Indirect Allorecognition in Acquired Thymic Tolerance

Induction of Donor-Specific Permanent Acceptance of Rat Islets by Adoptive Transfer of Allopeptide-Pulsed Host Myeloid and Thymic Dendritic Cells

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Pancreatic islet transplantation remains a promising approach to the treatment of type 1 diabetes. Unfortunately, graft failure continues to occur because of immunologic rejection, despite the use of potent immunosuppressive agents. It is therefore reasoned that induction of peripheral tolerance by the use of self–dendritic cells (DCs) as a vehicle to deliver specific target antigens to self–T-cells without ex vivo manipulation of the recipient is an attractive strategy in the treatment of type 1 diabetes. The finding that intrathymic inoculation of an immunodominant WF major histocompatibility complex (MHC) Class I (RT1.A^u) peptide five (P5) or P5-pulsed host myeloid DCs induces acquired thymic tolerance raises the possibility that adoptive transfer of allopeptide-primed host myeloid or lymphoid DCs might induce transplant tolerance. To address this hypothesis, we studied the effects of intravenous transfer of in vitro P5-pulsed syngeneic myeloid DCs or in vivo P5-primed syngeneic lymphoid (thymic) DCs on islet survival in the WF-to-ACI rat combination. In vivo primed thymic DCs isolated from ACI rats given intrathymic inoculation of P5 for 2 days were capable of in vitro restimulation of in vivo P5-primed T-cells (memory cells). In the first series of studies, we showed that intravenous—like intrathymic—inoculation of in vitro P5-pulsed host myeloid DCs induced donor-specific permanent acceptance of islets in recipients transiently immunosuppressed with antilymphocyte serum (ALS). We next examined whether thymic DCs isolated from animals that had been previously intrathymically inoculated with P5 could induce T-cell tolerance. The results showed that intravenous adoptive transfer of in vivo P5-primed thymic DCs led to donor-specific permanent acceptance of islets in recipients transiently immunosuppressed with ALS. This finding suggested that the thymic DCs take up and present P5 to developing T-cells to induce T-cell tolerance, thus providing evidence of a direct link between indirect allorecognition and acquired thymic tolerance. The second series of studies examined the mechanisms involved in this model by exploring whether in vivo generation of peptide-specific alloreactive peripheral T-cells by intravenous inoculation of P5-pulsed self-DCs was responsible for the induction of T-cell tolerance. Intrathymic inoculation of splenic T-cells obtained from syngeneic ACI rats primed with intravenous injection of P5-pulsed DCs with a high in vitro proliferative response to P5 in the context of self-MHC induced donor-specific permanent acceptance of islets from WF donors. In addition, the clinically relevant model of intravenous injection of P5-activated T-cells combined with transient ALS immunosuppression similarly induced transplant tolerance, which was then abrogated by thymectomy of the recipient before intravenous injection of the activated T-cells. These data raise the possibility that circulation of peptide-activated T-cells to the host thymus plays a role in the induction and possibly the maintenance of T-cell tolerance in this model. Our findings suggest that intravenous administration of genetically engineered host DCs expressing alloMHC peptides might have therapeutic potential in clinical islet transplantation for the treatment of autoimmune diabetes. *Diabetes* 50:1546–1552, 2001

Type I diabetes is a T-cell mediated autoimmune disease in which the immune system, through autoreactive T-cells, attacks and destroys the insulin-producing β-cells of pancreatic islets (1,2). The incidence of the disease continues to rise in the pediatric age group (3). Although the use of exogenous insulin remains the most successful therapy for type 1 diabetes, it has not prevented the progression of secondary complications of the disease (4). Thus, it appears that transplantation of whole pancreas or pancreatic islets remains the alternative method for treatment of the disease and prevention of its complications. However, the hope that such an approach will lead to the cure and prevention of the complications of type 1 diabetes without the use of exogenous insulin has not been realized because of immunologic rejection of islet grafts and disease recurrence (5). In addition, at the present time, islet transplantation has only limited success; an analysis of the registry data show that only 8.2% of islet transplant recipients have

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Ag, antigen; ALS, antilymphocyte serum; APC, ag-presenting cell; DC, dendritic cell; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL-4, interleukin-4; MHC, major histocompatibility complex; MST, mean survival time; P5, peptide 5; STZ, streptozotocin; TCR, T-cell receptor.

achieved insulin independence for over a year (6). Therefore, the major goal of diabetes research at the present time is to develop new approaches that will prevent graft rejection and disease recurrence without the use of chronic immunosuppression, with its attendant risks of infections and malignancy. Hence, it is reasoned that such strategies might target specific components of the immune system that mediate rejection without compromising the host's ability to mount an immune response to other foreign antigens (Ags).

