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J Immunol 2003; 170:1846-1853; doi: 10.4049/jimmunol.170.4.1846
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Role of Double-Negative Regulatory T Cells in Long-Term Cardiac Xenograft Survival

Wen Hao Chen,* Megan S. Ford,* Kevin J. Young,* Myron I. Cybulsky,* and Li Zhang²*†

A novel subset of CD3⁺CD4⁻CD8⁻ (double negative; DN) regulatory T cells has recently been shown to induce donor-specific skin allograft acceptance following donor lymphocyte infusion (DLI). In this study, we investigated the effect of DLI on rat to mouse cardiac xenotransplant survival and the ability of DN T cells to regulate xenoreactive T cells. B6 mice were given either DLI from Lewis rats, a short course of depleting anti-CD4 mAb, both DLI and anti-CD4 treatment together, or left untreated. DLI alone did not prolong graft survival when compared with untreated controls. Although anti-CD4-depleting mAb alone significantly prolonged graft survival, grafts were eventually rejected by all recipients. However, the combination of DLI and anti-CD4 treatment induced permanent cardiac xenograft survival. We demonstrate that recipients given both DLI and anti-CD4 treatment had a significant increase in the total number of DN T cells in their spleens when compared with all other treatment groups. Furthermore, DN T cells harvested from the spleens of DLI plus anti-CD4-treated mice could dose-dependently inhibit the proliferation of syngeneic antidonor T cells. Suppression mediated by these DN T cells was specific for antidonor T cells as T cells stimulated by third-party Ags were not suppressed. These results demonstrate for the first time that a combination of pretransplant DLI and anti-CD4-depleting mAb can induce permanent survival of rat to mouse cardiac xenografts and that DN T regulatory cells play an important role in preventing long-term concordant xenograft rejection through the specific suppression of antidonor T cells. The Journal of Immunology, 2003, 170: 1846–1853.

The shortage of human organs and tissues has severely limited the application of transplantation for the treatment of human diseases. Therefore, xenotransplantation has been of increasing interest in the past decade. One of the major barriers to successful xenotransplantation has been the potent immune response of recipients against xenografts, which leads to rejection of transplanted organs. Due to the recent progress in overcoming hyperacute rejection (1, 2), acute and chronic xenograft rejections are now the most significant obstacle to successful xenotransplantation. Immunosuppressive agents, which effectively prevent cell-mediated rejection in allogeneic transplantation, are less effective in controlling the cellular immune response to xenografts (2, 3). Moreover, attempts at increasing the dose of immunosuppressive agents to avoid cellular xenograft rejection increase toxicity and render the recipient highly susceptible to infectious complications (2, 4). Therefore, the success of xenotransplantation will likely depend, at least in part, on finding ways of inducing specific hypersensitiveness or tolerance across xenogeneic barriers rather than relying entirely on nonspecific immunosuppressive agents (2, 4). To achieve this goal, it is important to understand the mechanisms leading to long-term xenograft survival.

Various approaches that are very effective at inducing tolerance to allografts have been used to enhance xenograft survival (5–8). For instance, treatment with nondepleting anti-CD4 mAb has been clearly demonstrated to induce infectious donor-specific allogeneic transplantation tolerance (9). Treatment of recipients with anti-CD4 mAb either alone, or in combination with anti-CD8 mAb, has also been shown to enhance xenogeneic heart and islet graft survival (7, 8, 10). Similarly, blocking costimulatory signals through B7-CD28 or CD40-CD40 ligand, which leads to permanent allogeneic heart and skin graft acceptance (11–15), can also enhance survival of xenoislet, skin, and cardiac grafts (16, 17). However, compared with what is seen in allogeneic transplantation models, these treatments do not produce robust tolerance to xenografts, they either require relatively long-term treatment or are unable to induce permanent graft survival. Furthermore, the mechanisms involved in the prolongation of xenograft survival by these treatments remain to be determined.

