# Both Infiltrating Regulatory T Cells and Insufficient Antigen Presentation Are Involved in Long-Term Cardiac Xenograft Survival

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# Both Infiltrating Regulatory T Cells and Insufficient Antigen Presentation Are Involved in Long-Term Cardiac Xenograft Survival[1]

**Wenhao Chen,*† Jun Diao,* Stanislaw M. Stepkowski,§ and Li Zhang[2]*†‡**

**We have previously shown that pretransplant donor lymphocyte infusion (DLI) together with transient depletion of $CD4^+$ T cells could induce permanent rat-to-mouse heart graft survival, whereas depleting $CD4^+$ T cells alone failed to do so. In this study, we investigated the mechanism leading to long-term xenograft survival. We found that peripheral $CD4^+$ T cells from DLI/anti-CD4-treated mice could mount rat heart graft rejection after adoptive transfer into B6 $CD4^{-/-}$ mice. Infusing donor-Ag-loaded mature dendritic cells (DCs) could break long-term cardiac xenograft survival in DLI/anti-CD4-treated mice. Interestingly, when the number and phenotype of graft-infiltrating cells were compared between anti-CD4- and DLI/anti-CD4-treated groups, we observed a significant increase in both the number and suppressive activity of $\alpha\beta$-$TCR^+CD3^+CD4^-CD8^-$ double negative regulatory T cells and decrease in the numbers of $CD4^+$ and $CD8^+$ T cells in the xenografts of DLI/anti-CD4-treated mice. Moreover, there was a significant reduction in MHC class II-high DCs within the xenografts of DLI/anti-CD4-treated recipients. DCs isolated from the xenografts of anti-CD4- but not DLI/anti-CD4-treated recipients could stimulate $CD4^+$ T cell proliferation. Our data indicate that functional anti-donor T cells are present in the secondary lymphoid organs of the mice that permanently accepted cardiac xenografts. Their failure to reject xenografts is associated with an increase in double negative regulatory T cells as well as a reduction in Ag stimulation by DCs found within grafts. These findings suggest that local regulatory mechanisms need to be taken into account to control anti-xenograft T cell responses.** ***The Journal of Immunology,*** **2007, 179: 1542–1548.**

Use of organs from other species may be one of the solutions for relieving the critical shortage of human organs for transplantation. Following transplantation, organ xenografts undergo several types and stages of immune rejection, including hyperacute, acute vascular, and cell-mediated rejection (1, 2). Ab and complement are the primary mediators of hyperacute and acute vascular rejection, which can be overcome or reduced by various approaches (3–5). In addition to overcoming hyperacute and acute vascular rejection, the success of organ xenotransplantation also requires a better understanding of cell-mediated rejection and developing approaches to prevent it. Cells from both innate and adaptive immune systems are involved in rejecting organ xenografts (2). T cells, a major mediator of allograft rejection, have been demonstrated to be important in xenograft rejection. It is believed that mechanisms of T cell responses to discordant xenografts are fundamentally similar to those involved in allograft rejection (2). However, the nonspecific immunosuppressive drugs used to prevent allograft rejection are less effective in controlling T cell responses to organ xenografts (2). Therefore, seeking other approaches to down-regulate T cell responses or induce T cell tolerance are required to achieve long-term organ xenograft survival.

One of the mechanisms of tolerance induction in allo-transplantation models suggests an important role for regulatory T cells (Tregs)[3] to actively control the pathologic immune responses (6). Recently, $CD4^+CD25^+$ and $CD8^+CD28^-$ Tregs have been shown to suppress xenogeneic T cell responses in vitro (7, 8). It is not clear whether these Tregs are involved in the prevention of organ xenograft rejection in vivo. $\alpha\beta TCR^+CD3^+CD4^-CD8^-$ double negative (DN) Tregs have been shown to contribute to the maintenance of immunologic self-tolerance (9), the prevention of transplant rejection (10, 11), and graft-vs-host disease (12) in various animal models. DN Tregs that suppress Ag-specific T cell responses can also be isolated from the peripheral blood of humans (13). We have previously shown that pretransplant donor lymphocyte infusion (DLI) can induce permanent allogeneic skin graft survival in single MHC class I mismatched models (10). Infusion of the DN Tregs obtained from the spleen of those allograft-tolerant mice leads to significant prolongation of donor-specific allograft survival in mice (10). Moreover, DN Tregs can also suppress T cell responses to xenografts. Using a rat-to-mouse concordant cardiac xenotransplantation models, we have shown that $CD4^+$, but not $CD8^+$, T cells are essential for organ xenograft rejection (14). Transient $CD4^+$ T cell depletion using anti-CD4 depleting mAb plus pretransplant DLI, but not anti-CD4 mAb treatment alone, induced permanent rat heart survival in mice (15).

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[3] Abbreviations used in this paper: Tregs, regulatory T cells; DN, double negative; DLI, donor lymphocyte infusion; GICs, graft-infiltrating cells; GI, graft-infiltrating; DCs, dendritic cells; MHC-II, MHC class II.

The goal of this study was to further delineate the mechanisms by which DLI/anti-CD4 treatment leads to long-term organ xenograft survival with a focus on the role of graft-infiltrating cells (GICs). We found that DN Tregs were significantly increased within the xenografts of DLI/anti-CD4 treated mice and retained their suppressive function. In addition, graft-infiltrating (GI) dendritic cells (DCs) from DLI/anti-CD4-treated mice expressed lower levels of MHC class II (MHC-II) and costimulatory molecules, and had impaired ability to stimulate naive T cell proliferation when compared with GI-DCs from anti-CD4-treated mice. To our knowledge, this is the first report that shows that both the presence of suppressive DN Tregs and "arrested" maturation of DCs within organ xenografts may contribute to long-term cardiac xenograft survival.