One such strategy that has recently received attention is the deliberate introduction of foreign Ags into the adult thymus to specifically reeducate the immune system to accept foreign alloantigens as self without compromising the ability of the immune system to recognize and react to other Ags. This hypothesis is based on the finding that the avidity/affinity of the T-cell receptor (TCR)−major histocompatibility complex (MHC) Class I/self-peptide interactions regulate positive and negative selection events of T-cells in the thymus (7–9). Our initial reasoning that the presentation of alloMHC peptides by host ag-presenting cells (APCs) to developing T-cells in the thymus might induce acquired systemic tolerance is supported by the finding that intrathymic inoculation of the alloMHC Class I peptide with the immunodominant epitope induces transplant tolerance (10,11). These results were confirmed by our most recent finding that intrathymic inoculation of self−dendritic cells (DCs) loaded with a single donor (WF rat) immunodominant MHC Class I (RT1.A^u) peptide five (P5; residues 93–109) induced transplant tolerance to cardiac and islet allografts (12,13). The latter finding raises the possibility that adoptive transfer via the intravenous route of in vitro or in vivo alloMHC peptide−primed host DCs might induce tolerance to islets and further clarify the underlying mechanisms of tolerance induction in this model. Furthermore, understanding the mechanisms of tolerance induction might lead to the development of novel strategies for the prevention of graft rejection and the cure of type 1 diabetes. In the current study, we have demonstrated that adoptive transfer of P5-primed myeloid or lymphoid (thymic) self-DCs induces tolerance to islet allografts. Our data also show that in vivo P5-activated peripheral T-cells induced by intravenous inoculation of P5-primed self-DCs that continuously circulate through the thymus play an important role in the induction and possibly in the maintenance of Ag-specific tolerance. These data provide the first formal evidence that recognition of in vivo activated T-cells by developing T-cells in the thymus induces acquired systemic tolerance.

RESEARCH DESIGN AND METHODS

Animals. ACI (RT1^a), WF (RT1^u), and Brown Norway (BN; RT1^n) rats weighing 200–250 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Naive and transiently immunosuppressed with antilymphocyte serum (ALS), streptozotocin (STZ)-induced diabetic ACI rats were recipients of intrathymic or intravenous injection of WF MHC Class I (RT1.A^u) P5 (residues 93–109)−pulsed host DCs or in vivo P5-activated splenic T-cells. Then, 7 days later, the animals were transplanted with intraportal injection of 1,200–1,500 islets obtained from WF (donor) or BN (third-party) rats. Diabetes was induced by a single intravenous injection of STZ (60 mg/kg body wt). The animals were considered diabetic if blood glucose levels remained >350 mg/dl for at least 10 days.

Reagents. Immunodominant P5 (residues 93–109) was synthesized for us by Genosys Biotechnologies (Woodlands, TX) using the RT1.A^u sequences, as previously described (10). Fluorescein isothiocyanate (FITC)−conjugated rat monoclonal antibodies OX8 (CD8), W3/25 (CD4), OX18 (MHC Class I), RT1.A^a (ACI MHC Class I), OX6 (MHC Class II), OX19 (CD5, pan T-cells), OX62 (integrin marker), CD45RA (leukocyte common Ag), OX33 (B-cell marker), ED1 (rat monocyte/macrophages), ED2 (monocyte subsets), and ED3 (macrophage subsets), and mouse IgG were purchased from Harlan Bioproducts (Indianapolis, IN). Rat monoclonal antibodies OX8 (anti-CD8), OX6 (anti−MHC Class II), OX33 (anti−B-cells), and W3/25 (anti-CD4) were purchased from Accurate Chemical (Westbury, NY). Rat recombinant interleukin-4 (IL-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF) were purchased from R&D Systems (Minneapolis, MN).

Culture of DCs from bone marrow and thymus. Bone marrow cells removed from rat femurs, tibias, and humeri were depleted of erythrocytes with hypotonic buffered Tris−ammonium chloride (0.83%; pH 7.21). The cells were washed twice in complete medium consisting of RPMI-1640 (Gibco, Grand Island, NY) supplemented with 5% fetal calf serum (FCS), 5 μg gentamicin, 2 mmol/l L-glutamine, and 10 mmol/l HEPES buffer (Gibco). The culture technique used has been previously described (12,13). Thymic DCs were isolated from the thymus using a modified technique described by Khoury et al. (14). Briefly, collagenase-digested thymic cells were filtered through 60-gauge stainless steel mesh and washed with RPMI-1640 with 10% FCS. The cells were depleted of Fc^+ and plastic-adherent cells by panning on normal serum-coated petri dishes. The resulting cells were then cultured at a concentration of 3×10^6/ml in gelatin-coated, 75-cm^2 Falcon tissue culture flasks using complete medium containing 1.0 ng/ml rGM-CSF and 1.0 ng/ml rIL-4 only, without a maturation stimulus. Cultures were fed every second day by exchanging half medium for fresh GM-CSF− and IL-4−containing medium. DCs used in these studies were harvested on day 8 of in vitro bone marrow culture.

Fluorescence-activated cell sorter analysis. Myeloid and thymic ACI DCs were stained using FITC-conjugated monoclonal antibodies to determine the surface markers exhibited by the cells. The samples were analyzed on a FACScan (Becton Dickinson).

