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Development of Infectious Tolerance After Donor-Specific Transfusion and Rat Heart Transplantation

Masaaki Kataoka, Julie A. Margenthaler, Grace Ku, and M. Wayne Flye

Regulatory cells developed after donor-specific-transfusion (DST)-induced acceptance of a LEW heart transplanted into a DA rat. Both DST and the cardiac transplant were necessary to generate the regulatory cells. This donor-specific tolerance can then be transferred into a new DA recipient by adoptive transfer of lymphocytes from the DST-treated long term survivor (LTS) in a dose-dependent manner. The effectiveness of tolerance did not diminish over five generations of adoptive transfer, thus supporting its infectious nature. Although both spleen and lymph node cells were equally effective, graft-infiltrating lymphocytes were more potent. A high level of indirect CTL activity and MLC proliferation were observed in lymphocytes from LTS. In vivo tracking of adoptively transferred CFSE-labeled splenocytes from LTS showed equivalent FACs proliferation and a higher percentage of graft-infiltrating lymphocytes 7 days after heart transplantation, compared with adoptively transferred naive splenocytes. Adoptive transfer of CD8\(^+\)-depleted LTS splenocytes resulted in 100% subsequent LEW allograft acceptance; whereas CD4\(^+\) depletion decreased acceptance to 40%, and depletion of both CD4 and CD8 resulted in 0% acceptance. When positively selected CD4\(^+\) or CD8\(^+\) cells were adoptively transferred, 100% or 62.5% of LEW cardiac allografts survived, respectively. In conclusion, DST alone promotes a donor-specific infectious tolerance of a heart graft that can be adoptively transferred to subsequent naive allograft recipients despite the undiminished in vitro immunological response to donor Ag. Although both CD4\(^+\) and CD8\(^+\) populations are responsible for the regulatory mechanism in DST-induced tolerance, the CD4\(^+\) population appears to dominate.

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Establishment of tolerance to a well-functioning transplant with limited specific inductive therapy is a major goal of organ transplantation. Populations of lymphocytes with a regulatory effect specific to the donor transplant Ags can be generated in mice and rats treated perioperatively with a course of anti-CD4 and anti-CD8 mAbs (1–13). The cardinal feature of this phenomenon is that once a tolerant state is established, it can be perpetuated by the adoptive transfer of regulatory cells into subsequent recipients without additional mAb treatment. This condition has been termed infectious tolerance.

There is now a large body of evidence that the responsible regulatory lymphocyte population is a CD4\(^+\) T cell (2, 4–6, 11–13). However, there is evidence that CD8\(^+\) T lymphocytes can also exert regulatory activity (14–20). Thus, the regulatory cells contributing to the tolerant state appear to be of varying phenotypes, and their mechanism of action remains obscure. Therefore, a careful identification of the regulatory cells participating in tolerance induction will lead to a better understanding of the underlying regulatory mechanisms and then be used to actively enhance graft acceptance.

Donor-specific transusions (DST)\(^{1}\) induce both experimental and clinical donor-specific allograft tolerance (15, 18, 19, 21–34). However, the mechanisms of graft acceptance after DST are still unclear. The presence of donor-specific CTL in cardiac or renal allografts accepted after DST (21, 22, 28) indicates that clonal deletion of alloreactive T cells probably does not occur. Thus, other cells appear to be necessary to regulate these potentially reactive cells.

Recently, we have found that tolerance induced by DST, without mAb treatment, in LEW to DA rat heart transplantation can subsequently be transferred to a naive animal by the adoptive transfer of the tolerant recipient’s splenocytes (35). In this study, we characterize the regulatory cells in this DST-induced infectious tolerance.

Materials and Methods

Animals

Inbred male rats, weighing 200–250 g, were purchased from Harlan Sprague-Dawley (Indianapolis, IN), maintained under standard conditions, and allowed to drink water and eat rodent chow ad libitum. LEW (RT1\(^{l}\)) and DA (RT1\(^{a}\)) rats served as donor and recipient, respectively, for cardiac transplantation and Brown Norway rats (BN; RT1\(^{b}\)) were used as third-party donor controls. The care and use of laboratory animals conform to the National Institutes of Health and Washington University guidelines.