## Materials and Methods

### *Animals*

C57BL/6 (B6) and B6.129S2-*Cd4*$^{tm1Mak}$/J (B6 CD4$^{-/-}$) mice were purchased from The Jackson Laboratory. Lewis rats were obtained from Harlan Breeders and used either between 11 and 13 days of age as graft donors or between 6 and 8 wk for in vitro studies. All mice were kept in the animal facility at the University Health Network (Toronto, Canada) and were used according to the institutional guidelines.

### *Cardiac transplantation*

Heterotopic cardiac transplantation was performed using the techniques described previously (15). The survival of graft was monitored daily by palpation.

### *Isolation of CD4$^+$ or CD8$^+$ T cells*

CD8$^+$ or CD4$^+$ T cells were depleted in vivo by i.p. injection of 400 $\mu$g/mouse depleting anti-CD8 mAb (YTS169) or anti-CD4 mAb (YTS191.1). One day later, cells were obtained from the secondary lymphoid organs of these mice, passed through a nylon wool column to enrich the CD4$^+$ or CD8$^+$ T cell population. When needed, CD4$^+$ T cells were further purified using anti-CD4 mAb (BD Biosciences) and magnetic MicroBeads (Miltenyi Biotech) with 95–99% purity.

### *Isolation and culture of splenic DCs*

B6 spleens were cut into small pieces and digested with collagenase D and DNase 1 (Roche). CD11c$^+$ cells were positive selected using anti-CD11c-coated immunomagnetic beads (Miltenyi Biotech), and then labeled with anti-CD3, anti-CD19, and anti-DX5 biotin allophycocyanin and PE-conjugated anti-CD11c. CD11c$^+$CD3$^-$CD19$^-$DX5$^-$ cells were sorted on a MoFlo high-performance cell sorter using Summit acquisition and analysis software (DakoCytomation), and cultured for 14 h in 24-well plates in 2 ml of RPMI 1640 supplemented with 10% FCS, 1000 U/ml GM-CSF, and 5 ng/ml IL-4. To prepare Lewis protein solution, $1 \times 10^8$/ml Lewis splenocytes in PBS was subjected to three cycles of freezing and thawing followed by centrifugation at $3000 \times g$ for 15 min. Where applicable, 200 ul of the resultant supernatant was added to DC culture.

### *Isolation of GICs, GI-DN T cells, or GI-DCs*

Cardiac xenografts were perfused with saline, cut into small pieces, and incubated in a solution of 0.2 U/ml collagenase (Sigma-Aldrich) and 0.2 U/ml dispase (Valeant Pharmaceuticals) in $\alpha$-MEM at 37°C for 40 min. After incubation, the cells were gently pressed through a stainless steel mesh and then filtered. Tissue cells and RBC were removed from cell suspensions using Lympholyte M high-density solution (Cedarlane Laboratories). GICs were stained for surface markers. To isolate GI-DN T cells, GICs were incubated for 30 min with CD4- and CD8-depleting mAbs (RL 172-4 and 3.168, respectively) followed by incubation with rabbit C' (Cedarlane Laboratories). Complete depletion of CD4$^+$ and CD8$^+$ T cells were confirmed by flow cytometry analysis. The remaining cells (CD3$^+$ CD4$^-$CD8$^-$) were further purified using biotin-conjugated anti-CD3 mAb (BD Biosciences) and SA-magnetic microbeads. To isolate GI-DCs, CD11c$^+$ cells were sorted from GICs on a MoFlo high-performance cell sorter using Summit acquisition and analysis software (DakoCytomation).

### *MLR, suppression assays, and DC-T cell cocultures*

In MLR, varying numbers of purified CD4$^+$ T cells from naive or transplanted B6 mice were used as responder cells, and cocultured with irradiated Lewis splenocytes ($3 \times 10^5$ cells/well) in $\alpha$-MEM supplement with 10% FCS. In suppression assays, enriched naive CD4$^+$ or CD8$^+$ T cells ($1 \times 10^4$ cells/well) were used as responder cells, and stimulated with irradiated Lewis splenocytes ($3 \times 10^5$ cells/well) in $\alpha$-MEM supplement with 10% FCS and 50 U/ml rIL-2. Varying numbers of GI-DN T cells were used as putative suppressors. After 4 days of incubation, cultured cells were labeled with 1 $\mu$Ci/well of [$^3$H]TdR, harvested 16 h later and counted in a beta-scintillation counter (TOPCOUNT; Packard Instrument). Suppression was calculated using the following equation: % suppression = $1 - (E/R)$, where $E$ is the cpm of each well and $R$ is the cpm of responder alone.

In DC-T cell cocultures, enriched naive CD4$^+$ T cells ($1 \times 10^4$ cells/well) were cultured with varying numbers of GI-DCs. After 3 days of incubation, cells were labeled with 1 $\mu$Ci/well of [$^3$H]TdR, harvested 16 h later, and counted in a beta-scintillation counter (TOPCOUNT; Packard Instrument).

### *Abs and flow cytometry*

FITC-conjugated anti-CD3 (clone 145-2C11), anti-I-A$^b$ (AF6-120.1), anti-CD40 (HM40-3), anti-CD80 (16-10A1), and anti-CD86 (GL1); PE-conjugated anti-CD11c (HL3) and anti-CD4 (GK1.5); PE-conjugated anti-rat CD45 (OX-1); PE-Cy5-congugated anti-CD8 (53-6.7); biotinylated anti-CD3 (145-2C11), anti-CD19 (1D3), and anti-CD49b/Pan-NK (DX5) were purchased from BD Biosciences. PE-Cy5-conjugated streptavidin (eBioscience) was used as the secondary reagent for biotinylated Abs. Flow cytometric analysis was performed using an EPICS XL-MCL flow cytometer (Corixa).