Mixed lymphocyte reaction. Purified ACI and Lewis T-cells were obtained from splenic leukocytes using magnetic beads (Dynabead M-450; Dynal, Great Neck, NY), as previously described (12). Briefly, 10^8 splenic leukocytes were incubated with an equal mixture of OX33 (antibody against B-cells) and OX6 (anti−MHC Class II) monoclonal antibodies for 1 h at 4°C with continuous rotation. The cells were washed three times with complete medium and further incubated with goat anti-mouse IgG magnetic beads for 1 h at 4°C. Only washed, negatively selected T-cells were used in this study. The T-cells were >98% positive for OX19 and OX18. The 5×10^5 responding T-cells obtained from syngeneic (ACI) or allogeneic (Lewis) rats were cultured with different numbers of γ-irradiated (3,000 rads from a ^{137}Cs source) stimulator myeloid or thymic ACI DCs. The plates were cultured at 37°C with 5% CO_2 for 4 days and then treated with 18-h [^{3}H]thymidine pulse before harvesting and scintillation counting.

Allopeptide presentation assay. Myeloid or thymic ACI DCs were incubated with 100 μg/ml P5 for 4 h at 37°C, then washed twice in complete medium. The peptide-pulsed DCs were then co-cultured with various dosages of syngeneic (ACI) T-cells for 4 days at 37°C with 5% CO_2 before determination of [^{3}H]thymidine incorporation after 18-h [^{3}H]thymidine pulse.

Recipient pretreatment. ACI recipients were given either intrathymic injection of in vitro P5-pulsed myeloid ACI DCs (10^6) or in vivo lymphoid (thymic) ACI DCs (5×10^6) combined with or without intraperitoneal injection of 0.5 ml rabbit anti-rat lymphocyte serum (Sera Lab; Accurate Chemical, Westbury, NY) 7 days before islet transplantation. The peptide or peptide-pulsed host DCs dosage was dissolved in 100 μl of phosphate-buffered saline, and 50 μl of the solution was injected into each lobe of the recipient's thymus without any leaks (10). In a second set of studies, ACI recipients were pretreated with intravenous administration of P5-pulsed myeloid self-DCs or in vivo P5-primed thymic self-DCs 7 days before islet transplantation. Thymectomized recipients of P5-activated peripheral syngeneic T-cells served as controls for naïve recipients of in vivo P5-primed peripheral T-cells.

Thymectomy. Complete thymectomy was performed 2 weeks before intravenous injection of in vivo P5-primed syngeneic peripheral T-cells. Briefly, a midline incision was made in the anterior neck, the pretracheal muscles were retracted, and an upper partial sternotomy was performed to expose the thymus. The entire thymus was carefully dissected and excised with any adherent fatty tissue.

Islet isolation and transplantation. Fresh islets were isolated from WF or BN donor rats using collagenase digestion and Ficoll separation, as previously described (13). Then ~1,200–1,500 fresh islets were transplanted intraportally into each STZ-induced diabetic ACI rat (blood glucose >300 mg/dl for at least 10 days). Blood glucose was monitored daily for 1 month and subsequently two times per week after transplantation. Rejection of islets was considered complete when blood glucose exceeded 200 mg/dl on two consecutive measurements.

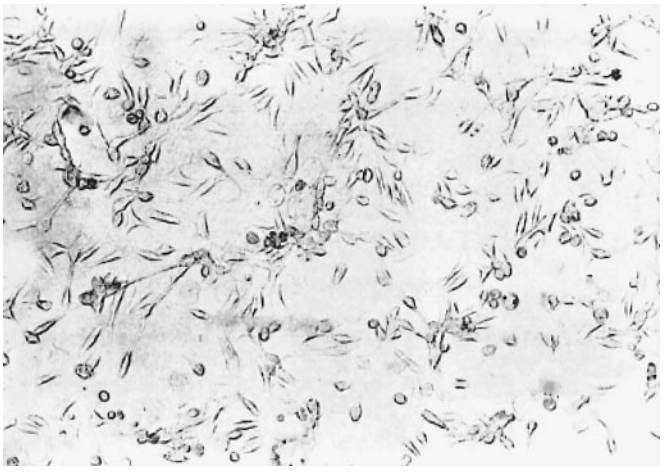

FIG. 1. Photomicrograph of ACI thymic DCs in liquid culture supplemented with rat rGM-CSF and rIL-4 on day 6 of culture. Original magnification ×400.

RESULTS

Morphology and surface markers of rat myeloid and thymic DCs. The photomicrographs of ACI rat myeloid and thymic DCs using rat rGM-CSF and rIL-4 show the characteristic large cells with oblong, twisted nuclei and irregular cytoplasm that have sheet-like processes or veils (Fig. 1). Analysis of surface markers showed that the DCs expressed high levels of MHC Class I and Class II molecules and ED2, with low levels of ED1, CD80, and OX62, as measured by intensity. There was no expression of CD45RA, ED3, OX19, OX33, CD4, or CD8 (not shown) (12,13). Although the number of cells that expressed MHC Class II was >95%, the expression of ED2 and ED1 suggested possible contamination with monocytes/macrophages.

