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Tolerance to Antigen-Presenting Cell-Depleted Islet Allografts Is CD4 T Cell Dependent

Marilyne Coulombe,* Huan Yang,* Leslie A. Wolf,† and Ronald G. Gill2*

Pretreatment of pancreatic islets in 95% oxygen culture depletes graft-associated APCs and leads to indefinite allograft acceptance in immunocompetent recipients. As such, the APC-depleted allograft represents a model of peripheral alloantigen presentation in the absence of donor-derived costimulation. Over time, a state of donor-specific tolerance develops in which recipients are resistant to donor APC-induced graft rejection. Thus, persistence of the graft is sufficient to induce tolerance independent of other immune interventions. Donor-specific tolerance could be adoptively transferred to immune-deficient SCID recipient mice transplanted with fresh immunogenic islet allografts, indicating that the original recipient was not simply “ignorant” of donor antigens. Interestingly, despite the fact that the original islet allograft presented only MHC class I alloantigens, CD8+ T cells obtained from tolerant animals readily collaborated with naive CD4+ T cells to reject donor-type islet grafts. Conversely, tolerant CD4+ T cells failed to collaborate effectively with naive CD8+ T cells for the rejection of donor-type grafts. In conclusion, the MHC class I+II- islet allograft paradoxically leads to a change in the donor-reactive CD4 T cell subset and not in the CD8 subset. We hypothesize that the tolerant state is not due to direct class I alloantigen presentation to CD8 T cells but, rather, occurs via the indirect pathway of donor Ag presentation to CD4 T cells in the context of host MHC class II molecules. The Journal of Immunology, 1999, 162: 2503–2510.

Several experimental manipulations have demonstrated that the immunogenicity of pancreatic islets and other endocrine tissues can be reduced to achieve prolonged allograft survival with little or no host immune suppression (1–6). We have demonstrated that pretreatment of C57BL/6 (B6, H-2b) islets in 95% oxygen culture leads to indefinite allograft acceptance in untreated BALB/c (H-2d) recipients (7). These pretreated grafts are composed essentially of MHC class I+ II- islet parenchymal cells and are devoid of detectable hemopoietic APCs and donor vasculature (8, 9). Allograft survival of such cultured islets appears to be due to reduced tissue immunogenicity rather than reduced donor MHC expression. Transgenic expression of the costimulatory molecule B7-1 (CD80) negated the benefit of organ culture, while, conversely, increasing donor islet MHC Ag expression by IFN-γ treatment did not lead to the rejection of APC-depleted grafts (9). As such, the APC-depleted islet allograft represents a model of peripheral alloantigen presentation in the absence of appropriate costimulation required for initiating rejection. Such signal 1 Ag presentation in the absence of costimulation potentially leads to T cell inactivation or anergy (10, 11) and has been proposed to contribute to the development of T cell tolerance in vivo following costimulation blockade (12–14).

In the early post-transplant period, rejection of high-oxygen cultured islet allografts can be triggered by host immunization with donor-type spleen cells as a source of APCs, indicating that the grafts express recognizable alloantigens (15, 16). Previous studies show that this induced rejection response is mediated by CD8+ T cells (17, 18), an expected result given that islet parenchymal cells express class I, but undetectable class II, MHC Ags (19, 20). Such animals at this point appear to be immunologically ignorant, showing neither tolerance nor immunity to the islet allograft (21, 22). However, recipients of APC-depleted islet allografts gradually transition into a state in which the established grafts are no longer susceptible to donor APC-induced rejection (7, 15, 23, 24). Previous studies show that this time-dependent change is due to the development of a nondeletional form of donor-specific tolerance (7, 25).

