mice showed no further division in the spleen (Fig. 6C) or peripheral LNs and dLNs (data not shown) on day +7. These data indicate that donor ECDI-SP infusions induce a state of unresponsiveness (anergy) in T cells with direct alloantigen specificity.

Consistent with this notion, in long-term protected mice by donor ECDI-SP infusions (>100 d after the initial transplant and with functioning islet grafts), transfer of LPS-activated, but not unactivated, BALB/c (donor) BMDCs (therefore presenting to and activating T cells with direct allospecificities) broke the established tolerance and precipitated graft rejection (Fig. 6D), suggesting that such a state of unresponsiveness of T cells with direct alloantigen specificity can be reversed when appropriate stimuli are present.

**Donor ECDI-SP infusions induce Tregs in secondary lymphoid organs and the islet graft**

As a third mechanism for tolerance (in addition to clonal deletion and anergy), immunoregulation was next examined. To do so, Foxp3-GFP knock-in mice were used as islet graft recipients, and CD4^+Foxp3^+ cells in secondary lymphoid organs (SLOs) and the islet were examined on 20 d after transplant in recipients with or without donor ECDI-SP infusions. As shown in Fig. 7 (representative dot plots, with summary bar graph with \( p \) values listed to indicate statistical significance), recipients treated with donor ECDI-SPs displayed higher percentages of CD4^+Foxp3^+ cells (among total CD4^+ cells) in the spleen, the dLNs, as well as the graft as compared with untreated recipients. Therefore, donor ECDI-SP infusions led to an early increase of CD4^+Foxp3^+ Tregs in all SLOs and the graft. This finding is consistent with our previous observation that tolerance by this regimen is abrogated by treatment of recipients with a depleting anti-CD25 Ab (PC61) at the time of the first donor ECDI-SP infusion (15, 21).

**Discussion**

Ag-coupled ECDI-SPs have been used to induce tolerance to autoantigens and alloantigens in several disease models, including multiple sclerosis, autoimmune diabetes, and allogeneic islet transplantation (10–12, 15), and they have recently been shown to be effective in controlling allergic airway disease and food allergy (22). Therefore, this tolerance strategy has high potential for translating to clinical practice for various applications, including transplantation. However, the alloreactive T cell repertoire is distinct from the autoreactive T cell repertoire. First, the precursor frequency is likely to be much larger (23). Second, recipient T cells may be directly activated by alloantigens presented on donor MHCs by donor passenger leukocytes, or indirectly activated by alloantigens processed and cross-presented on recipient MHCs. Therefore, successful tolerance strategies for transplant will need to be able control both pathways effectively.

In the present report, we attempt to dissect the mechanisms of donor-specific graft protection provided by donor ECDI-SP infu-
We demonstrate that donor ECDI-SP infusions target both pathways of Ag presentation via distinct mechanisms. Recipient CD11c+ DCs internalizing donor ECDI-SPs selectively upregulate negative costimulatory molecules. Indirect presentation of alloantigens to recipient T cells by these CD11c+ DCs results in an initial expansion followed by profound contraction of T cells with indirect Ag specificities. This pattern of short-term, unsustained T cell effector responses may also suggest the inability of these T cells to develop into long-lived memory cells (24). Alternatively, donor ECDI-SPs anergize T cells with direct Ag specificities.

**FIGURE 4.** Donor ECDI-SP infusions deplete T cells with indirect alloantigen specificity. (A) Schematic treatment plan. CFSE-labeled 45.2+ TEa T cells ($4 \times 10^6$) were adoptively transferred into 45.1 congenic recipients on day −8, followed by standard tolerance induction with ECDI-SP infusions (days −7, +1) and islet transplantation (day 0). TEa T cells were examined on days −4, 0, and +7 as indicated. In some experiments, DT injection was performed on day −9 to deplete CD11c+ DCs from the CD11c-DTR mice. (B) Plots were gated on total CD4+ T cells. Percentages of CD45.2+ TEa T cells in the spleen (left panels), the kidney (draining) LNs (middle panels), and the graft (right panels) are shown. +tol, with ECDI-SP infusions; −tol, without ECDI-SP infusions. Data shown are representative of three to five individual experiments. (C) Summary of data from three to five individual experiments performed as in (B). The averages of percentages of 45.2+ (TEa) cells among total CD4+ cells were calculated and are depicted in the bar graph, and $p$ values are listed to indicate statistical significance. (D) CD11c-DTR mice were injected with DT to deplete CD11c+ cells (+DT) or not (−DT) prior to injection of CD4+ TEa cells and ECDI-SPs as schematically shown in (A). TEa T cells were examined in the spleen on days −4 and 0 by gating on Vo2+ cells. Left panels, Representative dot plots. Right panel, Histogram overlay. (E) Long-term tolerated mice by ECDI-SP infusions (>100 d graft survival after initial islet transplantation) were adoptively transferred with LPS-activated B6 BMDCs pulsed with donor (BALB/c) or a third-party (SJL) lysate. Graft survival was monitored by blood glucose measurements.