Pretransplant donor lymphocyte infusion (DLI)³ is another approach that has been shown to improve allograft survival in renal transplant patients (18, 19). Experimental and clinical evidence indicate that under certain conditions DLI can induce specific tolerance to donor alloantigens while retaining immune responses to third-party Ags (19–23). When used in combination with other therapies such as anti-CD4 and anti-CD40 ligand, DLI is also able to prolong skin and islet xenograft survival (24). However, the use of both DLI and anti-CD4 mAb treatment to induce cardiac xenograft survival has not previously been attempted.

Although the underlying mechanisms of donor-specific transplant tolerance following DLI are unclear, several models have implicated the involvement of regulatory T cells (25–28). Recently, we have identified and cloned a novel subset of αβ-TCR⁺CD4⁻CD8⁻ double-negative (DN) regulatory T cells from mice that permanently accepted donor-specific skin allografts after

³Abbreviations used in this paper: DLI, donor lymphocyte infusion; DN, double negative; MST, mean survival time.

Received for publication October 3, 2002. Accepted for publication December 11, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by a grant from the Roche Organ Transplantation Research Foundation (Grant 239855245) and Canadian Institutes of Health Research (MOP 14431).

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pretransplant DLI (26, 29). The DN regulatory T cells are able to specifically kill activated syngeneic antidonor CD8+ T cells and enhance donor-specific skin allograft survival when adoptively transferred into naive syngeneic mice (26). It is uncertain whether regulatory T cells, including DN regulatory T cells, can also play a part in preventing xenograft rejection.

The objectives of this study were to establish a model in which permanent xenograft survival can be achieved without the need of long-term nonspecific immunosuppressive therapy and to understand the mechanisms involved. We demonstrate that a combination of pretransplant DLI and a short course of depleting anti-CD4 mAb result in permanent acceptance of concordant cardiac xenografts. Furthermore, DLI leads to a significant increase in the number of recipient-derived DN regulatory T cells which can specifically suppress antidonor T cells. These findings indicate, for the first time, that DN regulatory T cells are involved in DLI-mediated xenotransplantation tolerance.

**Materials and Methods**

**Animals**

C57BL/6 (B6) and BALB/c mice were purchased from Harlan Breeders (Indianapolis, IN) and used between 12 and 15 days of age. Adult Lewis and Wistar Furth (WF) rats were purchased from Harlan Breeders and used between 8 and 10 wk of age. All of the animals were kept in the animal facility at the University Health Network (Toronto, Canada).

**DLI and depleting CD4+ T cells in vivo**

Lymphocytes were collected from the spleen of Lewis rats, and single-cell suspensions were prepared by gently pressing the spleen against a fine metal screen. The RBC were depleted using RBC lysis buffer. The remaining lymphocytes were injected i.v. with 4 × 10^6 cells 2 days before transplantation. Recipient mice were injected i.p. with 400 μg/mouse YTS191.1 (anti-CD4-depleting) mAb (30) on days −2, 0, 3, and 7 of transplantation with or without DLI.

**Cardiac transplantation**

Heart grafts from 12- to 15-day-old Lewis rats were heterotopically transplanted into the abdomen of 8- to 10-wk-old B6 recipients as described previously (31). Briefly, donor rats and recipient mice were anesthetized with an i.p. injection of 65 mg/kg pentobarbital. Donor hearts were chilled by perfusing the inferior vena cava with cold heparinized saline before ligation of the vena cava and pulmonary veins. The donor pulmonary artery and aorta were left open for anastomoses. The grafts were stored in 4 °C saline for <20 min. After exposing the inferior vena cava and abdominal aorta, the recipient's donor aorta and main pulmonary artery were anastomosed with recipient aorta and vena cava, respectively. The survival of the graft was monitored daily by palpation of the heart. Grafts were considered rejected when the heart beat could no longer be detected and confirmed by autopsy. Survival was expressed as mean survival time (MST) ± SD.

**Histology**

Cardiac xenografts from B6 mice were harvested, fixed in 10% buffered Formalin, embedded in paraffin, and sectioned. Sections were stained with H&E and examined under light microscopy by a cardiac pathologist.