An unusual feature of this model system is that the peripheral allograft itself is sufficient to trigger tolerance in the absence of other immune manipulations of the host. It is plausible that donor-reactive CD8+ T cells are gradually rendered unresponsive due to an encounter with the MHC class I-bearing allograft devoid of costimulatory activity. However, in previous studies we could not detect any defect in the graft-destructive CD8+ T cell subset in tolerant animals (25). Tolerant animals were comparable to control animals regarding: 1) CTL precursor frequency, 2) antidonor proliferative and cytotoxic activity, 3) donor-specific cytokine production, and 4) the ability of in vitro-primed T cells to reject donor-type islet grafts after adoptive transfer in vivo (25). Since signal 1 Ag presentation by the donor did not appear to result in the intrinsic paralysis (anergy) of relevant, graft-destructive T cells, it was unclear what change in donor-reactive T cells occurred to account for the tolerant state. Here, to further investigate the nature of tolerance in this model, we used an adoptive transfer system to determine whether tolerance was due to altered function of donor-reactive CD4 T cells, CD8 T cells, or both. Results show that tolerance to the MHC class I+, class II- islet allograft paradoxically resides in the CD4+ T cell subset and not in the graft-destructive CD8+ T cell subset.

*Barbara Davis Center for Childhood Diabetes/University of Colorado Health Sciences Center, Denver, CO 80262; and †Laboratory of Public Health, Virology/Serology Branch, North Carolina State, Raleigh, NC 27611

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2 Address correspondence and reprint requests to Dr. Ronald G. Gill, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, 4280 East Ninth Ave., Box B-140, Denver, CO 80262. E-mail address: ron.g.gill@uchsc.edu

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Materials and Methods

Animals
Male C57BL/6ByJ (B6, H-2\(^{b}\)) and CBA/J (H-2\(^{a}\)) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used as pancreatic islet donors. BALB/cByJ (BALB/c, H-2\(^{b}\)) mice, obtained from The Jackson Laboratory, were used as islet recipients. SCID C.B-17scid/scid (SCID, H-2\(^{k}\)) mice, provided by L. Schultz, were bred at the Barbara Davis Center rodent facility and used as islet and adoptive transfer recipients.

Induction of diabetes
Diabetes was induced in C.B-17scid mice with a single i.p. injection of streptozotocin (225 mg/kg; Calbiochem, Behring, La Jolla, CA). Nonfasting whole blood glucose was measured using a MediSense blood glucose meter (MediSense, Cambridge, MA), three times per week. The criteria for selecting recipients for islet transplantation was a minimum of two consecutive blood glucose values \(>20\) mM.

Islet isolation and transplantation
Islets were isolated from adult C57BL/6 or CBA mouse pancreata by collagenase (type V, Sigma, St. Louis, MO) digestion (26) and Ficoll purification (27). APC-depleted B6 islets were generated by cyclophosphamide pretreatment of donors to reduce the load of islet-associated passenger leukocytes followed by a 7-day culture period in 95% O\(_2\)/5% CO\(_2\) (7). For other experiments, fresh islets containing donor leukocytes were prepared from untreated donors and transplanted immediately after isolation. Streptozotocin-induced diabetic BALB/c mice were grafted with 400 cultured, APC-depleted islets beneath the left kidney capsule (7). Fresh islets were hand-picked for transplantation and coalesced within a capsule composed of 7–9 \(\mu\)l of recipient blood. The clot containing the recipient blood was then placed beneath the left kidney capsule of diabetic recipients.

Generation of tolerant animals
Donor-specific tolerance to APC-depleted (cultured) C57BL/6 pancreatic islets was allowed to develop spontaneously in immunocompetent allogeneic BALB/c mice. Since none of the BALB/c animals spontaneously rejected the B6 APC-depleted islet graft, it was essential to confirm that an active tolerant state had developed. This is especially important since previous studies show that tolerance to APC-depleted islet allografts is a time-dependent process (7). Ninety days after transplantation, recipients received a secondary cultured B6 islet graft beneath the contralateral (right) kidney capsule (7). This secondary donor-type graft served as a sentinel graft to detect systemic alteration in antidonor reactivity (7). Animals then were actively immunized with donor-type APCs 120 days after graft rejection and the ability of these animals to reject allo- or autologous islet allografts was measured for each section. This observation implies that there is an essential collaboration between CD4 and CD8 T cells for the initiation of islet allograft rejection. Ninety days after transplantation, recipients were considered provisionally tolerant (25) and were used as spleen cell donors for adoptive transfer experiments.