Donor-specific permanent acceptance of islets by adoptive transfer of in vitro alloMHC peptide–pulsed host myeloid DCs. Our finding that immunodominant alloMHC WF P5-pulsed ACI DCs induces acquired thymic tolerance in the WF-to-ACI rat combination raised the possibility that intravenous administration of alloMHC peptide–pulsed myeloid self-DCs might migrate to and colonize the host thymus in vivo to provide tolerogenic signals to the developing T-cells. We therefore studied the effects of adoptive transfer of P5-pulsed host myeloid DCs on islet survival and further confirmed that intrathymic injection of P5-pulsed host DCs combined with ALS transient immunosuppression on day −7 before relative to islet transplantation led to donor-specific graft acceptance in the WF-to-ACI rat combination (Table 1, groups V and VI). Extension of this finding to a more clinically relevant model of intravenous inoculation of P5-pulsed host myeloid DCs resulted in permanent graft survival (Table 1, group IX). In contrast, similarly prepared recipients rejected the third party (BN) islets in an acute fashion (Table 1, group X).

Donor-specific permanent acceptance of islets by in vivo alloMHC peptide–primed thymic DCs. Our previous findings that intrathymic injection of P5 (10,11) and P5-pulsed DCs (12,13) induced acquired systemic tolerance raised the question of the importance of allopeptide presentation by the indirect pathway in the thymus during tolerance induction. To provide direct evidence for the role of thymic DCs during acquired thymic tolerance in this model, thymic DCs were isolated from the thymus of ACI rats 2 days after intrathymic inoculation of the immunodominant WF MHC Class I P5. In our initial studies, we evaluated the ability of thymic DCs isolated from such pretreated animals to take up, process, and present the allopeptides in vitro to syngeneic T-cells. In vitro restimulation of purified P5-primed syngeneic (ACI) T-cells obtained 7 days after in vivo priming with P5-pulsed thymic DCs showed a high proliferative response to both in vitro P5-pulsed thymic DCs and in vivo P5-primed thymic DCs compared with the response of naïve syngeneic T-cells (Fig. 2). Furthermore, as Fig. 2 shows, γ-irradiated ACI thymic DCs have a high allostimulatory capacity that parallels our observation with the myeloid DCs (12,13).

Having established the ability of lymphoid DCs isolated from the thymus of animals pretreated for 48 h with intrathymic inoculation of P5 to take-up and present alloMHC peptide to syngeneic T-cells, we then examined whether intravenous injection of the in vivo P5-primed thymic DCs could induce T-cell tolerance. Table 2 shows that although intravenous transfer of in vivo P5-primed thymic DCs significantly prolonged graft survival, it did not result in permanent graft survival (Table 2, group IV). In contrast, three of four recipients of intravenously administered in vivo P5-primed syngeneic thymic DCs combined with ALS transient immunosuppression permanently (>200 days) accepted donor-specific islets (Table 2, group V), a finding

TABLE 1
Effect on islet allograft survival of adoptive transfer of alloMHC peptide–pulsed myeloid self-DCs

Group	Immunosuppression (intraperitoneal ALS on day −7)	Inoculation of P5-pulsed self-DCs (route)	Islet donor	Survival time (days)	MST ± SD (days)
I	—	—	WF	7, 7, 8, 8, 8, 8	7.7 ± 0.5
II	—	—	BN	7, 8, 8, 8, 8, 9	8.0 ± 0.6
III	0.5 ml	—	WF	11, 12, 14, 16, 18	14.2 ± 2.8
IV	0.5 ml	—	BN	12, 13, 14, 15, 15	14.3 ± 1.3
V	0.5 ml	10^6 P5-pulsed DC (i.t.)	WF	72, 58, >200 (9 animals)	>200
VI	0.5 ml	10^6 P5-pulsed DC (i.t.)	BN	14, 15, 15, 16	15.0 ± 0.8
VII	—	10^6 P5-pulsed DC (i.v.)	WF	11, 12, 13, 11, 16	12.6 ± 2.1
VIII	—	2×10^6 P5-pulsed DC (i.v.)	WF	12, 12, 14, 16	13.5 ± 2.3
IX	0.5 ml	2×10^6 P5-pulsed DC (i.v.)	WF	25, >200 (4 animals)	>200
X	0.5 ml	2×10^6 P5-pulsed DC (i.v.)	BN	13, 14, 15, 16	14.5 ± 1.3

ACI recipients were pretreated with intrathymic (i.t.) injection of 1×10^6 P5-pulsed myeloid self-DCs (groups V and VI) or intravenous (i.v.) injection of P5-pulsed myeloid self-DCs (groups VII, VIII, IX, and X) with or without intraperitoneal 0.5 ml ALS 7 days before intraportal injection of 1,200–1,500 WF (donor) or BN (third party) islets.