### *Statistical analysis*

All statistical analyses were performed using the unpaired Student's $t$ test. Values of $p < 0.05$ were considered significant.

## Results

### *Anti-donor CD4$^+$ T cells, but not xenogeneic hemopoietic chimerism, are present in xenograft-accepted recipient mice*

In our Lewis rat to B6 mouse cardiac xenotransplantation model, treatment with DLI plus anti-CD4 mAb resulted in permanent xenograft survival (15). To investigate whether this treatment would facilitate the generation of hemopoietic chimerism, leukocytes were isolated either from the spleen and lymph nodes of naive B6 mice, or from DLI/anti-CD4-treated B6 recipient mice 30 days after transplantation, and stained with PE-conjugated anti-rat CD45 mAb (Fig. 1*A*, *right*). Leukocytes from the blood of Lewis rats were used as a positive control (Fig. 1*A*, *left*). No increase in CD45$^+$ rat cells in the secondary lymphoid organs of DLI/anti-CD4-treated mice was found on day 30 post grafting when compared with naive B6 mice. This result suggests that development of hemopoietic rat chimerism in recipient mice is unlikely the mechanism leading to the acceptance of rat cardiac xenografts.

As depletion of CD4$^+$ T cells was transient in our model, the number of CD4$^+$ T cells gradually returned to normal levels in the peripheral lymphoid organs of both anti-CD4-treated alone and DLI/anti-CD4-treated xenografted B6 recipients (15). However, these newly released CD4$^+$ T cells could reject Lewis hearts in anti-CD4- (mean survival time of 65 days; $n = 5$) but not DLI/anti-CD4-treated B6 recipients (graft survival >200 days in >90% recipients; $n = 12$) (15). To understand the mechanisms involved in DLI/anti-CD4-induced long-term xenograft survival, we examined the status of anti-donor CD4$^+$ T cells in xenograft-accepted recipients. We purified CD4$^+$ T cells from the spleen and lymph nodes of anti-CD4- or DLI/anti-CD4-treated B6 mice 30 days after receiving a Lewis heart, and assessed their ability to respond against donor-Ags in vitro and in vivo. As shown in Fig. 1*B*, purified CD4$^+$ T cells from both anti-CD4- and DLI/anti-CD4-treated xenografted mice proliferated in a similar pattern as naive CD4$^+$ T cells upon in vitro stimulation with irradiated Lewis

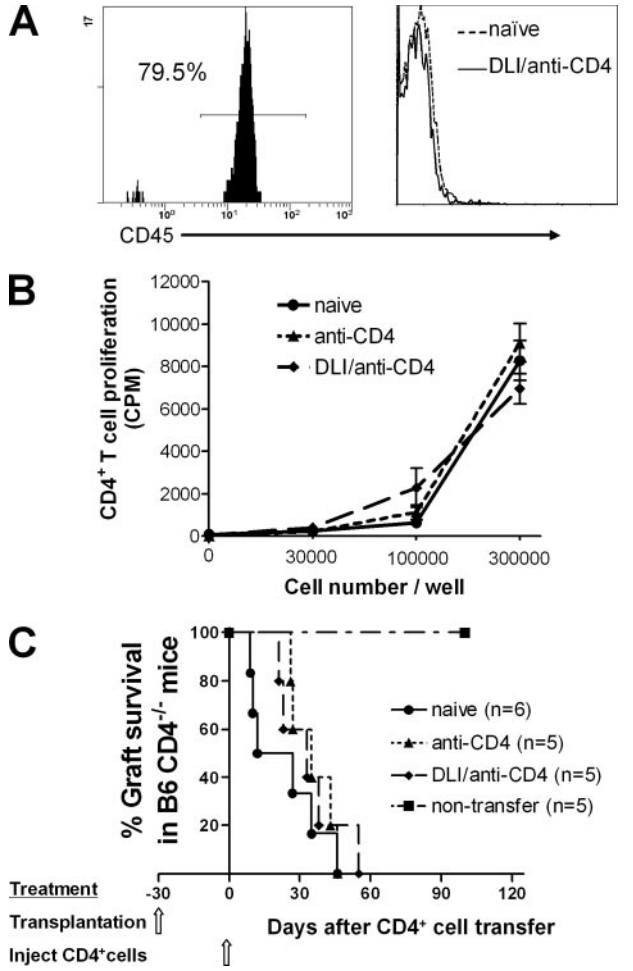


**FIGURE 1.** Functional anti-donor $CD4^+$ T cells, but not xenogeneic hemopoietic chimerism, are present in DLI/anti-CD4-treated B6 xenograft recipients. B6 mice were i.p. injected with 400 $\mu$g/mouse YTS 191.1 (anti-CD4-depleting) mAb on days −2, 0, 3, and 7 with or without i.v. injection of $4 \times 10^7$ Lewis splenocytes/mouse (DLI) on day −2, and transplanted with Lewis heart grafts on day 0. *A*, Leukocytes were collected from the peripheral blood of Lewis rats (*left*), the spleen and lymph nodes of either DLI/anti-CD4-treated B6 recipients 30 days after transplantation (*right*, solid line), or naive B6 mice (*right*, dashed line). These leukocytes were labeled with PE-conjugated anti-rat CD45 mAb and analyzed using a flow cytometer. The histograms show the percentages of $CD45^+$ cells. *B*, $CD4^+$ T cells were purified from the spleens and lymph nodes of naive (■), anti-CD4- (▲), or DLI/anti-CD4- (♦) treated B6 mice at 30 days after transplantation. Varying numbers of purified $CD4^+$ T cells were cocultured with irradiated Lewis splenocytes. After 4 days of incubation, cell proliferation was measured by [$^3$H]TdR incorporation. *C*, B6 $CD4^{-/-}$ mice were transplanted with Lewis heart grafts. At day 30 after transplantation, the recipient mice were either left untreated (■) or adoptively transferred with $2.5 \times 10^6$/mouse purified $CD4^+$ T cells from the naive B6 mice (■) or from recipients treated with anti-CD4 (▲) or DLI/anti-CD4 (♦). Graft survival was monitored by daily palpation.