Spleen cell fractionation
Spleen cells from tolerant or naive control BALB/c mice were depleted of CD4\(^+\) T cells by a 30-min incubation with anti-CD4 mAb (10 \(\mu\)g/ml GK1.5 ascites (28), rat IgG2b) followed by a 1-h incubation with 10 \(\mu\)g/ml anti-rat Ig (Boehringer Mannheim, Indianapolis, IN) and rabbit complement (Low Tox-M, Accurate Chemical, Westbury, NY) at 37°C. CD8\(^+\) T cells were depleted by treatment with anti-CD8 IgM mAb (supernatant from A2H4 (415 cells/293) and complement (1 : 1 at 37°C). B cells were not depleted in these experiments. Cell viability after depletion was confirmed by measuring Con A-induced proliferation and by phenotyping of peripheral blood from reconstituted SCID mice. Depletion of T cell subsets was confirmed by flow cytometry using FITC-labeled anti-CD4 and anti-CD8 (PharMingen, San Diego, CA). As determined on an EPICS Elite ESP flow cytometer (Coulter, Miami, FL), the extent of depletion of CD4\(^+\) T cell subsets in control and tolerant groups, relative to that in unfractionated populations, was 95.1–99.3%. Similarly, depletion of CD8\(^+\) T cells from control and tolerant spleen populations was 97.7–99.4%.

Adoptive transfer of tolerance
Streptozotocin-induced diabetic C.B-17scid mice (scid, H-2\(^{k}\)) were grafted with 450 immunogenetic (untreated) donor-type (B6) or third party (CBA, H-2\(^{a}\)) islets. We found that inducing diabetes and islet grafting C.B-17scid mice was a higher risk procedure than in corresponding immune-competent BALB/c mice; 10–15% of such animals had to be sacrificed within several days after grafting due to recipient morbidity. Therefore, to maximize the efficient use of tolerant animals in adoptive transfer experiments, diabetic SCID mice were islet grafted before lymphoid cell transfer to ensure islet graft function and recipient viability. This protocol raised the possibility that donor-derived APCs could turn over within the SCID host before lymphoid reconstitution, resulting in decreased immunogenicity of the graft. However, pilot experiments demonstrated that transferring naive BALB/c cells into islet-grafted C.B-17scid recipients either 3–5 days before islet transplantation or 2–10 days after islet grafting did not affect the tempo of rejection triggered by the transferred cells (data not shown). Therefore, in the present study there was no correlation between the time of graft rejection and the day of spleen cell reconstitution relative to islet grafting (p = NS in all groups). Unfractionated spleen cells (3 \(\times\) 10\(^7\)) from tolerant or control BALB/c mice were adoptively transferred i.p. to scid recipients. This number of splenic cells was derived from initial titration studies in which 3 \(\times\) 10\(^7\) spleen cells were found to be an excess dose for transferring efficient graft rejection to SCID mice within 30 days. In additional experiments, 1.5 \(\times\) 10\(^7\) CD8-depleted (CD\(^{4-}\), plus 1.5 \(\times\) 10\(^7\) CD4-depleted (CD\(^{8-}\)) spleen cells from tolerant and/or control mice were cotransferred to islet-grafted scid mice. Grafts functioning \(\geq60\) days after lymphocyte transfer were removed by nephrectomy of the graft-bearing kidney to confirm that the maintenance of euglycemia was graft dependent.

Histological examination of islet grafts
Islet graft-bearing kidneys, removed following graft rejection or by nephrectomy, were fixed in 10% (v/v) formaldehyde in aqueous phosphate buffer. Paraffin-embedded tissue sections were stained with Harris' hematoxylin-eosin (Fisher Scientific, Pittsburgh, PA) or stained for insulin using diaminobenzidine chromogen (Dako, Carpinteria, CA), a 20 µl mixture of ultra streptavidin peroxidase detection kit (Signet Laboratories, Dedham, MA), followed by diaminobenzidine chromogen (Signet) as a substrate. Sections stained for insulin were counterstained with Gill's hematoxylin (Fisher Scientific). The degree of mononuclear cell infiltration and islet tissue damage was determined for each section.