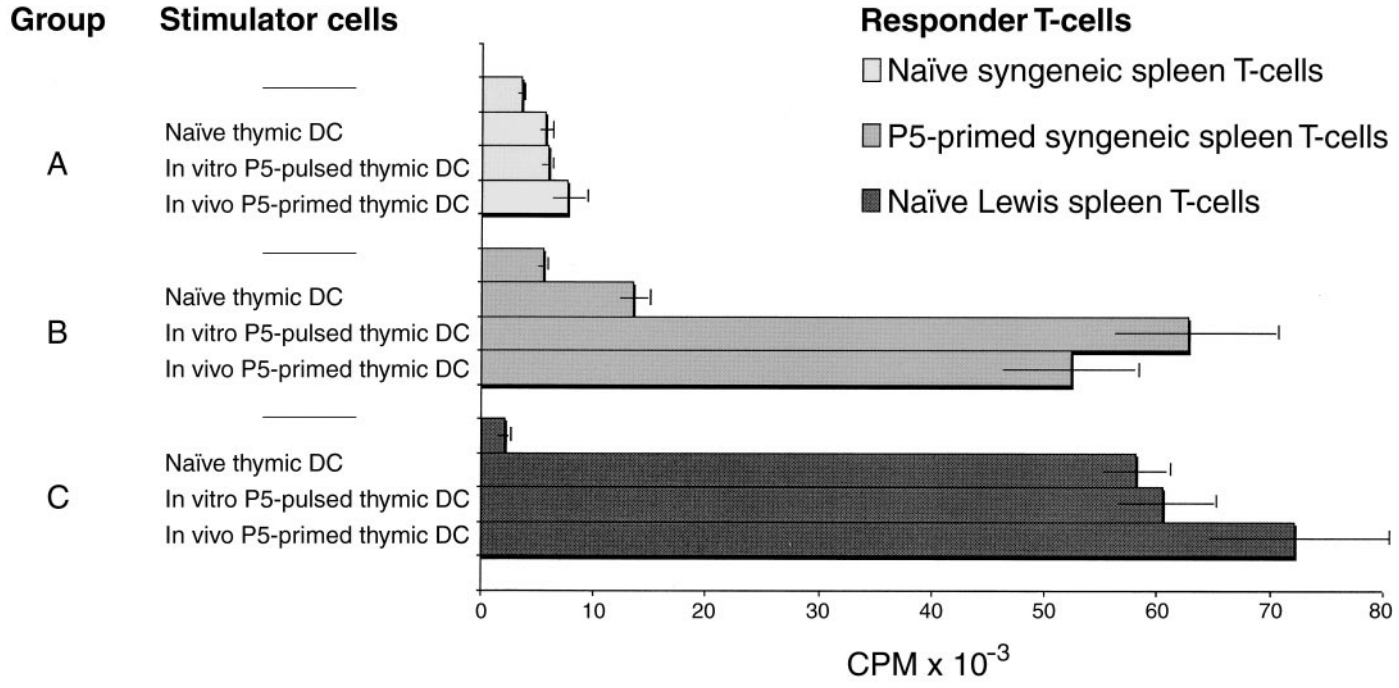

FIG. 2. *Groups A* and *B*: Allopeptide presentation by ACI thymic DCs to syngeneic T-cells. In vitro pulsed thymic DCs were incubated with 100 μg/ml RT1.A^u peptide for 4 h and washed twice in complete medium. In vivo P5-primed thymic DCs were obtained from the thymus of animals pretreated with intrathymic inoculation of 300 μg P5 for 48 h. The P5-pulsed DCs were co-cultured with naïve or in vivo P5-primed syngeneic splenic T-cells and incubated at 37°C in 5% CO_2/95% air for 4 days. The cells were pulsed for 18 h with [^{3}H]thymidine before harvesting and scintillation counting. These data obtained from a single experiment were reproducible in three separate experiments. *Group C*: Allostimulatory capacity of ACI thymic dendritic cells in mixed lymphocyte reaction. Purified Lewis T-cells were cultured with γ-irradiated (3,000 rads) ACI thymic DCs, as previously described.

that was consistent with the results obtained in recipients of intrathymic inoculation of 300-μg P5 (Table 2, groups I and II). In contrast, third-party (BN) islets were acutely rejected by similarly prepared animals (Table 2, groups III and VI).

Donor-specific permanent acceptance of islets by adoptive transfer of in vivo alloMHC peptide−primed syngeneic T-cells. Based on our observation that intravenous inoculation of P5-pulsed syngeneic myeloid DCs and in vivo P5-primed syngeneic lymphoid (thymic) DCs induced donor-specific acceptance of islets, we speculated that the intravenously administered P5-pulsed DCs might migrate to the recipient thymus to present the donor Ags to immature T-cells, which could then trigger the elimination, inactivation, or suppression of allopeptide reactive T-cells. On the other hand, there remained the possibility that intravenous administration of P5-pulsed host DCs might induce the generation of Ag-specific alloreactive peripheral T-cells through the indirect pathway of allorecognition. We explored the latter possibility—that in vivo generation of peptide alloreactive peripheral T-cells that circulate to the thymus could be responsible for the induction of T-cell tolerance in this model—because only in vivo−activated T-cells reenter the thymus (15).