splenocytes. This finding indicates that anti-donor $CD4^+$ T cells are present in the periphery of DLI/anti-CD4-treated xenografted recipient mice 30 days after transplantation and are able to proliferate in vitro upon donor-Ag stimulation.

We have recently shown that B6 $CD4^{-/-}$ mice are not able to reject Lewis heart grafts. However, adoptive transfer of $2.5 \times 10^6$ of naive $CD4^+$ T cells is sufficient to cause rejection of Lewis heart grafts in $CD4^{-/-}$ mice (14). To study whether $CD4^+$ T cells from DLI/anti-CD4-treated xenografted mice can also mount xenograft rejection, Lewis hearts were transplanted into B6 $CD4^{-/-}$ mice. Thirty days after transplantation, each B6 $CD4^{-/-}$ recipient was infused with $2.5 \times 10^6$ $CD4^+$ T cells that were purified from either anti-CD4- or DLI/anti-CD4-treated wild-type B6 mice that had been grafted 30 days earlier with Lewis hearts. This time point was selected because $CD4^{-/-}$ mice had accepted their grafts, so there was no major inflammatory response in recipients and to match the time when $CD4^+$ T cells were isolated from wild-type B6 recipients after transplantation. Graft survival was compared with xenografted B6 $CD4^{-/-}$ mice that were injected with the same number of naive $CD4^+$ T cells or uninjected control transplant recipients. As shown in Fig. 1*C*, B6 $CD4^{-/-}$ mice infused with $CD4^+$ T cells were able to mount xenograft rejection in a similar fashion regardless of the origin of the $CD4^+$ T cells. These data demonstrate that anti-donor $CD4^+$ T cells are present in DLI/anti-CD4-treated xenografted mice 30 days after transplantation and are fully capable of responding to xenoantigens both in vitro and in vivo upon adoptive transfer.

### *Infusion of donor-Ag-loaded mature B6 DCs breaks long-term cardiac xenograft survival*

The presence of anti-donor T cells in xenograft-accepted mice suggested that the inability of rejecting cardiac xenografts by anti-donor T cells might reflect insufficient Ag stimulation to these cells in DLI/anti-CD4-treated mice. To test this possibility, $CD11c^+$ DCs that did not express T (CD3), B (CD19), and NK (DX5) cell markers (16) were sorted from naive B6 splenocytes and cultured with or without Lewis proteins. After a 14-h culture, both Lewis-Ag-loaded (Fig. 2*A*) and control (Lewis-Ag-unloaded, data not shown) DCs expressed significantly higher levels of MHC-II ($I\text{-}A^b$) and costimulatory molecules than before culture. A total of $2 \times 10^6$ mature B6 DCs from either Lewis-Ag-loaded or control cultures were adoptively transferred into DLI/anti-CD4-treated B6 recipients that had received Lewis hearts 30 days previously. B6 recipients that were infused with Lewis-Ag-loaded but not control mature B6 DCs rejected the accepted Lewis hearts (Fig. 2*B*). Together, these data demonstrate that anti-donor T cells in DLI/anti-CD4-treated mice are capable of rejecting Lewis heart grafts when appropriately stimulated, and suggest that the mechanisms leading to long-term xenograft survival in these mice may be due to insufficient stimulation/activation of anti-donor T cells and/or suppression of anti-donor T cells by Tregs.

### *Reduction of $CD4^+$ and $CD8^+$ T cells is concomitant with increased numbers of DN T cells in accepted xenografts*

The finding that DLI/anti-CD4-treated mice had functional anti-donor T cells in the spleen and lymph nodes, yet retained their cardiac xenografts permanently, provoked us to further investigate the mechanisms that could prevent anti-donor T cell to function within the grafts. We first examined the phenotype and number of GI-T cells. As shown in Fig. 3*A*, xenografts from both treatment groups contained a significant number of infiltrating cells. At 30 and 60 days after transplantation, the number of GI-$CD4^+$ T cells within the xenografts of DLI/anti-CD4-treated mice was significantly lower than that of anti-CD4-treated mice (Fig. 3*B*). Similar findings hold true for GI-$CD8^+$ T cells (Fig. 3*C*). As both $CD4^+$ $CD25^+$ and DN Tregs have been shown to be able to suppress xenogeneic T cell responses in vitro (7, 15), we examined whether these Tregs were present in xenografts. A small proportion (2.06–2.9% of total GI T cells) of $CD4^+CD25^{high}$ T cells could be detected in xenografts, but no significant difference was seen between the two treatment groups (data not shown). In contrast, the