Statistics
Mann-Whitney U and Fisher's exact tests were used to determine the significance of graft survival data in adoptive transfer studies.

Results
Adoptive transfer of efficient islet allograft immunity requires both CD4\(^+\) and CD8\(^+\) T cells
The goal of this study was to determine the phenotype of T cells responsible for the maintenance of tolerance to APC-depleted islet allografts. Previous studies show that the rejection of islet allografts involves the participation of both CD4 and CD8 (30) T cells. This observation implies that there is an essential collaboration between CD4 and CD8 T cells for the initiation of islet allograft immunity. To confirm the requirement for this collaboration in adoptive transfer studies, initial experiments set out to determine the phenotype(s) of T cells necessary for reconstituting islet allograft immunity in immune-deficient C.B-17scid mice. BALB/c lymph node plus spleen cells were depleted of either CD4 or CD8 T cells and adoptively transferred to congeneric C.B-17scid mice. SCID recipients then were grafted with untreated allogeneic C57BL/6 (B6) islet allografts. The data presented in Fig. 1 show that C.B-17scid mice that are not reconstituted with BALB/c lymphoid cells fail to reject B6 islet allografts and show no sign of mononuclear cell infiltration (not shown). Reconstitution of SCID mice with unseparated BALB/c lymphoid cells led to the rejection of B6 islet allografts with a complete destruction of islet architecture and residual scarring and mononuclear cell infiltration (not shown). However, depletion of either CD4\(^+\) or CD8\(^+\) T cells from the BALB/c lymphoid inoculum prevented acute islet allograft rejection in the majority of SCID recipients. Such grafts demonstrated focal, peri-islet accumulation of mononuclear cells without disruption of islet architecture, with strong staining of insulin granules as indicated by aldehyde-fuchsin (not shown). The viability of T cell-depleted BALB/c lymphoid cells was indicated by the fact that recombining CD4-depleted (CD\(^{4-}\)) cells with CD8-depleted (CD\(^{8-}\)) cells reconstituted graft rejection in SCID recipients (Fig. 1).
1. T cells are required for the adoptive transfer of islet allograft rejection. In this adoptive transfer study, C.B-17scid mice were reconstituted with BALB/c spleen plus lymph node cells i.p. These cells populations were obtained from either untreated BALB/c mice (unfractionated) or BALB/c mice depleted of CD4+ or CD8+ T cells by in vivo treatment with mAbs (GK1.5 or 2.43, respectively). This initial depletion was followed by an additional round of depletion with the same Abs and complement in vitro. Four to seven days after reconstitution, 400 C57BL/6 (H-2b) islets were grafted beneath the kidney capsule of SCID recipients. Thirty days later, graft survival was assessed macroscopically and histologically. Only reconstitution with 3 × 10^7 unfractionated cells or with 3 × 10^7 CD4-depleted (CD8+) cells plus 3 × 10^7 CD8-depleted (CD4+) cells resulted in allograft rejection in this model. Significance was determined by Fisher’s exact test. Group 2 vs 3, p < 0.0001; group 2 vs 4, p = 0.0012; group 2 vs 5, p = NS; group 3 vs 5, p < 0.0001; group 4 vs 5, p = 0.0006.

1, group 5). Taken together, this study demonstrated that both CD4+ and CD8+ lymphoid cell populations are necessary to reconstitute efficient islet allograft rejection. This model system formed the basis of subsequent experiments to determine the phenotype(s) of T cells from tolerant animals responsible for the tolerant state.