We showed that peripheral T-cells obtained from ACI rat spleen primed with intravenous inoculation of P5-pulsed self-DCs develop a high proliferative response to P5 in context of self-MHC (Fig. 2), thus suggesting the presentation of in vivo alloMHC peptide to naïve syngeneic T-cells via the indirect pathway of allorecognition. Of in-

TABLE 2
Effect on islet allograft survival of adoptive transfer of thymic self-DCs isolated from syngeneic recipients of intrathymic inoculation of alloMHC P5

Group	Immunosuppression (intraperitoneal ALS on day −7)	Inoculation of in vivo P5-primed thymic-self-DCs (route)	Islet donor	Graft survival time (days)	MST ± SD (days)
I	—	300 μg P5 (i.t.)	WF	47, 50, 66, >200 (3 animals)	>200
II	0.5 ml	300 μg P5 (i.t.)	WF	>200 (8 animals)	>200
III	0.5 ml	300 μg P5 (i.t.)	BN	14, 15, 16, 18, 18	16.2 ± 1.8
IV	—	5×10^6 P5-primed thymic DC (i.v.)	WF	26, 27, 28, 29	27.5 ± 1.2
V	0.5 ml	5×10^6 P5-primed thymic DC (i.v.)	WF	72, >200 (3 animals)	>200
VI	0.5 ml	5×10^6 P5-primed thymic DC (i.v.)	BN	11, 11, 14, 15	12.8 ± 1.8

ACI recipients were pretreated with intrathymic (i.t.) inoculation of 300-μg P5 with or without ALS immunosuppression or pretreated with intravenous (i.v.) injection of in vivo 5×10^6 P5-primed thymic DC isolated from the thymus of ACI rats 2 days after i.t. injection of 300-μg P5. Each animal was transplanted with intraportal injection of 1,200–1,500 islets from WF (donor) or BN (third party) rats.

TABLE 3
Effect on islet allograft survival of adoptive transfer of in vivo alloMHC peptide–primed syngeneic T-cells

Group	ALS immunosuppression (0.5 ml on day −7)	Injection of in vivo P5-primed T-cells (route)	Islet donor	Survival time (days)	MST ± SD (days)
I	—	2×10^7 T-cells (i.v.)	WF	4, 4, 4, 5, 5, 5, 5, 5	4.6 ± 0.5
II	—	2×10^7 T-cells (i.v.)	BN	7, 8, 8, 8	7.8 ± 0.5
III	0.5 ml	2×10^7 T-cells (i.v.)	WF	>200 (6 animals)	>200
IV	0.5 ml	2×10^7 T-cells (i.v.)	BN	9, 11, 12, 14	11.5 ± 2.1
V	—	2×10^7 T-cells (i.t.)	WF	14, 15, 17, 21	16.8 ± 3.1
VI	—	2×10^7 T-cells (i.t.)	BN	7, 8, 8, 8, 9	8.0 ± 0.6
VII	0.5 ml	2×10^7 T-cells (i.t.)	WF	35, 38, >200 (6 animals)	>200
VIII	0.5 ml	2×10^7 T-cells (i.t.)	BN	10, 11, 11, 12	11.0 ± 0.8

ACI recipients were pretreated with intravenous (i.v.) or intrathymic (i.t.) injection of in vivo P5-primed syngeneic T-cells obtained from the spleen of ACI rats inoculated 7 days earlier with 2×10^6 P5-pulsed DC combined with or without 0.5 ml ALS intraperitoneal immunosuppression. Each recipient was transplanted with intraportal injection of 1,200–1,500 islets obtained from WF (donor) or BN (third party).

terest was our finding that thymic T-cells obtained from the same animals showed hyporesponsiveness to P5 (data not shown), thus suggesting elimination or inactivation of alloMHC peptide−reactive T-cells in the thymus.

Table 3 shows that intravenous transfer of in vivo P5-primed syngeneic T-cells on day −7 led to accelerated donor-specific islet rejection (mean survival time [MST]: 4.6 ± 0.5 [Table 3, group I] vs. 7.7 ± 0.5 days in controls [Table 1, group I]), thus confirming the sensitization of the peripheral T-cells to P5 in the primary host by the indirect pathway. In contrast, intrathymic inoculation of the in vivo P5-primed syngeneic T-cells significantly prolonged donor-specific islet survival (MST: 16.8 ± 3.1 days [Table 3, group V]; $P < 0.01$). It was interesting to observe that intravenous injection of in vivo P5-primed T-cells combined with 0.5 ml ALS transient immunosuppression on day −7 induced 100% permanent acceptance of donor islets (Table 3, group III). In contrast, similar treatment resulted in acute rejection of third-party (BN) islets (Table 3, group IV). These results were reproduced in recipients inoculated with intrathymic P5-primed T-cells combined with ALS transient immunosuppression (Table 3, group VII). Similarly treated animals rejected the third party (BN) grafts in an acute fashion (Table 3, groups VI and VIII).

Thymectomy abrogates the effect of adoptive transfer of in vivo allopeptide-primed syngeneic peripheral T-cells. To address the question of whether circulation of intravenously administered P5-activated peripheral syngeneic T-cells to the host thymus induces T-cell tolerance, we performed recipient thymectomy before intravenous transfer of P5-primed T-cells. Table 4 shows that thymectomy abrogated the induction of donor-specific unresponsiveness to islets. This observation confirmed that Ag-specific alloreactive peripheral T-cells that continuously circulate through the thymus play an important role in the induction of acquired systemic tolerance.