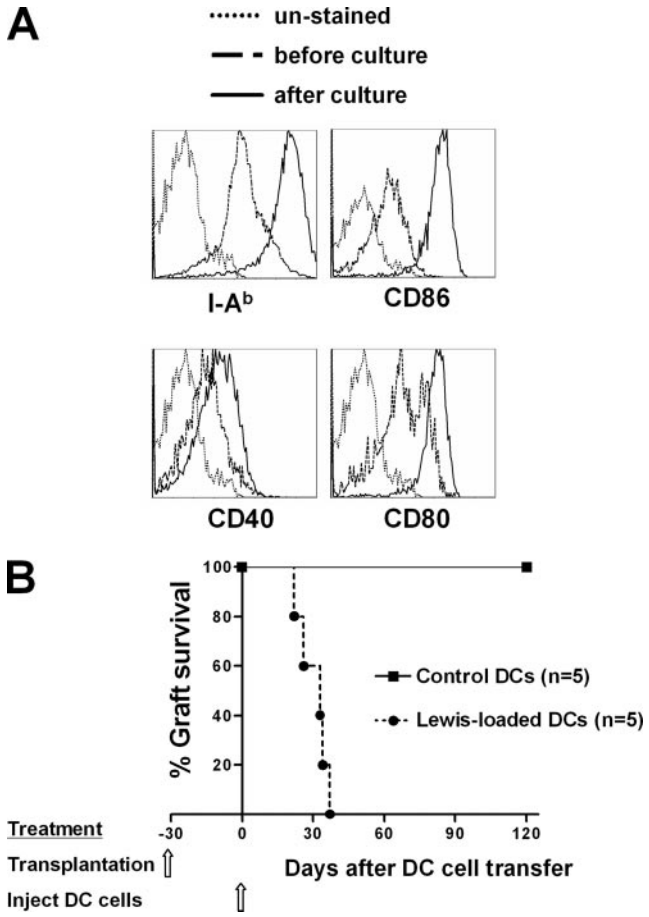


**FIGURE 2.** Infusion of xenoantigen-loaded mature B6 DCs break long-term xenograft survival. $CD11C^+CD3^-CD19^-DX5^-$ cells were sorted from the spleens of naive B6 mice, and cultured 14 h in the presence or absence of Lewis proteins in GM-CSF containing RPMI 1640 medium. *A*, Sorted B6 splenic DCs were labeled with FITC-conjugated anti-$I\text{-}A^b$, anti-CD40, anti-CD86, and anti-CD80, and were analyzed by flow cytometry. The overlay plots show the expression levels of $I\text{-}A^b$, CD40, CD86, and CD80 on sorted B6 splenic DCs before (dashed lines) and after culture with Lewis proteins (solid lines) in comparison with unstained cells (dotted lines). *B*, At 30 days after receiving a Lewis heart graft, DLI/anti-CD4-treated recipient B6 mice were adoptively transferred with $2 \times 10^6$ cultured B6 DCs that were pulsed with (●, Lewis-Ag loaded DCs; $n = 5$) or without (■, control DCs; $n = 5$) Lewis proteins. Graft survival was monitored by daily palpation.

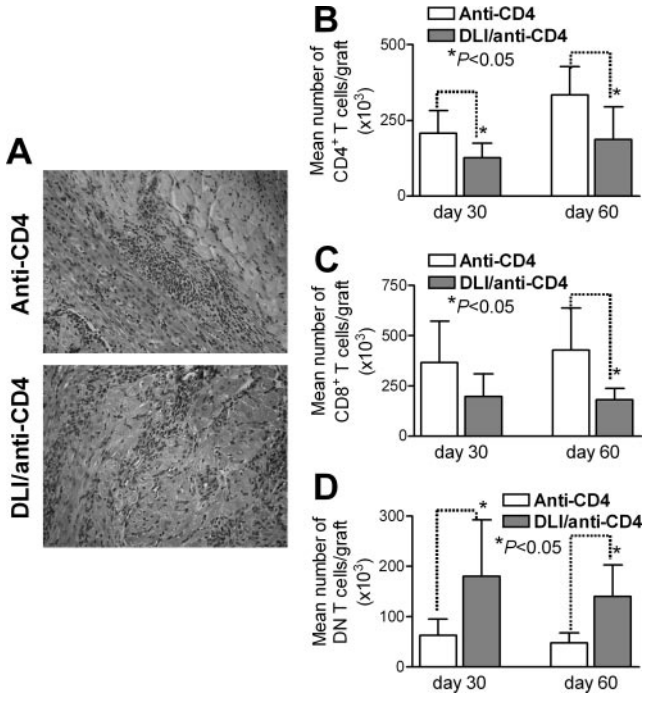


**FIGURE 3.** Decrease of $CD4^+$ and $CD8^+$ T cells and increase of DN T cells in accepted cardiac xenografts. *A*, Representative xenograft sections from anti-CD4- (*top*) and DLI/anti-CD4- (*bottom*) treated B6 recipients are shown (30 days after transplantation; H&E; magnification, ×200). *B–D*, GICs were collected from the xenografts of anti-CD4- (□; before graft rejection) and DLI/anti-CD4- (■) treated B6 recipients at 30 and 60 days after transplantation. Cells were stained with anti-CD3-FITC, anti-CD4-PE, and anti-CD8-PECy5. The percentages of $CD3^+CD4^+CD8^-$, $CD3^+CD4^-CD8^+$, and $CD3^+CD4^-CD8^-$ (DN) T cells were determined by flow cytometry analysis. The total numbers of $CD4^+$ (*B*), $CD8^+$ (*C*), and DN (*D*) T cells in cardiac xenografts were determined by multiplying the number of total GICs by the proportion of each T cell subset; *, $p < 0.05$ ($n = 5$; unpaired Student's *t* test).

number of DN T cells within the xenografts of DLI/anti-CD4-treated mice was significantly higher than that of anti-CD4-treated mice (Fig. 3*D*; $p < 0.05$).