DST

Single-cell suspensions of LEW splenocytes were prepared in ice-cold RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10 mmol/L HEPES and 1% penicillin-streptomycin. RBC were lysed with 0.83% Tris-ammonium chloride buffer at 37°C for 5 min, and the remaining cells were washed twice with RPMI 1640. Splenocytes (100 \(\times\) 10\(^6\) in 1 ml of PBS) were injected i.v. into each rat via the penile vein as a source of DST. Seven days after DST, a LEW to DA heart transplantation was performed to produce long term recipients to provide tolerant cells for adoptive transfer.

Cardiac transplantation and induction of primary tolerance

Heterotopic cardiac transplantation was performed using the modified technique of Ono and Lindsey (36). Briefly, the donor thoracic aorta and pulmonary artery were anastomosed to the infrarenal recipient aorta and inferior vena cava, respectively. Graft survival was assessed by daily palpation and rejection was confirmed histologically.
Adoptive transfer study

To determine the presence of regulatory cells, a naive DA recipient was gamma-irradiated with 300 or 450 rad, and 100 × 10⁶ splenocytes harvested from a tolerant DA rat bearing a LEW heart for >60 days were adoptively transferred on the following day. A LEW heart was transplanted 24 h later. Irradiation was performed with a 137Cs irradiator (MarK I, model 30; J. L. Shepherd & Associates, San Fernando, CA). In some experiments, positive selection or depletion of CD4⁺ and/or CD8⁺ cells were performed with MACS Rat CD4 and/or CD8 MicroBeads (Miltenyi Biotec, Auburn, CA) before adoptive transfer. The resulting cell population after MACS manipulation was characterized by flow cytometry.

MLC

Stimulator cells (2 × 10⁵/ml) and MMC-treated stimulator splenocytes (5 × 10⁶/ml) were cocultured in 200 µl of RPMI 1640 supplemented with 10% FBS, 1% l-glutamine, 100,000 U/L penicillin-streptomycin, 10 mM HEPES, 1% sodium pyruvate, 1% nonessential amino acids, and 1 × 10⁻³ M 2-mercaptoethanol (complete RPMI) in a 96-well U-bottom microtiter plate for 4 days at 37°C in a 95% air, 5% CO₂ humidified atmosphere. [³H]Thymidine (1.0 Ci/mM; ICN Pharmaceuticals, Costa Mesa, CA) was added 18 h before harvesting, and [³H]thymidine incorporation was determined in a liquid scintillation counter (model 1450; Microbeta, Gaithersburg, MD). Each sample was performed in triplicate. The mean of triplicate samples was calculated, and percent lysis stimulation/cpm from syngeneic stimulation.

Direct and indirect CTL assay

Freshly isolated splenocytes were used as effector cells in the direct CTL assay without in vitro stimulation. For indirect CTL, responder (5 × 10⁵) cells were incubated with 50 µg/ml mitomycin C (MMC) for 40 min at 37°C and washed four times with RPMI 1640.Responder cells (2.5 × 10⁵) and MMC-treated allogeneic or syngeneic splenocytes (2.5 × 10⁶) were cocultured in 200 µl of RPMI 1640 supplemented with 10% FBS, 1% l-glutamine, 100,000 U/L penicillin-streptomycin, 10 mM HEPES, 1% sodium pyruvate, 1% nonessential amino acids, and 1 × 10⁻³ M 2-mercaptoethanol (complete RPMI) in a 96-well U-bottom microtiter plate for 4 days at 37°C in a 95% air, 5% CO₂ humidified atmosphere. [³H]Thymidine (1.0 Ci/mM; ICN Pharmaceuticals, Costa Mesa, CA) was added 18 h before harvesting, and [³H]thymidine incorporation was determined in a liquid scintillation counter (model 1450; Microbeta, Gaithersburg, MD). Each sample was performed in triplicate. The mean of triplicate samples was calculated, and percent lysis stimulation/cpm from syngeneic stimulation.