Adoptive transfer of donor-specific tolerance to C.B-17scid mice

We have previously shown that donor-specific tolerance to an APC-depleted (cultured) allograft can be adoptively transferred to immune-deficient C.B-17scid mice bearing secondary APC-depleted islet allografts (7). In those studies nondiabetic scid recipients were then adoptively transferred into SCID mice bearing functionally restored islet capsules of SCID recipients. Thirty days later, graft survival was assessed macroscopically and histologically. Only reconstitution with 3 × 10^7 unfractionated cells or with 3 × 10^7 CD4-depleted (CD8+) cells plus 3 × 10^7 CD8-depleted (CD4+) cells resulted in allograft rejection in this model. Significance was determined by Fisher’s exact test. Group 2 vs 3, p < 0.0001; group 2 vs 4, p = 0.0012; group 2 vs 5, p = NS; group 3 vs 5, p < 0.0001; group 4 vs 5, p = 0.0006.

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As reported previously, original BALB/c recipients of APC-depleted B6 islet allografts were considered tolerant if they maintained a functioning graft >120 days and resisted induced rejection of the established graft after immunization with donor-type APCs (7). Spleen cells from either tolerant or naive BALB/c mice were then adoptively transferred into SCID mice bearing functioning islet allografts. The data presented in Fig. 2 show that spleen cells from naive BALB/c animals led to the rejection of both B6 (9 of 11) and CBA (7 of 7) islet grafts (p = NS). However, tolerant spleen cells failed to trigger the rejection of most donor-type B6 islet grafts (2 of 12), while rejecting the majority of third-party CBA grafts (7 of 8; p = 0.004). These results demonstrated that donor-specific tolerance to APC-depleted islet allografts could be adoptively transferred to secondary SCID mice. This result indicated that the original host was not simply ignorant of the APC-depleted islet allograft, since spleen cells confer tolerance to secondary fresh immunogenic islet transplants capable of triggering allograft rejection.

An essential issue of the current study centered on the phenotype(s) of donor-specific T cells responsible for allograft tolerance. Given that effective islet allograft rejection by BALB/c lymphocytes requires a collaboration between CD4 and CD8 T cells (Fig. 1), altered donor reactivity of either T cell subset alone could potentially result in the tolerant state. Therefore, spleen cells from tolerant BALB/c mice were depleted of either CD4 or CD8 T cells to determine whether either of these populations was required for tolerance in vivo. Spleen cells from tolerant or naive BALB/c recipients were fractionated into populations depleted of CD4+ cells or CD8+ cells, recombined in varied combinations, and transferred into SCID mice bearing either donor-type (B6) or third-party (CBA) islet allografts. When 1.5 × 10^7 CD4-depleted naive spleen cells were mixed with 1.5 × 10^7 naive CD8-depleted spleen cells and adoptively transferred to SCID mice bearing donor-type or third-party islet allografts, such cells were capable of reconstituting the rejection of both B6 and CBA islet allografts (seven of seven and four of four, respectively). The time course of graft rejection was similar to that of unfractionated cells; all grafts were
reconstituted with tolerant CD4+

mice (open squares) led to the rejection of all B6 grafts. In contrast, cotransfer of CD8-
scid animals (closed circles) did not. CD4-depleted (CD8+
subset was capable of readily collaborating with naive CD4+
1

islet grafts. Conversely, when tolerant CD8-depleted spleen
cells to mediate the rejection of both donor-type and third-party
islet grafts showed significantly prolonged survival (p < 0.001).

Allograft tolerance is CD4+
T cell dependent and CD8+
T cell independent

The phenotype(s) of T cells responsible for the tolerant state
could then be determined by experiments in which various com-
binations of fractionated subpopulations of naive and tolerant
spleen cells were mixed together for adoptive transfer (1.5 ×
10^7 of each), these populations reconstituted rejection of third-
party CBA grafts (five of five), yet did not reject most donor-type
(B6) islet grafts (one of six rejected; p = 0.02). These control
experiments indicated that the cell fractionation procedure itself
did not influence the ability of either naive or tolerant spleen cells
to transfer immunity or tolerance, respectively.