### *Suppression of anti-donor $CD4^+$ and $CD8^+$ T cells by graft-infiltrating DN Tregs*

We next studied whether GI-DN T cells could suppress anti-donor T cells. GI-DN T cells were isolated from either DLI/anti-CD4- or anti-CD4-treated mice 25–30 days post transplantation and their ability to suppress the in vitro proliferation of syngeneic $CD4^+$ or $CD8^+$ T cells upon donor-Ag stimulation was determined. As shown in Fig. 4, *A* and *B*, a 1:1 suppressor to responder ratio, the proliferation of both $CD4^+$ and $CD8^+$ T cells was reduced >50% by the GI-DN T cells from DLI/anti-CD4-treated mice. In contrast, no significant reduction in proliferation was observed by the GI-DN T cells from anti-CD4-treated group. GI-DN T from both treatment groups were able to suppress at a 2:1 ratio. However, the GI-DN T cells from DLI/anti-CD4-treaed mice were more potent suppressors than those from anti-CD4-treated mice. GI-DN T cells were anergic and did not proliferate upon donor-Ag stimulation in vitro. These results indicate that DN Tregs from accepted xenografts are able to suppress the anti-donor $CD4^+$ and $CD8^+$ T cell response in vitro.

### *Infiltrating DCs within xenografts of DLI/anti-CD4-treated mice remain in an immature state and fail to stimulate T cell proliferation*

Infusion of Lewis-Ag-loaded DCs alone resulted in the rejection of accepted Lewis heart grafts in DLI/anti-CD4-treated recipients, suggesting that the lack of sufficient Ag-stimulation by DCs may account for the inability of $CD4^+$ T cells to reject xenografts in DLI/anti-CD4-treated recipients. However, when the frequency and phenotype of DCs in the secondary lymphoid organs were examined, no significant difference was found between anti-CD4 alone and DLI/anti-CD4-treated groups (data not shown). We therefore analyzed the phenotype and frequency of DCs in xenografts. We found that at 30 days after transplantation, GI-DCs ($CD11c^+CD3^-CD19^-DX5^-$ cells) from DLI/anti-CD4-treated recipients expressed much lower levels of MHC-II molecules than that of anti-CD4-treated ones (Fig. 5*A*). Thus, the number of MHC-II-high DCs within the xenografts of DLI/anti-CD4-treated recipients was significantly decreased (Fig. 5*B*; $p < 0.05$). In addition, the overall expression levels of CD80, CD86, and

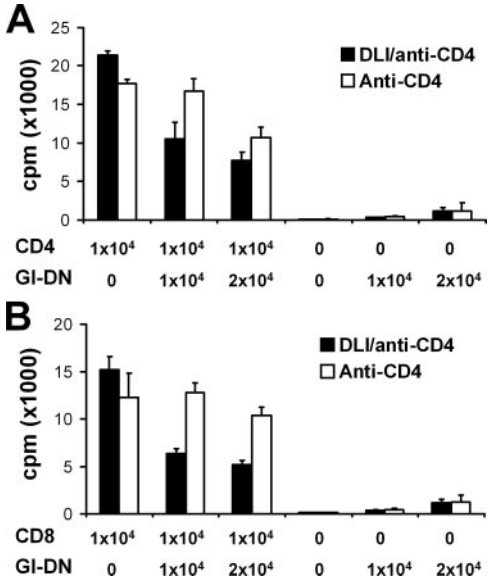


**FIGURE 4.** DN Tregs from accepted xenografts suppress proliferation of naive $CD4^+$ and $CD8^+$ T cells. Naive B6 $CD4^+$ (*A*) or $CD8^+$ (*B*) T cells were enriched as described in *Materials and Methods*, used as responders at $1 \times 10^4$/well, and stimulated by $3 \times 10^5$/well irradiated Lewis splenocytes. GI-DN T cells were purified from anti-CD4- (□) and DLI/anti-CD4- (■) treated B6 recipients at 25 to 30 days after transplantation and used as putative suppressors at the ratios as indicated. After 4 days of incubation, cell proliferation was measured by [$^3$H]TdR incorporation. This experiment was performed in triplicate cultures and repeated three times with similar results.

CD40 costimulatory molecules were also lower on GI-DCs from the DLI/anti-CD4-treated group than on DCs from anti-CD4-treated mice (Fig. 5*C*). These results imply that the majority GI-DCs remain in an immature state within the xenografts of DLI/anti-CD4-treated mice.

We further assessed whether the GI-DCs from the accepted xenografts could stimulate T cell proliferation. GI-DCs were isolated from the xenografts of anti-CD4- or DLI/anti-CD4-treated recipient mice 30 days after transplantation, and cocultured with $CD4^+$ T cells from naive B6 mice. We found that GI-DCs from anti-CD4-treated mice could stimulate $CD4^+$ T cell proliferation in vitro, whereas the ability of DCs from DLI/anti-CD4-treated grafts to stimulate naive $CD4^+$ T cell proliferation was significantly hampered (Fig. 5*D*). Together, these data indicate that DCs isolated from the accepted xenografts not only stay in an immature state, but also have an impaired ability to stimulate T cell responses.