In vivo tracking of adoptively transferred lymphocytes

Labeling of splenocytes with CFSE was performed (38) by resuspending splenocytes in PBS at 1 × 10⁶ cells/ml and incubating with CFSE (Molecular Probes, Eugene, OR) at a final concentration of 5 µM for 10 min at 37°C. Labeling was terminated by the addition of FBS (10% of total volume). Splenocytes were washed twice with complete RPMI and resuspended in PBS at 1 × 10⁶ cells/ml for i.v. injection. CFSE-labeled splenocytes (100 × 10⁶) were adoptively transferred into a 450-rad irradiated naive DA rat that was transplanted with a LEW heart 24 h later. Seven days after heart transplantation, splenocytes and heart graft-infiltrating lymphocytes (GIL) were isolated for flow cytometric analysis. As a control, irradiated (450 rad) and nonirradiated DA rats were adoptively transferred with CFSE-labeled naive DA splenocytes without heart transplantation, and 8 days later splenocytes were also isolated for flow cytometric analysis.

Isolation of GIL

Graft tissue was minced through a 50-µm pore size stainless steel sieve into RPMI 1640 supplemented with 10% FBS. The tissue suspension was then digested with 1 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) for 60 min at 37°C. After two washes, viable lymphocytes were separated on a Ficoll gradient (Histopaque-1077, Sigma-Aldrich). Cells were washed three times with RPMI 1640 supplemented with 10% FBS and used for additional experiments.

Flow cytometric analysis

Cells were incubated with Cy-Chrome-conjugated anti-rat CD4 mAb (mouse IgG2a, clone OX35), FITC-conjugated anti-rat CD8a mAb (mouse IgG1, clone OX8), and PE-conjugated anti-rat CD3 mAb (mouse IgG3, clone G4.18) (all from BD Pharmingen, San Diego, CA) for 30 min at 4°C. The cells were washed, resuspended in PBS, and analyzed by FACScan (BD Biosciences, Mountain View, CA) using CellQuest (BD Biosciences) software.

Results

DST-induced tolerance was serially transferable in irradiated recipients

Adoptive transfer of 100 × 10⁶ splenocytes from DST-treated LTS (graft accepted for >60 days) into a 300-rad (n = 5) or 450-rad (n = 10)-irradiated naive DA recipient resulted in the acceptance of all LEW cardiac allografts (mean survival time (MST) > 100 days in both groups), indicating the presence of donor regulatory cells (Fig. 1). To demonstrate donor specificity, third-party BN hearts were also transplanted into DA recipients that had been irradiated with 300 or 450 rad and given splenocytes from DA long term survivors (LTS) with a LEW heart. Rejection of all BN grafts in MST of 11.3 ± 0.6 days (300 rad, n = 3) and 23.8 ± 8.3 days.
adoptive transfer of $100 \times 10^6$ splenocytes from a DST-treated LTS rejected a LEW heart in MST of 8.3 ± 0.6 days ($n = 3$).

The transfer of tolerance was dose dependent (Fig. 2) because $\geq 30 \times 10^6$ splenocytes transferred tolerance to all 450-rad-irradiated recipients, but $1 \times 10^6$ and $10 \times 10^6$ splenocytes were insufficient to prevent acute rejection of the cardiac transplant (19.0 ± 2.8 or 13.0 ± 1.4 days, respectively). Because one of two 300-rad-irradiated recipients injected with $50 \times 10^6$ splenocytes rejected a LEW heart graft, a 450-rad dose was used in all subsequent experiments.