DISCUSSION

The presentation of self-peptides bound to MHC molecules plays a critical role in the process of negative and positive selection of mature T-cells in the developing thymus (7–8). Similarly, in adults, T-cell recognition of self-peptides continuously displayed on APCs may regulate the immune response to foreign Ags and contribute to the maintenance of self-tolerance in the periphery (16). Therefore, the mechanisms of self-Ag processing and presentation must be important in the induction of self-tolerance in the thymus, where self-Ags presented to the developing T-cells during ontogeny cause deletion of autoreactive T-cells. We therefore reasoned that the T-cell repertoire selection to the dominant determinants of allogeneic MHC peptides in the context of thymic self-MHC molecules might induce acquired thymic tolerance. The finding that intrathymic injection of alloMHC peptides induces acquired systemic tolerance (10,11,17–19) lent support to our hypothesis. Most recently, we showed that intrathymic inoculation of immunodominant alloMHC peptide−pulsed host DCs induces tolerance to rat cardiac and islet allografts (12,13). Based on these findings, we explored the possibility of inducing transplant tolerance to islets by using the clinically relevant model of adoptive transfer by intravenous route of allopeptides bound to self-DCs. The intravenous route is preferred because it is readily available compared

TABLE 4
Effect on islet survival of thymectomy before intravenous administration of in vivo allopeptide-primed syngeneic T-cells

Group	Immunosuppression (intraperitoneal ALS on day −7)	Intravenous injection of P5-primed T-cells	Islet donor	Graft survival (days)	MST ± SD (days)
I	—	—	WF	8, 8, 8, 8	8.0 ± 0.0
II	—	—	BN	6, 8, 9, 9	8.0 ± 1.4
III	0.5 ml	—	WF	11, 11, 12, 13	11.8 ± 1.0
IV	0.5 ml	2×10^7 T-cells	WF	12, 14, 14, 16	14.0 ± 1.6
V	0.5 ml	2×10^7 T-cells	BN	6, 7, 10	7.7 ± 2.1

ACI recipients were thymectomized 10 days before intravenous injection of 2×10^7 alloMHC peptide (P5)-primed T-cells obtained at 7 days from syngeneic rats inoculated intravenously with 2×10^6 P5-pulsed myeloid self-DC. The animals were transplanted with WF or BN islets 7 days after intravenous injection of P5-primed syngeneic T-cells combined with or without 0.5 ml ALS intraperitoneal immunosuppression.

with the intrathymic route. The results of our earlier studies, which showed that intrathymic inoculation of alloMHC peptides induces T-cell tolerance, led us to postulate that adoptive transfer of in vitro allopeptide-pulsed host myeloid DCs might induce donor-specific transplant tolerance. We have now shown in a more clinically relevant model that intravenous-like intrathymic inoculation of a single immunodominant alloMHC Class I P5-pulsed myeloid self-DC induces donor-specific permanent acceptance of islets.

Presentation of allopeptides either by recipient or donor MHC molecules displayed on the surface of APCs is essential in the induction of T-cell responses to transplantation Ags. T-cell immune responses to donor MHC Ags can occur through two distinct pathways of allorecognition: direct and indirect. In the direct pathway of allorecognition, the recipient T-cells recognize native allogeneic MHC molecules with bound peptides as intact structures on the surface of donor APCs (20,21). In the indirect pathway, donor MHC peptides are recognized in the context of self-MHC molecules (22,23). Although the finding that intrathymic injection of P5 (10,11) or P5-pulsed myeloid DCs (12,13) induces acquired thymic tolerance suggests an indirect pathway of allorecognition, there has been no direct evidence that thymic DCs are actually involved in the induction phase of acquired thymic tolerance. To address this question, we injected immunodominant WF MHC Class I ($RT1.A^u$) P5 (residues 93–109) into the thymus of ACI rats and harvested the thymic DCs 48 h later. The thymic DCs showed the characteristic veiled appearance of express MHC Class I and II, ED2, CD80, and OX62, as previously described (12–14,24). In addition, they had strong allostimulatory ability and presented alloMHC peptides to syngeneic T-cells in a similar fashion with myeloid DCs (13,14,24). Intravenous adoptive transfer of the in vivo P5-primed host thymic DCs specifically induced permanent acceptance of donor islets in ALS transiently immunosuppressed secondary syngeneic hosts. This finding was consistent with the results of intrathymic inoculation of P5 alone or in vitro P5-pulsed myeloid DCs. Our observation that in vivo P5-primed thymic DCs with in vitro allostimulatory ability induced donor-specific unresponsiveness to islets demonstrated that tolerance and immunogenicity are focused on the same epitope. The results showed that thymic DCs take up and present the intrathymically administered allopeptides to the developing T-cells to induce T-cell tolerance. This finding establishes a link between indirect allorecognition and presentation of allopeptides and acquired thymic tolerance. Our data parallel the previous observation by Khoury et al. (14) in the rat model of experimental autoimmune encephalomyelitis that intravenous injection of immunodominant peptide of myelin basic protein bound to self-thymic DCs prevents the development of the disease.