**Isolation of CD4+, CD8+, and DN T cells**

Spleen cells were collected from treated or naive mice. After lysing the RBC, the single-cell suspensions were passed through a nylon wool column. The nonbinding cells (>90% CD3+ T cells) were collected and incubated with either anti-CD4-depleting mAb (RL172-4) (32) or anti-CD8-depleting mAb (3.168) (32) at 4°C for 30 min. After washing with 1% BSA/PBS, the cells were incubated with rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) for 45 min to deplete CD4+ and/or CD8+ T cells. Some of the remaining spleen cells from treated mice were further purified using a cell sorter (Coulter Epics V; Coulter, Hialeah, FL) following staining with FITC-conjugated anti-CD3, PE-conjugated anti-CD4 mAb, or CyChrome-conjugated anti-CD8 mAb (BD Pharmingen, San Diego, CA) to obtain purified CD4+, CD8+, or DN T cells.

**Cell surface marker staining**

Single-cell suspensions of spleenocytes were collected from recipient mice at various time points after transplantation. After lysing the RBC, the remaining cells were triple stained with CD3-FITC, CD4-PE, or CD8-Cy-Chrome or with NK1.1-PE, CD3-Cy-Chrome, CD4-FITC, or CD8-FITC. All of the fluorescence-conjugated mAbs specifically recognizing the mouse CD3, CD4, CD8, or NK cells were obtained from BD Pharmingen. Data were acquired and analyzed on an Epics XL-MCL flow cytometer (Coulter).

**Mixed lymphocyte reaction**

Spleen cells that were depleted of either CD4+ T cells or CD8+ T cells or purified spleen CD4+ T cells or CD8+ T cells from both treated mice and naive mice were used as responder cells in MLR. Varying numbers of responder cells (1 × 10^3–3 × 10^4 cells/well) were cocultured in 96-well plates with irradiated (20 Gy) sex-matched spleenocytes (3 × 10^7 cells/well) from Lewis or WF rats, or BALB/c mice, depending on the experiment, in α-MEM supplemented with 10% FCS, 50 U/ml rIL-2, and 300 U/ml rIL-4. After 3 days of incubation, 1 μCi of [3H]Tdr was added to each well. Sixteen hours later, cells were harvested and counted in a beta scintillation counter (TOPCOUNT; Packard Instrument, Meriden, CT).

**Suppression assays**

Naive B6 splenocytes were depleted of either CD4+ T cells or CD8+ T cells, and used as responder cells (8000 cells/well), and cocultured in 96-well plates with irradiated (20 Gy) sex-matched spleenocytes (3 × 10^7 cells/well) from Lewis or WF rats, or BALB/c mice, in α-MEM supplemented with 10% FCS, 50 U/ml rIL-2, and 300 U/ml rIL-4. Splenic DN T cells from either DLI/anti-CD4-treated or anti-CD4 mAb-treated mice were used as putative suppressors in standard suppression assays. Serial dilutions of suppressor cells were added to the MLR. After a 4-day incubation, 1 μCi of [3H]Tdr was added to each well. Sixteen hours later, cells were harvested and counted in a beta scintillation counter. Suppression was calculated using the equation: % suppression = (E - R)/(E), where E is the cpm of each well and R is the cpm of responder alone.

**Results**

Pretransplant DLI plus a short course of anti-CD4 mAb treatment induces permanent cardiac xenograft survival

To understand the mechanisms leading to xenotransplantation tolerance, we first needed to establish a model in which long-term xenograft survival can be reliably achieved. Both pretransplantation DLI (18–23) and anti-CD4 mAb (7, 30, 33, 34) have been shown to be effective in enhancing allograft survival in man and several animal models. Therefore, we investigated the effect of DLI and anti-CD4 mAb treatment on Lewis to B6 concordant cardiac xenotransplantation. B6 mice were given a single dose of DLI at 2 days before transplantation. We found, unlike that seen in allotransplant models (20, 21, 35), pretransplant DLI alone could not prolong Lewis heart graft survival in B6 recipients (Fig. 1a). The MST of Lewis heart grafts was 8.5 days, which was similar to those of untreated mice (MST, 8.0 days). Histological analysis showed a typical acute rejection in the xenografts from both non-treated and DLI-treated mice (Fig. 1b, i and ii).