We then determined whether the tolerant state could be serially transferred to subsequent cardiac recipients with $100 \times 10^6$ LTS splenocytes from each generation (Table I). After the first adoptive transfer, those rats accepting a LEW heart graft for >100 days were used for further serial transfer experiments. When $100 \times 10^6$ splenocytes from these LTS recipients were transferred into another 450-rad-irradiated naive DA rat (second adoptive transfer), all of the LEW heart grafts transplanted 24 h later survived for >100 days. We have observed that even after the fifth serial adoptive cell transfer from these recipients, the cardiac graft was uniformly accepted for >100 days.

**Both LEW DST and a LEW heart allograft were necessary to generate regulatory lymphocytes**

The adoptive transfer of cells from a DA rat receiving only LEW DST, but no heart transplant, did not lead to LEW heart graft acceptance in any irradiated naive recipient. Splenocytes ($100 \times 10^6$) adoptively transferred from a DA rat 7 days after DST or a DA rat >60 days after DST treatment without LEW heart transplantation did not prevent rejection of LEW heart grafts in MST of 10.5 ± 5.4 and 5.6 ± 0.5 days, respectively (Fig. 3). Therefore,

**Table I. Survival of LEW cardiac allograft after serial adoptive transfer**

<table>
<thead>
<tr>
<th>Group</th>
<th>Source of Adoptively Transferred Cells</th>
<th>Survival of LEW Heart Graft (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1st adoptive transfer)</td>
<td>DA recipient &gt;60 days after DST and heart transplant</td>
<td>&gt;100 ($n = 10$)</td>
</tr>
<tr>
<td>2 (2nd adoptive transfer)</td>
<td>Group 1 DA recipient &gt;100 days after LEW heart transplantation</td>
<td>&gt;100 ($n = 6$)</td>
</tr>
<tr>
<td>3 (3rd adoptive transfer)</td>
<td>Group 2 DA recipient &gt;100 days after LEW heart transplantation</td>
<td>&gt;100 ($n = 6$)</td>
</tr>
<tr>
<td>4 (4th adoptive transfer)</td>
<td>Group 3 DA recipient &gt;100 days after LEW heart transplantation</td>
<td>&gt;100 ($n = 3$)</td>
</tr>
<tr>
<td>5 (5th adoptive transfer)</td>
<td>Group 4 DA recipient &gt;100 days after LEW heart transplantation</td>
<td>&gt;100 ($n = 3$)</td>
</tr>
</tbody>
</table>
DST alone was ineffective without the presence of a heart allograft in generating the regulatory cells of this infectious tolerance.

**DST-tolerant lymphocytes are not compartmentalized**

Zhai et al. (13) has reported that lymphocytes capable of transferring tolerance were compartmentalized only in the spleen, and were not present in the lymph nodes (LNs) of LTS after anti-CD4 mAb treatment. To determine whether this occurred in our DST tolerant model, 100 x 10^5 LN cells from DST-treated DA LTS were adoptively transferred into a 450-rad-irradiated DA recipient 24 h before a LEW heart transplant. All rats given 100 x 10^5 LN cells (n = 5) accepted cardiac allografts for >100 days, similar to that seen when 100 x 10^6 LTS splenocytes were transferred. We also confirmed that 50 x 10^6 LN cells or splenocytes could transfer tolerance into a 450-rad-irradiated DA rat. Therefore, both splenocytes and LN cells were equally effective in transferring tolerance to a DA heart graft.

**GIL were more effective for transfer of tolerance**

Considering that graft-infiltrating alloreactive recipient cells encounter the donor Ag at the graft site, it is possible that more regulatory cells reside in the graft. To examine this possibility, we transferred isolated GIL from a recipient accepting a LEW heart graft for >60 days into a 450-rad-irradiated DA rat (Fig. 4). Because only a small number of GIL can be harvested from long term accepted heart grafts, all of the GIL harvested from a single heart graft were adoptively transferred (0.3–3 x 10^6). However, two of the four subsequent LEW cardiac allografts were accepted. Because 30 x 10^6 LTS splenocytes were necessary to transfer tolerance, GIL were 10 to 100 times more effective in transferring tolerance. To avoid losing cells in GIL isolation, we harvested the tolerant graft 60 days after heart transplantation and retransplanted into a 450-rad-irradiated DA recipient. Three of four LEW retransplanted grafts were accepted. To examine whether lymphocyte populations changed in the GIL, we performed FACS analysis of GILs and splenocytes from LTS. The proportions of these subsets are similar in the two populations (31.84% vs 41.37% for CD4^+ T cells, 3.27% vs 4.12% for CD25^+CD4^+ T cells, and 10.72% vs 9.18% for CD8^+ T cells).