Donor-specific tolerance can be transferred with CD4+

scid animals (closed circles) did not. CD4-depleted (CD8+
subset and not in the CD8+
subset.

Histological examination of grafts

Fig. 4 shows representative histological sections of islet allografts
beneath the kidney capsule of C.B-17scid mice following adoptive
transfer of naive, tolerant, or mixtures of BALB/c spleen cells.
Donor-type B6 islet allografts beneath the kidney capsule of a
SCID recipient not receiving reconstituting cells show no detect-
able mononuclear cell infiltration (Fig. 4A). This is also true for
CBA grafts in nonreconstituted scid mice (not shown). Such grafts
functioned for >60 days, after which removal of the graft-bearing
kidney led to a reversion to hyperglycemia. When SCID recipients
of B6 islet grafts were reconstituted with a mixture of CD4+
plus CD8+
spleen cells from naive BALB/c spleen cells, the grafts were
acutely rejected, leaving a fibrotic lesion with residual mononu-
clear cells and the absence of intact islets (Fig. 4B). In contrast,
when SCID mice were reconstituted with the same mixture of
CD4+ plus CD8+ spleen cells from tolerant animals, donor-type
B6 islet grafts remained intact (Fig. 4C). These grafts did show
varying degrees of peri-islet mononuclear cell accumulations,
yet islets remained functional and stained strongly for insulin by
immunohistochemistry. Such apparently nondestructive cellular in-
filtrates are a common feature of many models of allograft toler-
ance (31, 32, 75). Most third-party CBA islet grafts in SCID
recipients were acutely rejected by all combinations of lymphoid
cells, with complete graft destruction and residual mononuclear
cell infiltration (not shown).

In experiments in which SCID mice were reconstituted with a
combination of CD8+ T cells from tolerant animals and CD4+
T cells from naive animals, both donor-type B6 (Fig. 4D) and
third-party CBA (not shown) islet grafts showed complete graft
destruction. Macroscopically, no islets were visible and an
inflammatory response as well as scar tissue were present. How-
ever, when SCID mice were reconstituted with a combination of
naive CD8<sup>+</sup> T cells and tolerant CD4<sup>+</sup> cells, donor-type B6 islet grafts remained intact (Fig. 4E) and continued to maintain euglycemia. The histological appearance of donor-type B6 islet grafts in this group was strikingly similar to that observed in animals reconstituted with fractionated tolerant spleen cells (Fig. 4C). Mononuclear cells accumulated at the graft site but usually did not lead to overt graft destruction. In contrast, these same cell populations were able to efficiently reject third party (CBA) grafts (Fig. 4F).

**Discussion**

Several mechanisms of tolerance to peripheral self Ags or to allogeneic transplanted tissues have been described, including clonal ignorance (21, 22, 33), clonal deletion (34–36), clonal anergy (21, 22, 33), clonal deletion (34–36), clonal anergy (21, 22, 33), clonal deletion (34–36), clonal anergy (21, 22, 33), clonal deletion (34–36), clonal anergy (21, 22, 33), clonal deletion (34–36), clonal anergy (21, 22, 33), clonal deletion (34–36), clonal anergy (21, 22, 33), clonal deletion (34–36), clonal anergy (21, 22, 33), clonal deletion (34–36), clonal anergy (21, 22, 33), clonal deletion (34–36), clonal anergy (21, 22, 33), clonal deletion (34–36), clonal anergy.
(10, 36–39), and active suppressive/regulatory processes (40–44). APC-depleted islet allografts provide an unusual opportunity to study the generation of peripheral allograft tolerance in adult animals. First, the allograft itself is sufficient to tolerate the host without additional therapeutic interventions to manipulate the immune response. This result itself implies that a spontaneous pathway of peripheral Ag presentation exists in adult animals that can lead to tolerance, including tolerance to alloantigens for which a very high T cell precursor frequency exists. Second, this form of allograft tolerance does not require donor hemopoietic chimerism of the host, in distinction to other forms of induced allograft tolerance in which such chimerism may facilitate the tolerant state (45–48). Thus, while donor hemopoietic chimerism certainly may contribute to allograft tolerance, the response to APC-depleted islet allografts indicates that such chimerism is not required for tolerance induction, a conclusion supported by other studies (49, 50).