**FIGURE 5.** IFN-γ contributes to the depletion of allospecific T cells and is obligatory for tolerance induced by donor ECDI-SP infusions. CFSE-labeled CD45.2+ TEa T cells ($4 \times 10^6$) were injected into CD45.1+ congenic B6 recipients 1 d prior to treatment with donor ECDI-SPs. (A) The spleen was obtained 72 h after the injection of ECDI-SPs and analyzed by FACS. Plots shown were gated on CD45.2+ cells. IFN-γ expression was increased in proliferating TEa T cells in response to ECDI-fixed donor cell infusion. (B) Neutralizing anti–IFN-γ mAb (500 µg) was injected on the day of ECDI-fixed donor cell infusion. The spleen and peripheral LNs were obtained 7 d after the injection of ECDI-SPs and analyzed by FACS. Plots shown were gated on CD4+ cells. Anti–IFN-γ treatment partially rescued the depletion of TEa T cells in the spleen and peripheral LNs. The averages of percentages of 45.2+ (TEa) cells among total CD4+ cells in the spleen and peripheral LNs with or without anti–IFN-γ treatment were calculated and are depicted in the bar graph, and $p$ values are listed to indicate statistical significance. (C) IFN-γ−/− recipients were not able to be tolerized by donor ECDI-SP infusions. IFN-γ−/− recipients were transplanted with islet allografts with or without donor ECDI-SP infusions. Graft survival was not different between IFN-γ−/− recipients with or without ECDI-SP treatment (*$p=0.411$). Data shown in (A) and (B) are representative of an average of three individual experiments.
ities, likely because of delivery of signal 1 in the absence of signal 2 (25). This notion is consistent with our observation that splenocytes after ECDI treatment are unable to upregulate B7-1, B7-2, and CD40 when cultured in vitro (J. Bryant, unpublished observations). Therefore, unlike the tolerance induced by ECDI-coupled peptide infusion in models of autoimmunity where the main mechanism of tolerance occurs through indirect Ag presentation to autoreactive T cells (10, 25), tolerance induction to allografts benefits from targeting the direct as well as the indirect pathways. Because different organ or tissue grafts may carry different types and numbers of donor passenger leukocytes, ECDI-SPs may have different efficacy in controlling the direct pathway in these different models. This is an important consideration for designing ECDI-SP–based tolerance strategy in other models of transplantation such as allogeneic cardiac transplantation or xenogeneic islet transplantation. Alternatively, indirect Ag presentation is one of the main contributors responsible for chronic rejection (26) and for Ab-mediated rejection (27, 28). Consistent with this notion is our previous report of the lack of anti-donor Ab production in tolerized mice treated with donor ECDI-SPs (examined on day ~4). (D) Long-term tolerized mice by ECDI-SP infusions (>100 d graft survival after initial islet transplantation) were adoptively transferred with unactivated donor (BALB/c) BMDCs, LPS-activated donor (BALB/c) BMDCs, or third-party (SJL) BMDCs. Graft survival was monitored by blood glucose measurements. Data shown in (A)–(C) are representative of at least three individual experiments.

We observed a significant early expansion of Foxp3+ Tregs in all SLOs as well as the islet graft in ECDI-SP–treated recipients (Fig. 7). One unresolved issue in this study is whether the observed increase of Foxp3+ Tregs arises from the direct or the indirect alloreactive T cell repertoires. Neither the TEa nor the 4C T cells in our hands were observed to express Foxp3 in any of the SLOs examined (data not shown). One possibility is that the TCR affinity of TEa or 4C T cells is such that in our system they do not become Foxp3-expressing Tregs. However, this does not exclude the possibility of other allospecific T cells with different affinities for donor Ags to become Foxp3+ Tregs. It is also possible that the initial increased Foxp3+ cells (Fig. 7) are not donor-specific, such as those seen to be induced in the presence of large numbers of apoptotic cells (29), and the donor specificity of this tolerance strategy is determined by its ability to control donor-specific effector T cell function seen with the 4C and TEa cells. It has also been observed by others that conversion of TEa T cells to Foxp3+ cells in vivo is tied to the timing of the injection of these cells in relationship to the injection of the toleragen (30). Furthermore, we observed that in contrast to the critical role of the CD25+Foxp3+ T cells during tolerance induction (15), in long-term tolerized recipients (>100 d), depletion of these cells by PC-61 no longer had an effect on graft survival (T. Kheradmand and X. Luo, unpublished observations), suggesting that the role of these cells during tolerance maintenance is less prominent. Therefore, we postulate that the TCR repertoire of CD25+Foxp3+ T cells induced by ECDI-SPs, as well as their role in the induction and mainte-
costimulatory molecules in our tolerance regimen, we have previously shown that PD-L1−/− mice were resistant to tolerance induction by donor ECDI-SP infusions (15). Lack of costimulation by B7-1/B7-2–CD28 interactions between APCs and T cells has been shown to induce a rapid but transient T cell activation that is characterized by production of IFN-γ and IL-10 but a lack of production of IL-2, IL-6, and TNF-α (38, 39). This distinct pattern of cytokine production is thought to subsequently contribute to apoptosis and deletion of the Ag-specific T cell population via both activation-induced cell death and passive cell death (40, 41). Our observation that TEa cells underwent initial expansion in cell numbers followed by rapid clonal contraction with donor ECDI-SP infusions in an IFN-γ–dependent fashion is consistent with this notion. The role of IFN-γ in tolerance induced by this regimen is further ascertained by the inability of IFN-γ−/− recipients to be tolerized. The source of the IFN-γ participating in tolerance induction in this study is not entirely clear. It is possible that the effector cells are the primary producers of IFN-γ, as shown in Fig. 5A, which then acts in an autocrine fashion and promotes activation-induced cell death via STAT1 and caspase-dependent pathways (19). Ultimately, elucidation of the precise mechanisms by which IFN-γ exerts tolerogenic effects in donor ECDI-SP infusions awaits studies utilizing cell-specific IFN-γ or IFN-γ receptor knockouts.