The underlying mechanisms of cellular interactions between intravenous inoculation of in vivo P5-primed host thymic DCs or in vitro P5-host myeloid DCs and the syngeneic naïve peripheral T-cells have not yet been defined. Understanding the factors that govern the in vivo processing and presentation of alloMHC peptides and the mechanisms that regulate the development of activated T-cells during acquired systemic tolerance induced by intravenous inoculation of P5-primed self-DCs might guide us in the design of future studies based on immune-targeted therapeutic strategies to cure autoimmune diabetes. In line with this reasoning, we explored the underlying mechanisms of tolerance induction in this model by examining whether in vivo generation of Ag-specific alloreactive T-cells that circulate through the thymus (15) is responsible for the induction of T-cell tolerance. In our initial studies, we tested the ability of intravenously administered P5-pulsed syngeneic myeloid host DCs to induce the generation of Ag-specific alloreactive peripheral T-cells and confirmed that indeed in vivo presentation of alloMHC peptides to naïve T-cells in context of self-MHC molecules leads to T-cell activation. This finding was consistent with other reports that have shown that adoptive transfer of in vitro tumor peptide–pulsed host DCs induce a strong cytotoxic T-cell response in vivo (25,26). Furthermore, our observation that intravenous administration of P5-activated T-cells alone leads to accelerated rejection of donor islets confirmed the in vitro finding of high mixed lymphocyte proliferative response of splenic T-cells obtained from animals pretreated with intravenous inoculation of P5 when restimulated with the initial priming allopeptide.

To explain the finding that intravenous inoculation of P5-pulsed host DCs induces T-cell tolerance, we hypothesized that in vivo generation of allopeptide-specific activated T-cells that circulate through the thymus might induce T-cell tolerance by either elimination through apoptosis or inactivation of Ag-specific alloreactive T-cells. To address this issue, STZ-diabetic ACI recipients of WF islets were pretreated with intrathymic injection of peptide-specific alloreactive T-cells combined with ALS-induced transient immunosuppression; in that study, we were able to demonstrate the induction of donor-specific unresponsiveness to islets. In addition, those results were reproduced in diabetic recipients intravenously injected with in vivo allopeptide-activated T-cells combined with ALS transient immunosuppression. These data demonstrate a direct link between in vivo generation of alloreactive T-cells induced by intravenous injection of P5-pulsed self-DCs and the induction of acquired thymic tolerance. To further evaluate the role of the host thymus in the induction of tolerance in this model, thymectomy was performed before intravenous transfer of allopeptide activated T-cells. The results showed that thymectomy abrogated the induction of tolerance. This finding led us to conclude that the underlying mechanism of tolerance induction by intravenous inoculation of P5-pulsed self-DCs is partially dependent on circulation of activated peripheral T-cells generated by indirect allorecognition to the native thymus. It is speculated that interaction of the peptide alloreactive T-cells with thymic DCs with specific TCRs of activated peripheral T-cells might lead to inactivation of the Ag-specific cells. The results of our preliminary studies that showed that intravenously administered, radiolabeled, P5-activated syngeneic T-cells reenter the thymus (unpublished data) confirmed our conclusion that the native thymus is required for induction of tolerance by adoptive transfer of alloMHC peptide-pulsed self-DCs. These data provide the first formal evidence that Ag-specific alloreactive peripheral T-cells that continuously circulate to the thymus play an important role in acquired systemic tolerance.

The finding that permanent graft survival is achieved

only in ALS transiently immunosuppressed syngeneic recipients of P5-primed self-DCs—unlike intrathymic inoculation of P5 alone, which led to 50% graft survival—raises the possibility that T-cell tolerance might be dependent on Ag load. Although the direct intrathymic inoculation of P5 might present sufficient Ag load to induce T-cell tolerance in the thymic microenvironment, intrathymic inoculation of P5-primed self-DCs requires additional recipient immunomodulation before similar results could be achieved. The use of ALS transient immunosuppression, which depletes or immunomodulates the mature peripheral T-cells, prevents graft rejection before the arrival of new thymic emigrants with Ag-specific tolerance in the peripheral lymphoid compartments. To make this model clinically relevant, we plan to identify the DC subsets that will induce T-cell tolerance. In human and murine models, three DC subsets have been identified, and their characterization is dependent on the cytokines used in the propagation of the DCs in culture (27). Although the subset designated DC1 is generated from myeloid DC precursor grown in GM-CSF/IL-4, DC2 generated from lymphoid DCs is not dependent on GM-CSF. The third DC subset, designated B-DC because the subset exhibits B-cell phenotype, is generated from the lymphoid DC precursor and requires GM-CSF/IL-4 or CD40L/IL-4 in culture (27). Identification and use of a specific DC subset in our future studies might further define which subsets have potential to prime or tolerize alloreactive T-cells.

In conclusion, we have confirmed that induction of acquired systemic tolerance by intrathymic inoculation of alloMHC peptide is attributable to indirect allorecognition in the thymus. Of interest is our finding that these results are reproducible in a clinically relevant model of intravenous transfer of allopeptide-pulsed host myeloid DCs. Furthermore, the underlying mechanism of T-cell tolerance induced by intravenous injection of allopeptide-primed myeloid or thymic DCs may, in part, be dependent on the circulation of in vivo–generated, peptide-specific alloreactive T-cells to the host thymus. Our data suggest that the use of host DCs as a vehicle to deliver specific target Ags to self–T-cells without ex vivo recipient manipulation is a simple, reproducible strategy that has great therapeutic potential in clinical islet transplantation for the treatment of type 1 diabetes.

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