Finally, this tolerance appears to be nondeletional in that previous studies show that tolerant animals exhibit normal reactivity to donor APCs in vitro, including reactivity to donor tissue-specific (islet cell) targets (25).

Tolerance to APC-depleted islet allografts occurs gradually, in that the host transitions from a state in which the recipient appears to be immunologically ignorant of the graft (22), being neither immune nor tolerant, to a state of donor-specific tolerance. The current study indicates that tolerance to APC-depleted allografts is not simply due to clonal ignorance, since spleen cells from tolerant animals fail to reject secondary fresh immunogenic grafts capable of activating recipient T cells. However, the fact that APC-depleted islet allografts are devoid of detectable donor-derived costimulatory activity both in vitro (51) and in vivo (7, 9, 22) raises that possibility that the graft gradually induces anergy in donor-reactive T cells by direct Ag presentation devoid of appropriate second signals required for activation (10). Since APC-depleted islet allografts express MHC class I but undetectable MHC class II alloantigens (8, 9), such an interaction with the graft would be expected to predominantly influence the CD8 T cell subset. However, previous data do not indicate an alteration in the in vitro activity of donor-specific CD8 T cells derived from tolerant animals (25).

The current study extends these observations by showing that donor-reactive CD8 T cells from tolerant animals remain functionally competent in that they can readily collaborate with naive CD4 T cells to trigger acute rejection of donor-type grafts. Such results do not support a mechanism by which CD8 T cells are rendered anergic by the peripheral allograft. Rather, such results appear more consistent with the hypothesis that the majority of donor-specific CD8 T cells remain essentially ignorant of the donor (21, 52), being neither activated nor inactivated by the peripheral islet allograft (53). The potential for peripheral Ags to directly induce T cell unresponsiveness due to the absence of costimulation remains a controversial subject. For example, other allograft models have employed costimulation blockade in vivo to achieve long term graft acceptance and tolerance. However, while blocking costimulation by CD80/CD86 with CTLA4-Ig can lead to long term cardiac allograft survival (13, 54) and tolerance to islet allografts (44) or xenografts (12), it has not been clearly demonstrated that such tolerance is the result of signal 1 T cell inactivation. In fact, a recent study indicates that recipient treatment with CTLA4-Ig can lead to an active regulatory response maintaining allograft tolerance (44). Thus, blocking or eliminating costimulatory signals may lead to unexpected responses that may not be due solely to the paralysis of Ag-specific T cells. The lack of detectable alteration in donor-specific CD8 T cells has led us to the proposition that direct presentation of allogeneic class I MHC Ags without costimulatory signals may be a null event rather than an inherently paralytic signal in vivo (53).

Surprisingly, tolerance to APC-depleted islet allografts appears to reside in the CD4 T cell subset. As such, these results are consistent with an increasingly wide variety of interventions that result in CD4 T cell-dependent allograft tolerance (40, 43, 55–59). CD4 T cells also appear to be a predominant population responsible for mediating neonatal tolerance (60), oral tolerance (83), and peripheral self tolerance (61–63). A key question is how the apparently MHC class I, class II islet allograft paradoxically leads to CD4 T cell-dependent tolerance. We hypothesize that the tolerance observed is the result of indirect Ag presentation in which graft-derived Ags are processed and presented by host-type APCs in association with class II MHC molecules as previously suggested by others (64). This form of Ag presentation would account for tolerance in the CD4 T cell subset. An alternate hypothesis is that low level donor MHC class II expression leads to an alteration of the direct alloreactive CD4 T cell repertoire. This appears somewhat unlikely, since both flow cytometric (9) and ultrastructural analysis (19) of cultured islet allografts failed to detect donor MHC class II expression. Also, previous studies found that primed alloreactive CD8, but not CD4, T cells were capable of recognizing and rejecting cultured islet allografts (17). However, it remains formally possible that MHC class II expression below detection levels contributed to the tolerant state. Future studies using MHC class II-deficient islet donors would test this later possibility.