## Discussion

DLI or donor-specific transfusion either alone or in combination with anti-CD4 or anti-CD154 mAbs has been shown to induce long-term allo- and xenograft survival in various models (10, 15, 17). The mechanism by which DLI induces operational transplantation tolerance remains elusive. The promotion of chimerism was suggested as a possible mechanism by which DLI or donor-specific transfusion modulates allogeneic immune responses and leading to graft acceptance (18). However, we did not identify donor leukocytes in the secondary lymphoid organs of DLI/anti-CD4-treated mice 30 days after xenotransplantation (Fig. 1*A*). Thus, the establishment of stable chimerism is not the major mechanism to induce long-term xenograft survival in our model. Gordon et al. (17) have previously shown that host $CD4^+$ T cells were eliminated in xenograft-accepted mice treated with donor-specific transfusion and anti-CD154 mAb. In contrast, in this study, we found

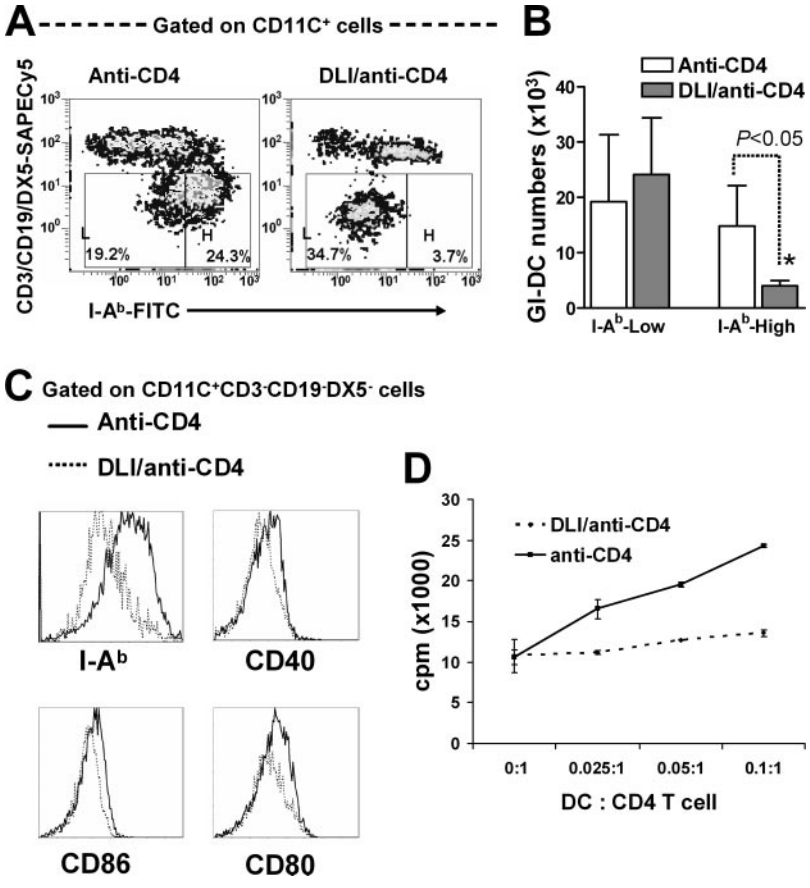


**FIGURE 5.** DCs from the xenografts of DLI/anti-CD4-treated mice remain in an immature state and were unable to stimulate T cell proliferation in vitro. *A–C*, GICs were collected from the xenografts of anti-CD4- and DLI/anti-CD4-treated B6 recipients at 30 days after transplantation, stained with FITC-conjugated anti-I-$A^b$, anti-CD40, anti-CD80, and anti-CD86, PE-conjugated anti-CD11c, and biotinylated anti-CD3, anti-CD19, and anti-DX5 followed by PE-Cy5-conjugated streptavidin. The GICs were analyzed by flow cytometry. *A*, Dot plots shown were gated on $CD11c^+$ cells, and the percentage of $CD11c^+CD3^-CD19^-DX5^-$I-$A^{b-low}$ and $CD11c^+CD3^-CD19^-DX5^-$I-$A^{b-high}$ cells are indicated. *B*, The total number of graft-infiltrating $CD11c^+CD3^-CD19^-DX5^-$I-$A^{b-low}$ or $CD11c^+CD3^-CD19^-DX5^-$I-$A^{b-high}$ cells was determined by multiplying their percentages by the total number of GICs $*$, $p < 0.05$ ($n = 5$, unpaired Student's *t* test). *C*, The overlay plots show the expression levels of I-$A^b$, CD40, CD86, and CD80 on $CD11c^+CD3^-CD19^-DX5^-$ infiltrating cells from anti-CD4- (solid lines) and DLI/anti-CD4- (dashed lines) treated recipient mice. Data are representative of five independent experiments. *D*, Enriched naive $CD4^+$ T cells from B6 mice were used as responders at $10^4$/well. Graft-infiltrating $CD11c^+$ cells were sorted from anti-CD4- (solid line) and DLI/anti-CD4- (dashed line) treated recipient mice 30 days after transplantation and used as stimulator cells at ratios as indicated. After 3 days of coculture, cell proliferation was measured by [$^3$H]TdR incorporation. The data are shown as the average of three replicate cultures. The experiment has been repeated two times and similar results were obtained.

that functional anti-donor $CD4^+$ T cells were present in the periphery of DLI/anti-CD4-treated xenografted mice (Fig. 1, *B* and *C*). When appropriately stimulated by infusion of donor-Ag-loaded mature B6 DCs, these anti-donor T cells were capable of rejecting accepted xenografts (Fig. 2). These data indicate that there is no apparent defect in anti-donor T cells within the secondary lymphoid organs of DLI/anti-CD4-treated xenografted mice.

Recent studies of Tregs suggest a nondeletional regulatory mechanism that helps to maintain peripheral tolerance to foreign grafts (19, 20). Indeed, we found that recipient DN Tregs were significantly increased in the secondary lymphoid organs of DLI/anti-CD4-treated xenografted mice, and could suppress the in vitro proliferation of recipient mouse T cells against rat donor xenoantigens (15). Adoptive transfer of DN Tregs from mice accepting xenografts could suppress the proliferation of xenoreactive $CD4^+$ T cells and prevent $CD4^+$ T cell-mediated xenograft rejection (14).