**LTS lymphocytes do not suppress MLC proliferation**

When 2.5 x 10^5 responder LN cells from 1) naive DA, 2) DST-treated DA LTS, or 3) DA 7 days after DST without heart transplant were cultured with 2.5 x 10^6 MMC-treated LEW (allogeneic), DA (syngeneic), or BN (third-party) stimulator splenocytes for 4 days, there was no significant difference in the proliferative response among these three groups (Fig. 5). To determine whether lymphocytes from 2) DST-treated DA LTS or 3) DA 7 days after DST (suppressed 1) naive DA lymphocytes in vitro, 1.25 x 10^5 naive DA lymph node cells mixed with the same number of lymph node cells from 2) DST-treated DA LTS or 3) DA 7 days after DST (total 2.5 x 10^5 responder cells) were cocultured with 2.5 x 10^5 MMC-treated stimulator splenocytes for 4 days. There was no suppressive effect by lymphocytes from DST-treated DA LTS or DA 7 days after DST on the normal DA response to LEW rat Ags (Fig. 5).

**Cytotoxicity of splenocytes from DST-treated LTS**

The development of indirect CTL activity by splenocytes was examined in the following groups: group 1, naive DA; group 2, DA rejecting a LEW heart 12 days after transplantation without DST-pretreatment; group 3, DA 7 days after LEW DST without LEW heart transplant; group 4, LEW DST-treated DA LTS (>60 days); and group 5, LTS after the first adoptive transfer (Fig. 6A). CTL activity observed in the splenocytes from DST-treated LTS (group 4, 70% at E:T of 100:1) was equal to that seen in an untreated animal rejecting a graft (group 2) or treated only with DST (group 3) and higher than that seen with nontransplanted naive DA.
splenocytes (group 1). The indirect cytotoxicity of splenocytes from a LTS after the first adoptive transfer (group 5) was also equivalent to that of naive DA splenocytes (group 1). The specificity of CTL generated from DST-treated LTS splenocytes was donor specific in that cytotoxicity was observed against LEW Con A blasts but not against DA or BN blasts (Fig. 6B). No direct

FIGURE 6. Cytotoxicity of splenocytes from DST-treated DA LTS. A, Generation of CTL from the spleens of naive DA (A, group 1), DA rejecting a LEW heart 12 days after transplantation without DST pretreatment (B, group 2), DA 7 days after LEW DST without LEW heart transplant (×; group 3), LEW DST-treated DA LTS (>60 days; E, group 4), and LTS after the first adoptive transfer (E, group 5). Effector splenocytes were incubated with MMC-treated naive LEW splenocytes for 7 days before culturing with LEW As-F4 target cells in CTL assay. B, Specificity of DST-treated LTS (>60 days) splenocyte cytotoxicity. Effector splenocytes were incubated with MMC-treated naive LEW splenocytes for 7 days and splenic Con A blast from LEW (●), DA (○), and BN (▲) were used as targets. C, Direct cytotoxicity of splenocytes from naive DA (A, group 1), DA rejecting a LEW heart 12 days after transplantation without DST pretreatment (B, group 2), DA 7 days after LEW DST without LEW heart transplant (×; group 3), LEW DST-treated DA LTS (>60 days; E, group 4), and LTS after the first adoptive transfer (E, group 5) against LEW As-F4 target cells. Results are representative of three separate experiments.