We reasoned that the failure of DLI alone to protect xenografts could be due to the strong immune response to xenogenic proteins. Therefore, we investigated whether depleting recipient CD4+ T cells at the time of transplantation could enhance xenograft survival. B6 mice were given a short course of depleting anti-CD4 mAb on days −2, 0, 3, and 7 either alone or in combination with a single dose of DLI on day −2 before transplantation. Each mouse was transplanted with a Lewis heart graft on day 0 and graft survival was monitored daily. We found that treatment with anti-CD4 mAb alone significantly prolonged the survival of Lewis heart grafts (MST, 65 ± 19.7 days) compared with untreated mice (MST, 8.0 ± 0.6 days, p < 0.01). However, late-stage graft rejection was not prevented and all grafts were eventually rejected.
DLI/anti-CD4-treated recipients, even at were largely intact in the xenogeneic heart grafts harvested from bvi plantation (Fig. 1). Although the histology sections of DLI/anti-CD4-treated mice compared with syngeneic cardiac transplant controls (data not shown), there was no evidence of myocyte injury or fibrosis (Fig. 1). These data demonstrate that a short course of depleting anti-CD4 mAb can prolong xenograft survival but failed to induce tol-

Prolongation of xenograft survival by adoptive transfer of splenocytes from DLI/anti-CD4-treated recipients

The depletion of CD4+ T cells following anti-CD4 mAb treatment is transient, which may explain why all anti-CD4-treated mice eventually rejected xenografts. However, most of the DLI/anti-CD4-treated mice permanently accepted Lewis heart grafts (Fig. 1a), suggesting that the function of newly released antidonor T cells is inhibited in vivo by regulatory T cells. To test this hypothesis, spleen cells were harvested from both anti-CD4- and DLI/anti-CD4-treated recipients 25 days after transplantation and adoptively transferred into naive B6 mice. Lewis hearts were transplanted into recipients 1–2 days later. Naive B6 mice were transplanted with a Lewis heart without any pretreatment and served as controls. Fig. 2 shows that xenocardiac graft survival was prolonged significantly in the recipients that were infused with splenocytes from DLI/anti-CD4-treated mice (MST, 17.5 ± 5.4 days) compared with nontreated controls (MST, 8.0 ± 0.6 days, p < 0.01). In contrast, adoptive transfer of splenocytes from anti-CD4-treated mice did not result in prolongation of xenograft survival compared with controls (Fig. 2). These data indicate that significant numbers of regulatory cells exist in the spleen of DLI/anti-CD4-treated but not anti-CD4-treated mice. Since the only difference between the two treatment groups is DLI, this suggests that DLI may promote the generation of recipient regulatory cells.

Increase of NK1.1+ DN T cells in the spleens of tolerant recipients

Various subsets of regulatory T cells have been identified in different models, including CD4+ (36–39), CD8+ (40–42), CD4+ CD8− (DN) T cells (26, 29, 43, 44), and NK+ T cells (45, 46). To identify which subset of T cells may be responsible for the long-term cardiac xenograft survival, we next studied the in vivo kinetics of T cell subsets following xenotransplantation. B6 mice were treated with either DLI/anti-CD4 or anti-CD4 mAb alone followed by Lewis heart transplantation. Splenocytes were harvested from both groups of mice at various time points after transplantation and the total numbers of CD4+, CD8+, and DN T cells