However, we also observed a significant number of GICs in long-term surviving cardiac xenografts from DLI/anti-CD4-treated mice (15). These findings raised a question whether local regulatory mechanisms are also involved in preventing cardiac xenograft rejection. Several previous studies have shown that Tregs can infiltrate allografts (21–23). In this study, we found that the number of DN Tregs in DLI/anti-CD4-treated accepted xenografts was significantly increased compared with anti-CD4-treated ones (Fig. 3*D*). Moreover, these GI-DN Tregs could suppress both anti-donor $CD4^+$ and $CD8^+$ T cells in vitro (Fig. 4). Consistent with these findings, the numbers of GI-$CD4^+$ and $CD8^+$ T cells were significantly lower when compared with those of anti-CD4-treated mice (Fig. 3, *B* and *C*). Together, these data suggest that GI-DN Tregs may contribute to the reduction of anti-donor T cells in accepted xenografts and prevent xenograft rejection.

Recently, we (10) and others (13) have demonstrated that both murine and human DN Treg-mediated suppression is Ag-specific. When irradiated Lewis rat splenocytes were used as stimulators, the ability of DN Tregs from the periphery of Lewis heart-accepted mice to suppress the in vitro proliferation of recipient mouse T cells was stronger than when Wistar Furth rat or BALB/c mouse splenocytes were used as stimulators (15). This finding is consistent with the results obtained from allogeneic transplantation models (10, 11, 24). Furthermore, when DN Tregs were purified from the spleens of DLI/anti-CD4-treated B6 mice, which accepted Lewis heart grafts, and were coinjected with naive $CD4^+$ T cells into $CD4^{-/-}$ xenograft recipients, the majority of recipient mice achieved long-term survival of Lewis, but not Wistar Furth, cardiac xenografts (14). These data indicate that DN Tregs suppress anti-donor T cells in an Ag-specific fashion.

In addition to an increase in DN Tregs in the accepted xenografts, we also found that the majority DCs remained in an immature state within the xenografts of DLI/anti-CD4-treated mice in contrast to those in anti-CD4-treated mice (Fig. 5, *A–C*). This result indicates, for the first time, that GI-DC maturation status correlates to organ xenograft survival. Nevertheless, the role of GI-DCs in organ xenotransplantation and their relationship with the increased number of DN Tregs remain to be determined. Tesar et al. (25) have recently indicated that recipient mice devoid of secondary lymphoid organs were able to reject porcine skin xenografts without delay, and that recognition of xenoantigens could occur within the xenograft itself. In contrast, DCs within nonlymphoid tissues also play a predominant role in local reactivation of primed and memory T cells (26, 27). Therefore, "arrested" maturation of DCs in xenografts may lead to insufficient priming and reactivation of anti-donor T cells at this site and contribute to long-term xenograft acceptance. Indeed, we found that GI-DCs that have stayed at an "arrested" maturation status have impaired ability to stimulate $CD4^+$ T cell proliferation (Fig. 5*D*). Whether GI-DCs with altered maturation status migrate to the secondary lymphoid organs of xenograft recipients is unknown. As infusion of donor-Ag-loaded mature DCs can trigger rejection of accepted grafts (Fig. 2), it suggests that insufficient Ag-presentation to anti-donor T cells may also occur systemically. Thus, further characterizing the fate of GI-DCs within the accepted xenografts, such as their migration to lymphoid organs and subsequent Ag presentation to T cells, may unveil the nature of the hyporesponsiveness of anti-donor T cells within DLI/anti-CD4-treated xenograft recipients.

It is also unclear why GI-DCs within DLI/anti-CD4-treated mice stayed in a less-mature state than those within of anti-CD4-treated recipients. Multiple factors may be involved in determining the maturation status of DCs in an inflammation site. Proinflammatory cytokines (28, 29) and CD40/CD154 interactions (30) may prompt the maturation process of DCs. In contrast, anti-inflammatory cytokines may inhibit DC maturation (31). How these factors integrate to adjust the maturation status of DCs within an inflammatory site, such as a transplanted organ, is largely unknown.

The most interesting observation of this study is that accepted xenografts contain suppressive DN Tregs and DCs with arrested maturation status. The relationship between these coexisting phenomena is not clear. Several reports suggested that immature DCs can induce Tregs both in vitro (32) and in vivo (33). Ochando et al. (34) reported that treatment with DLI and anti-CD154 could induce long-term allogeneic cardiac graft survival. GI plasmacytoid DCs from accepted allografts could migrate to the spleen and induce $CD4^+CD25^+FoxP3^+$ Tregs. In contrast, an in vitro study has shown that Tregs can also modulate the maturation and Ag-presenting function of DCs (35). Thus, DCs and Tregs may interact with each other to down-regulate immune responses locally. The cause and effect of the coexistence of DN Tregs and DCs with arrested maturation status within accepted xenografts require further investigation.

In summary, we demonstrate that long-term organ xenograft survival can be achieved in the presence of fully functional anti-donor T cells in the periphery of the recipients. We suggest that the local regulatory mechanisms may contribute to the prevention of xenograft rejection through insufficient Ag-stimulation and suppression of anti-donor T cells within xenografts. Undoubtedly, further characterization of cell-cell interactions within an inflammation site, such as a transplanted organ xenograft, will provide new insights into the control of pathogenic T cell responses.

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## Disclosures

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