FIGURE 7. In vivo tracking of adoptively transferred splenocytes in DA recipients. Eight days after CFSE-labeled naive DA splenocytes were adoptively transferred into a nonirradiated (A) and 450-rad-irradiated (B) DA rat, splenocytes were isolated for FACS analysis. CFSE-labeled splenocytes from naive DA (C and E) or DST-treated LTS (D and F) were adoptively transferred into 450-rad-irradiated naive DA recipient and transplanted 24 h later with a LEW heart. Splenocytes (C) and GIL (E) were isolated from a recipient that received CFSE-labeled naive splenocytes. Splenocytes (D) and GIL (F) were isolated from a recipient that received CFSE-labeled splenocytes from DST-treated LTS. Results are representative of two separate experiments.
cytotoxicity (without in vitro stimulation) was observed with freshly isolated splenocytes from any of groups 1–5 (Fig. 6C).

Tracking of adoptively transferred splenocytes in recipients

Adoptively transferred CFSE-labeled naive DA splenocytes were barely detectable (0.20%) in the spleen of nonirradiated naive DA recipients 8 days after adoptive transfer without heart transplantation (Fig. 7A). The percent of adoptively transferred DA splenocytes detected in DA recipients irradiated with 450 rad before adoptive transfer increased to 3.07% (Fig. 7B). Adoptively transferred splenocytes from naive DA or DST-treated DA LTS settling in the recipient spleen were further increased (12.99 and 13.48%, respectively) when a DA recipient was transplanted with a LEW heart (Fig. 7, C and D). The similar proliferation of adoptively transferred naive DA T cells (2.95 and 1.57%, respectively) when a DA recipient was transplanted with a LEW heart, respectively, whereas only 62.5% of the LEW allografts were accepted when 10^6 unfractionated DST-treated LTS splenocytes uniformly transferred tolerance (Fig. 8B). Because CD4+ and CD8+ cells comprise 40% or 10% of splenocytes, respectively, we transferred 40 x 10^6 CD4+ cells or 10 x 10^6 CD8+ cells to reflect the actual number of these populations in 100 x 10^6 unfractionated splenocytes. Adoptive transfer of 40 x 10^6 (n = 6) or 10 x 10^6 (n = 3) CD4+ cells resulted in the acceptance of all transplanted LEW hearts, respectively, whereas only 62.5% of the LEW allografts were accepted when 10 x 10^6 CD8+ cells (n = 8) were transferred. These results confirmed that although CD4+ T cells are the primary regulatory population that transfers infectious tolerance, CD8+ cells also provide a less potent regulatory population.

Both CD4+ and CD8+ T lymphocytes contribute to immune regulation

We examined the relative contribution of CD4+ or CD8+ cells to transfer infectious tolerance. Splenocytes harvested from DST-treated LTS were depleted of CD4+ and/or CD8+ cells with magnetic beads before transfer to a 450-rad-irradiated DA recipient (Fig. 8A). Thirty million depleted splenocytes were used because 3 x 10^7 unfractionated DST-treated LTS splenocytes uniformly transferred tolerance (Fig. 2). When CD8+ -depleted splenocytes were transferred, all (n = 5) of the transplanted LEW cardiac allografts were accepted. However, when CD4+ -depleted splenocytes (n = 10) were transferred, only 40% of LEW allografts were accepted. The depletion of both CD4 and CD8 populations removed all T lymphocytes and resulted in the rejection of all (n = 5) allografts in a MST of 14.0 ± 2.3 days. We also adoptively transferred positively selected CD4+ or CD8+ T cells (Fig. 8B). Because CD4+ or CD8+ cells comprise 40% or 10% of splenocytes, respectively, we transferred 40 x 10^6 CD4+ cells or 10 x 10^6 CD8+ cells to reflect the actual number of these populations in 100 x 10^6 unfractionated splenocytes. Adoptive transfer of 40 x 10^6 (n = 6) or 10 x 10^6 (n = 3) CD4+ cells resulted in the acceptance of all transplanted LEW hearts, respectively, whereas only 62.5% of the LEW allografts were accepted when 10 x 10^6 CD8+ cells (n = 8) were transferred. These results confirmed that although CD4+ T cells are the primary regulatory population that transfers infectious tolerance, CD8+ cells also provide a less potent regulatory population.