FIGURE 2. Adoptive transfer of splenocytes from tolerant recipients prolongs xenograft survival. Splenocytes were collected from DLI/anti-CD4 mAb (●, n = 4) or anti-CD4 mAb-alone (▲, n = 3) treated recipients on day 25 after grafting and adoptively transferred into naive B6 recipients (4 × 107 cells/mouse). One or 2 days later, the splenocyte recipients were transplanted with Lewis heart grafts. Graft survival was significantly (p < 0.05) enhanced in recipient mice that had been adoptively transferred with cells from the DLI/anti-CD4 treatment group when compared with those that received cells either from the anti-CD4 mAb-alone treatment group or cells from the nontreated group (●, n = 6).
were compared. As expected, the total number of CD4\(^+\) T cells was drastically reduced in the spleens of both groups of recipients during the first few weeks of treatment due to the infusion of depleting anti-CD4 mAb. The number of CD4\(^+\) T cells gradually returned to normal levels (Fig. 3a, middle). The total number of CD8\(^-\) T cells increased until day 7, at which time the CD8\(^-\) T cell numbers decreased steadily to below pretransplant levels in both treatment groups (Fig. 3a, top). Interestingly, when the total number of CD3\(^+\)CD4\(^-\)CD8\(^-\) (DN) T cells was examined, the DLI/anti-CD4-treated mice had a significantly higher number of DN T cells in the spleen compared with anti-CD4 mAb-treated animals at all time points after transplantation (Fig. 3a, bottom).

Since both NK1.1\(^+\) and NK1.1\(^-\) DN T cells can function as regulatory cells (26, 29, 43–46), spleen cells from both treatment groups were also stained with mAb that specifically recognize CD3, CD4, CD8, and NK1.1 60 days following transplantation to identify whether regulatory NK1.1\(^+\) DN T cells or regulatory NK1.1\(^-\) DN T cells were activated and proliferated following the treatments. Fig. 3b shows that the NK1.1\(^+\) DN T cell population remained low 60 days following xenotransplantation and did not differ between the two treatment groups. However, the NK1.1\(^-\) DN T cell population was significantly increased in the DLI/anti-CD4 mAb treatment group compared with the anti-CD4 mAb-alone treatment group. Together, these results demonstrate that the combination of DLI and a short course of anti-CD4-depleting mAb leads to a significant increase in recipient peripheral NK1.1\(^-\) DN T cells, and suggest that pretransplant DLI may facilitate activation and proliferation of recipient DN T cells.

**Proliferation of splenic CD8\(^-\) and CD4\(^+\) T cells to donor Ag is specifically reduced in the presence of DN T cells**

To assess the function of DN T cells, we determined whether the presence of splenic DN T cells can affect the proliferation of syngeneic CD4\(^+\) or CD8\(^-\) T cells to donor-specific and third-party Ags in vitro. Spleen cells were collected from naive, anti-CD4-treated, or DLI/anti-CD4-treated mice at 60 days after transplantation and passed through nylon wool columns. The enriched T cell population was further depleted of either CD4\(^+\) or CD8\(^-\) T cells, leaving populations of either CD4\(^+\) plus DN T cells or CD8\(^-\) plus DN T cells. These populations were used as responder cells and stimulated by irradiated donor-specific (Lewis), third-party xenogeneic (WF), or allogeneic (BALB/c) spleen cells. As shown in Fig. 4, both CD4\(^+\) and CD8\(^-\) T cells from naive or anti-CD4-treated mice proliferated well in responding to all strains of stimulator cells. Although a normal proliferation was observed when stimulated by third-party xenoreactive or alloreactive, the proliferation of the mixtures of both CD8\(^-\) plus DN and CD4\(^+\) plus DN T cells from DLI/anti-CD4 mAb-treated recipients was significantly reduced in response to Lewis stimulators (p < 0.01, Fig. 4).

**No difference in responding to Ag stimulation in vitro between naive and DLI/anti-CD4-treated CD4\(^+\) and CD8\(^-\) T cells**