The alloantigen-specific TCR repertoire represents up to 5–10% of the total host TCR repertoire (23). Studies using sensitive MHC class II tetramer technology reveal that T cell clones expressing TCRs specific for different foreign peptide/MHC II complex ligands in general do not have cross-reactivities (42, 43). Therefore, tolerizing the entire alloantigen-specific T cell repertoire by individually tolerizing each alloreactive T cell clone may be impractical. Alternatively, APCs interacting with allospecific T cells may simultaneously express multiple allopeptide/MHC complexes of interest. Consequently, inducing tolerogenic APCs in vivo by regimens such as ours has the potential to tolerize multiple alloreactive T cell clones whose peptide/MHC complex ligands are coexpressed on the same APCs. Therefore, regimens targeting APCs may allow more efficient induction of tolerance via mechanisms including linked suppression (44, 45) and epitope spreading (46).

In conclusion, donor ECDI-SP infusions target host allogeneic responses via a multitude of mechanisms, including clonal deletion, anergy, and immunoregulation, thereby providing potent donor-specific allograft protection. Our studies highlighted multiple advantages of this strategy for transplant tolerance over other regimens: 1) generalized T cell depletion is not required, as ECDI-SPs effectively deplete and anergize donor-specific T cells; 2) generalized costimulation blockade is not required, as ECDI-SPs ensure defective positive costimulatory interactions (47) and enhance PD-L1/2–mediated negative costimulation; 3) adoptive transfer of ex vivo-expanded Tregs is not required, as ECDI-SPs directly promote enhanced Treg function in vivo (15); 4) ECDI-SPs allow pretransplant donor Ag presentation to a quiescent immune system promoting preemptive tolerization (48, 49), thereby allowing engraftment of transplanted tissues/organs in the presence of greatly reduced alloreactivity and immunosuppressive drug toxicity; and finally 4) as autoantigen-coupled leukocytes are known to restore tolerance in animal models of autoimmunity, both allo- and autoantigens can be coupled to carrier cells for tolerance induction to transplanted tissue/organs where both alloimmunity and recurrent autoimmunity may be detrimental to the graft. We therefore think that this simple form of negative vaccination has significant potential for clinical translation to human transplantation.
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Disclosures

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Supplementary FIG.1. Depletional therapies selectively delete macrophages, B cells and dendrites cells that have taken up the ECDI-SPs. Macrophages, B cells and dendritic cells were individually depleted in B6 mice (I-A^b^+) as described in Materials and Methods prior to the injection of PKH-labeled donor (BALB/c) ECDI-SPs. 18 hours after the injection of PKH-labeled donor ECDI-SPs, mice were sacrificed and the spleens were examined for I-A^b^+PKH^+^ cells under various depletional conditions. Histograms were all gated on I-A^b^+PKH^+^ cells for examination of the F4/80^+^ (A), B220^+^ (B) and CD11c^+^ (C) populations. A. Clodronate-loaded liposome treatment significantly depleted F4/80^+^ macrophages among I-A^b^+^ cells that had taken up PKH^+^ donor cells. B. Anti-CD20 treatment significantly depleted B220^+^ B cells among I-A^b^+^ cells that had taken up PKH^+^ donor cells. C. Diphtheria toxin (DT) treatment of CD11c-DTR mice significantly depleted CD11c^+^ dendritic cells among I-A^b^+^ cells that had taken up PKH^+^ donor cells.
Supplementary Figure 1

Gated on I-A^b+PKH^+ cells

A

No Treatment
Macrophage Depletion

% of Max

F4/80

B

No Treatment
B Cell Depletion

% of Max

B220

C

No Treatment
Dendritic Cell Depletion

% of Max

CD11c