Discussion

In this study, we have shown that the tolerance to a major MHC-mismatched cardiac allograft induced by LEW DST alone can be adoptively transferred by DA LTS splenocytes and LN cells to a lightly irradiated naive DA recipient. Waldmann’s group induced a similar state of acceptance for mouse skin grafts across multiple minor histocompatibility barriers and for mouse heart transplants across a complete MHC histocompatibility barrier by using a combination of nondepleting anti-CD4 and anti-CD8 mAbs (1–5). That they could adoptively transfer this tolerant state over 10 generations (4) emphasized the ability of the regulatory cells to infect rather than to merely suppress graft-rejecting recipient cells. Qin et al. (2) showed that naive mouse splenocytes not expressing human (h) CD2 infused into hCD2+/CBA-transgenic mice tolerized to B10.BR skin allografts remained tolerant to B10.BR skin if the tolerant hCD2+/CBA cells were Ab depleted only after 2 weeks of coexistence. Therefore, the “infection” of transferred hCD2+ cells occurred before the tolerant hCD2+ cells were eliminated. Our study, which used only DST, without Ab treatment, also results in tolerance to rat heart grafts that can be transferred over at least five generations. This suggests that the mAbs may induce infectious tolerance by evoking a natural inherent regulatory immune mechanism and are not an artifact of the therapeutic drugs. Because the splenocytes initially tolerized by DST are diluted out in serial adoptive transfers, the regulatory cell must be amplified in the recipient to perpetuate subsequent tolerance by either direct clonal expansion or active infectious recruitment of recipient cells.

Our study found equal transfer of tolerance by lymph node cells and splenocytes. This contrasts with the reported compartmentalization of the tolerogenic rat lymphocytes in the spleen, but not in lymph nodes, in recipients given peritransplant nondepleting anti-CD4 mAb (RIB 5/2) (13). This difference may reflect the different
methods of primary tolerance induction. Zhai et al. (13) exposed the recipient rat to a skin graft 1 week before heart transplantation under the cover of 10 injections of anti-CD4 mAbs from the day of skin grafting to 3 wk after heart transplantation. In contrast, we gave only a single preoperative DST 7 days before heart transplantation.

Recently, the ability of GIL to transfer tolerance has been reported (20, 39, 40). Sawitzki et al. (39) reported that GIL in the tolerant rat kidney graft induced by anti-CD4 mAbs are enriched and could prevent the rejection of a subsequent fresh B10.BR skin graft by transfused naive CBA/Ca splenocytes. Our transfer of tolerance with GIL isolated from tolerant heart grafts shows the presence of regulatory cells in the GIL. We also found that GIL are much more effective than splenocytes or LN cells in transferring tolerance.

Quigley et al. (23) reported that DA lymph node cells harvested 7 days after LEW DST suppressed proliferation to LEW stimulator cells in 4-day MLC. In contrast, our MLC assays showed no suppression of proliferation by DA lymph node cells from either DST-treated LTS or DA 7 days after DST. However, in vivo tracking of adoptively transferred CFSE-labeled splenocytes showed equivalent proliferation of adoptively transferred DST-treated LTS and naive DA splenocytes in the spleen of the recipient. A higher percent of DST-treated LTS splenocytes was observed in heart GIL 7 days after heart transplantation than naive splenocytes. Thus, the proliferation of splenocytes from DST-treated LTS in response to donor Ag occurs in recipients and expands the regulatory population.