There are at least two possibilities that can explain the reduced antidonor responses in DLI/anti-CD4-treated mice. First, antidonor T cells might be unresponsive following treatment with DLI/anti-CD4. Second, the treatment might activate other T cells, such as DN regulatory T cells, which in turn could have suppressed the function of antidonor T cells. To determine whether DLI/anti-CD4 treatment leads to an unresponsiveness of recipient xenoreactive T cells, both CD8\(^-\) and CD4\(^+\) T cells were purified from the spleen of DLI/anti-CD4-treated xenograft recipients at 60 days after transplantation. The ability of these purified T cells to proliferate to donor-specific and third-party Ag stimulation was compared with purified CD8\(^-\) and CD4\(^+\) T cells from naive mice. As shown in Fig. 5, there is no difference in the ability between naive and DLI/anti-CD4-treated T cells to proliferate to xenograft stimulation. Neither purified naive nor DLI/anti-CD4-treated CD4\(^+\) T cells were able to proliferate upon encountering Lewis Ag, presumably due to the lack of recipient APC in the culture which are required for the indirect pathway of Ag presentation. On the other hand, purified CD8\(^-\) T cells from both naive and DLI/anti-CD4-
treated recipient mice proliferated equally well to Lewis Ag stimulation. These findings indicate that treatment with DLI/anti-CD4 does not induce clonal anergy in recipient T cells and that the reduced proliferation seen in Fig. 4 may be due to suppression by regulatory T cells.

Spleenic DN T cells are able to suppress proliferation of syngeneic CD4−/− and CD8− T cells in an Ag-specific manner.

To directly address the question of whether DLI/anti-CD4-treated recipient T cells could suppress the proliferation of naive xenoreactive T cells, DN T cells were isolated from the spleens of DLI/anti-CD4-treated and anti-CD4 mAb-treated B6 xenograft recipient mice 25 days after transplantation and used as putative regulatory cells in a standard suppression assay. The ability of these DN T cell populations to suppress the proliferation of naive CD4+ or CD8+ B6 splenocytes was compared with those obtained from anti-CD4 mAb-treated xenograft recipients. As shown in Fig. 6, both DN T cell populations were able to inhibit the proliferation of CD4+ (Fig. 6a) and CD8+ (Fig. 6b) antidonor T cells.
dose-dependent manner when equal numbers of DN T cells were used. However, the suppression was more pronounced using DN T cells obtained from DLI/anti-CD4-treated mice. Interestingly, the ability of DN T cells from DLI/anti-CD4 mAb-treated mice to suppress naive B6 CD8+ T cells was decreased when irradiated WF rat splenocytes or allogeneic BALB/c mouse splenocytes were used as stimulators (Fig. 6c). These data indicate that DN T cells are able to suppress the proliferation of both CD4+ and CD8+ T cells to xenogeneic Ags in an Ag-specific and dose-dependent manner. This finding is consistent with the results obtained from an allogeneic skin transplantation model (26, 29) and suggests that DLI can induce permanent acceptance of cardiac xenografts, at least partially, through the activation of recipient DN regulatory T cells.

Discussion

One of the major barriers toward understanding the mechanisms of long-term xenotransplantation tolerance has been the lack of good animal models in which long-term xenograft survival can be reliably achieved. Although various approaches were shown to be successful in inducing long-term xenograft graft survival (16, 24), reports of achieving permanent xenograft survival are rare. In a Syrian hamster to rat model, treatment with anti-CD4 mAb can enhance xenograft graft survival, but all of the grafts were eventually rejected during therapy (8). There is one report in which the injection of CBA/Ca mice with anti-CD4 and anti-CD8 mAbs induced long-term survival of PVG rat heart grafts (7). The mechanism by which anti-CD4 and anti-CD8 mAb treatment leads to acceptance of xenogeneic graft is not clear. Although the combination of DLI with anti-CD4 mAb treatment has been shown to be effective in the induction of long-term tolerance to allogeneic heart grafts (47), this approach has not yet been used to induce long-term cardiac xenograft survival. In this study, we demonstrated that the transient depletion of recipient CD4+ T cells leads to a significant prolongation of concordant xenograft survival. However, permanent acceptance of a Lewis rat cardiac xenograft by B6 mice can only be achieved by the combination of a short course of depleting anti-CD4 mAb treatment with a single dose of pretransplantation DLI. This finding indicates that it is possible to achieve long-term xenograft survival without the need for continuous immunosuppressive therapy. It also provides a model in which the cellular and molecular mechanisms involved in the maintenance of long-term xenograft survival can be investigated.