If CD4 reactivity to processed graft-derived Ags does contribute to tolerance, it should not be assumed that an indirect response to allogeneic Ags is inherently tolerogenic. Numerous studies have suggested that this form of Ag presentation can contribute to allograft rejection (65–71). CD4 T cells have been shown to contribute to the rejection of class I-deficient (72) or MHC class II-deficient (73) skin allografts through an apparent indirect response to donor Ags. It should be noted, however, that differing tissues appear to demonstrate different requirements for rejection. For example, while CD4 T cells are sufficient for skin allograft rejection (74), they do not appear to be sufficient for islet allograft rejection (30) where CD8 T cells are also required. Also, while class I-deficient skin (72, 76) allografts acutely reject, class I-deficient islet allografts are permanently accepted (76). Thus, the contribution of CD4 T cells to allograft immunity and tolerance may vary according to the organ/tissue grafted. Although the varied roles for CD4 T cells in allograft immunity remain to be clarified, studies clearly indicate that CD4 T cells may facilitate graft acceptance rather than destruction when activated under appropriate conditions. This finding is analogous to the results of numerous studies of responses to parasites in which CD4 T cells can either promote or impair parasite elimination (77, 78). To date, we have been unable to detect obvious changes in antidonor reactivity in tolerant animals. For example, tolerant animals demonstrate normal antidonor proliferative and cytotoxic responses in vitro, and we have not observed cytokine deviation, such as Th1-like vs Th2-like responses, that distinguish tolerant from naive animals (25) (our unpublished findings). We are also currently examining the nature of CD4-dependent indirect alloreactivity in tolerant animals to determine whether an active, regulatory response is responsible for the tolerant state, as has reported in other systems (41, 43, 44, 79). Also, we do not yet know whether the perturbation in CD4 T cells seen in our model is due to an active regulatory response or is due simply to the lack of insufficient T cell help for CD8 T cells (38). We are currently attempting to distinguish between these two possibilities.

A final point of speculation is that the model of APC-depleted islet allografts inadvertently recapitulates features of peripheral
self-tolerance. It is increasingly apparent that self-tolerance can be maintained despite the persistence of peripheral autoreactive T cells. One explanation for this coexistence of peripheral self Ags with corresponding self-reactive T cells may be that such autoreactive T cells are ignorant of peripheral Ags expressed on the surface of most tissue parenchymal cells that are devoid of co-stimulatory activity (21, 33). However, purified CD4 T cells expressing high levels of CD45RB (CD45RB<sup>high</sup>) can spontaneously transfer autoimmunity in both rat (80) and mouse (81) models, suggesting that at least some autoaggressive cells are not ignorant of self Ags in vivo. Importantly, the cotransfer of CD4 T cells with low CD45RB expression (CD45RB<sup>low</sup>) can prevent the expression of autoimmune triggered by CD45RB<sup>high</sup> T cells (80, 81). Thus, T cells exist that are capable of actively regulating potentially pathogenic, self-reactive T cells. This theme of regulatory CD4 T cells is also seen in autoimmune diseases such as diabetes (82) and experimental allergic encephalomyelitis (83). Interestingly, as noted above regarding allograft tolerance, most studies concerning self tolerance implicate CD4 T cells as the predominant regulatory population. It is possible that regulatory T cell responses in part are a consequence of peripheral Ags that enter the pool of Ags spontaneously processed and presented by hemopoietic APCs. When such Ags are presented under appropriate conditions, perhaps as in the absence of tissue damage or inflammation (84), such a response may result in protective, rather than destructive, immunity. From the perspective of transplantation immunology, this would comprise the indirect pathway of graft Ag presentation by host APCs. We are currently exploring the possibility that some forms of self tolerance and induced allograft tolerance involve similar immune pathways leading to CD4 T cell-dependent regulatory responses.

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