Recently, Lin et al. (41) reported that when adoptively transferred into tolerant mice, CD8+ T cells monospecific for the tolerated transplant Ag could proliferate and accumulate to the same extent as in a naive host but, in contrast, could not cause graft rejection, express IFN-γ, or generate CTLs. They suggested that the tolerance was mediated by regulatory T cells that censored immune effector functions rather than suppressing the induction of T cell responses. Unlike their Ab-induced tolerance, our DST-induced tolerance generated CTL after in vitro stimulation. Equal numbers of graft-infiltrating cells (32) and an equivalent level of anti-donor cytotoxic activity (21, 22, 28) have been reported for DST-treated recipients compared with untreated recipients. Our results showed that DST-induced LTS splenocytes generated cytotoxicity after in vitro stimulation, equal to that seen in splenocytes from a rat rejecting a graft without DST pretreatment or a rat treated with DST alone but without heart transplantation. Together, these findings suggest that, after initial preoperative DST stimulation and subsequent heart transplantation, persisting CTL precursors (CTLp) are regulated to achieve tolerance. Generation of our indirect cytotoxicity in splenocytes from DST-treated LTS reflects the in vitro differentiation of LTS CTLp into effector CTL by release from this in vivo regulation.

The method used for inducing tolerance may also influence the phenotype of the regulatory T cells imparting infectious tolerance. Waldmann’s group showed that tolerant cells selectively depleted of CD4+ cells failed to transfer the mAb-induced tolerance (2, 4, 5). Wood’s group found that pretreatment with DST alone was not sufficient to establish the tolerance and the addition of depleting anti-CD4 mAb was necessary to induce a regulatory population of murine lymphocytes (CD45RBlowCD25+CD4+ cells) that could adoptively transfer the donor specific tolerance to a subsequent naive mouse heart recipient (10–12). In skin-sensitized rats tolerized with a nondepleting anti-CD4 mAb (RIB 5/2), Onodera et al. (6) showed that the depletion of adoptively transferred CD4+, but not CD8+, cells prevented the development of infectious tolerance. Regardless of the animal model used, CD4 cells have uniformly been reported to exert regulatory effect.

Our failure to induce infectious tolerance by depletion of both CD4+ and CD8+ splenocytes indicates that the regulatory cells reside within the T cell population. CD8-depleted splenocytes or positively selected CD4+ cells uniformly transferred tolerance also clearly identified CD4+ cells as regulatory cells. However, we also found that CD4-depleted cells or positively selected CD8+ cells could transfer tolerance to 40 or 62.5% of the naive recipients, respectively. Recently, Nicolls et al. (42) reported that the ability to transfer established tolerance to islet allograft induced by anti-CD154/anti-LFA-1 therapy was only partially inhibited by depleting CD4 T cells from tolerant cells. The suppressive effect of CD8+ T cells has been previously reported in several transplantation models (14–20). Padberg et al. (14) demonstrated that both CD4+ and CD8+ T cells from LTS, induced by DST plus hyperimmune serum treatment, suppressed rat heart transplant rejection when adoptively transferred to a naive recipient. Oluwole et al. (15) showed that adoptive transfer of 5 × 106 LEW DST induced LTS ACI CD8+ T cells prolonged subsequent LEW heart graft survival in an unmodified ACI recipient. Douillard et al. (18) found that the expansion of a CD8+ clone bearing the Vβ18-DJβ1-Jβ2.7 TCR gene rearrangement specific for donor MHC was detected in each LEW.1A rat that was tolerized to LEW.1W hearts after DST. They also reported that anti-TCR Vβ18-DJβ1-Jβ2.7 DNA vaccination abolished this DST induced allograft tolerance (19). Recently, Zhou et al. (20) reported the generation of CD8α regulatory GIL in a renal allograft after oral administration of donor splenocytes. They showed that although those CD8+ graft-infiltrating cells expressed increased direct CTL activity, they could adoptively transfer allograft tolerance into a naive recipient.

In conclusion, despite the enhanced in vitro immunological reactivity against donor Ag, rats treated with DST alone accept a donor-specific heart transplant and develop an infectious tolerance similar to that achieved in previous studies using in vivo mAb administration. We found that both CD4+ and CD8+ populations were responsible for the regulatory mechanism in DST-induced tolerance, with the CD4+ population playing the major role. This infectious tolerance can be expanded and serially transferred to subsequent naïve cardiac recipients.

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