Numerous pieces of evidence indicate that regulatory T cells play an important role in the induction and maintenance of tolerance to self- and alloantigens (38, 39, 48–51). The role of regulatory T cells in down-regulating xenogeneic immune responses has not, however, been previously investigated. We found that the total numbers of CD4+ and CD8+ T cells are similar between DLI/anti-CD4-treated recipient mice that permanently accepted xenografts and anti-CD4 mAb-treated recipient mice that rejected xenografts (Fig. 3a). This finding indicates that DLI-induced long-term xenograft survival cannot be explained solely by clonal deletion. The ability of both CD8+ and CD4+ T cells from DLI/anti-CD4-treated mice to proliferate to donor Ags in vitro is similar to those from naive mice (Fig. 5), suggesting that the antidonor T cells are not anergic. Furthermore, adoptive transfer of spleen cells from DLI/anti-CD4- but not anti-CD4-alone-treated mice into naive B6 mice significantly prolonged xenograft survival (Fig. 2). Together, these findings strongly suggest that pretransplant DLI may facilitate the induction of regulatory cells which can inhibit the function of donor-reactive T cells and protect xenografts from rejection in the long term.

We and others have shown previously that pretransplant DLI alone can induce permanent skin and cardiac allograft survival in single MHC class I- or class II-mismatched transplantation models (20, 21, 26, 35, 52). In this study, we found that DLI when given alone, has no beneficial effect on concordant cardiac xenograft survival. Permanent cardiac xenograft survival can only be achieved by combining DLI with a short course of anti-CD4 mAb treatment (Fig. 1). Furthermore, although adoptive transfer of splenocytes from DLI/anti-CD4-treated mice in the absence of anti-CD4 treatment could prolong xenograft survival, it failed to induce permanent acceptance of the xenografts (Fig. 2). It is well known that CD4+ T cells play an important role in xenograft rejection (1, 2). Many studies have reported that treatment with anti-CD4 mAb, either alone (7, 30, 33, 34) or in combination with DLI (47, 53), can lead to prolongation or permanent acceptance of allografts. The mechanisms by which anti-CD4 mAb treatment enhances graft survival varies in different transplantation models (54–58).

We and others have demonstrated in single MHC class I- or class II-mismatched transplantation models that pretransplant DLI leads to activation of recipient DN regulatory T cells (29, 63). Furthermore, DN regulatory T cells and clones can specifically suppress and kill antidonor T cells in vitro and prolong allogeneic skin graft survival when adoptively transferred into naive animals (26). In this study, we also demonstrate that DN T cells can suppress the proliferation of recipient T cells to donor-specific, but not third-party allo- or xenoantigens in a dose-dependent manner (Fig. 6). These data strongly suggest that
in this model DN regulatory T cells play a role in the maintenance of long-term xenograft survival. Direct demonstration of the significance of DN T cells in the maintenance of long-term xenograft survival would require selective depletion of DN regulatory T cells in vivo. This experiment is presently not possible due to the lack of a mAb that can selectively recognize and deplete DN regulatory T cells in vivo.

In multicenter studies, pretransplant blood transfusion has been shown to enhance allograft survival (18–23), but can also sensitize patients to donor HLA alleles, precluding the possibility of subsequent transplants from donors that carry the same Ags (23). This presensitization was one of the major reasons leading to the abandonment of this once widely used procedure. In this study, we found that pretransplant DLI can be a double-edged sword, causing either accelerated rejection or long-term graft survival depending on when it is given. When pretransplant DLI is given 2 days before xenotransplantation, it does not sensitize recipients but induces long-term graft survival when accompanied by a short course of rapamycin on when it is given. When pretransplant DLI is given 2 days before xenotransplantation, it does not sensitize recipients but induces long-term graft survival when accompanied by a short course of rapamycin. This presensitization was one of the major reasons leading to the abandonment of this once widely used procedure. In this study, we found that pretransplant DLI can be a double-edged sword, causing either accelerated rejection or long-term graft survival depending on when it is given. When pretransplant DLI is given 2 days before xenotransplantation, it does not sensitize recipients but induces long-term graft survival when accompanied by a short course of rapamycin.


