Donor-lymphocyte infusion induces tolerance by activating systemic and graft-infiltrating double negative regulatory T cells

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This work is supported by Canadian Institutes of Health Research (L. Zhang, MOP 14431).

Short title: DLI and graft-infiltrating DN regulatory T cells.
Scientific Heading: Transplantation
Abstract

Pretransplantation donor lymphocyte infusion (DLI) can lead to specific tolerance to allografts in mice, primates and humans. We and others have demonstrated a role for regulatory T cells in DLI-induced donor specific transplantation tolerance. It is nonetheless unclear how regulatory T cells are activated, and where they execute their function. Here we demonstrate, in both transgenic and normal mice, that pretransplant DLI is required for the activation of $\alpha\beta$-T cell receptor (TCR)$^+$CD3$^+$CD4$^-$CD8$^-$ double negative (DN) regulatory T cells in the periphery of recipient animals. More interestingly, DLI promotes the DN regulatory T cells to preferentially migrate to donor-specific allogeneic skin grafts, and form a majority of graft infiltrating T cells in accepted skin allografts. Furthermore, both recipient-derived peripheral as well as graft infiltrating DN T cells are able to suppress and kill anti-donor CD8$^+$ T cells in an antigen-specific manner. These data indicate that DLI may induce donor-specific transplantation tolerance by activating recipient DN regulatory T cells in the periphery, and by promoting the migration of regulatory T cells to the donor-specific allogeneic skin grafts. Our results also demonstrate that DN regulatory T cells can eliminate anti-donor T cells both systemically and locally, suggesting that graft-infiltrating T cells can be beneficial to graft survival. Email: lzhang@transplantunit.org.
Introduction

Induction of tolerance to an allogeneic graft without the need for non-specific immunosuppression is the goal of transplantation therapy. There are many experimental models in which tolerance can be induced by pretransplantation donor lymphocyte infusion (DLI)\(^1\)-\(^7\), and the effects of DLI have also been documented in renal, cardiac, and bone marrow transplant recipients in the clinic\(^8\)-\(^14\). Several mechanisms have been postulated to explain DLI-induced tolerance, including mixed allogeneic chimerisms\(^15\)-\(^18\), deletion of donor-reactive T cells\(^19\)-\(^23\), induction of clonal anergy\(^24\), immune deviation\(^24\)-\(^26\), and regulatory T cells\(^4\),\(^27\)-\(^32\). Regulatory T cells have also been demonstrated to play an important role in preventing autoimmune diseases\(^33\)-\(^43\) and allograft rejection\(^27\)-\(^29\),\(^32\),\(^44\),\(^45\).

We have demonstrated that the pre-transplantation infusion of L\(^d\) or bm1 single MHC class I locus mismatched donor lymphocytes leads to the permanent or significantly prolonged survival of donor-specific but not third party skin allografts in both transgenic and normal mice\(^14\),\(^32\),\(^46\),\(^47\). Recently, we have identified and cloned a novel αβ-TCR\(^+\)CD4\(^-\)CD8\(^-\) double negative (DN) regulatory T cell from mice that permanently accepted donor-specific skin allografts after one dose of a single class I locus-mismatched DLI\(^31\),\(^32\),\(^48\). We demonstrated that infusion of DN regulatory T cell clones into syngeneic naïve animals led to a significantly prolonged survival of allogeneic skin grafts in a dose-dependant and antigen-specific manner\(^32\). Our recent data also indicate that adoptive transfer of DLI-activated DN T cells can significantly prolong skin graft survival in a single class II mismatched model\(^49\). These studies indicate the importance of DN regulatory T cells in preventing allograft rejection. However, the mechanism by which DLI induces donor-specific transplantation tolerance remains elusive. The relationship between DLI and
regulatory T cells is not known. Additionally, where regulatory T cells mediate their function in vivo is unclear.

The goal of the present study was to determine whether DLI is required for activation of antigen-specific DN regulatory T cells, and where these DN regulatory T cells execute their function in vivo. Our data here provide the first direct in vivo evidence that DLI promotes the activation and function of recipient peripheral DN regulatory T cells. The activated DN regulatory T cells preferentially infiltrate donor-specific skin allografts and are cytotoxic to anti-donor CD8+ T cells. Graft infiltrating cells have traditionally been used as a criteria for the diagnosis of graft rejection. Our findings illustrate that graft-infiltrating T cells can be beneficial to graft survival, and highlights the importance of determining the identity of graft-infiltrating cells when using their existence as a criteria for graft rejection.

Materials and Methods

Mice

C57BL/6 (B6, H-2b), SJL (H-2s), (B6xBALB/c)F1 (H-2bd, L^d+) and BALB/c H-2-dm2 (dm2, a BALB/c L^d loss mutant, H-2 D^d+, K^d+, L^d) mice were purchased from Jackson Laboratories (Bar Harbor, ME). A breeding stock of 2C transgenic mice (on B6 background) was kindly provided by Dr. Dennis Y. Loh. 2C (H-2^bd) transgenic mice carry functionally re-arranged TCR α- (one copy) and β-chain (eight copies) transgenes from a cytotoxic T cell clone 2C, which is specific for L^d MHC class I antigen. The 2C clonotypic TCR is recognized by the monoclonal antibody (mAb) 1B2 (hybridoma kindly
provided by Dr. Herman Eisen, MIT). 2C mice were bred with dm2 mice to obtain (2Cxdm2)_{F1} (H-2^{b/d}, L^{d-}, 1B2^{+}) mice.

*Donor lymphocyte infusion and tail skin grafting.*

(2Cxdm2)_{F1} and (B6xdm2)_{F1} mice (both L^{d-}) were used as recipients, and infused intravenously with 4x10^7 lymphocytes from sex matched L^{d+} (B6xBALB/c)_{F1} mice as described previously^32. 7 days after DLI, each recipient mouse received 2 sex-matched skin grafts from (B6xBALB/c)_{F1} (L^{d+}, donor-specific) and SJL (H-2^s, third party control) mice^52. Briefly, a piece of donor tail skin about 1x0.5 cm^2 and a thickness including the epidermis and most of the dermis was removed with a sharp scalpel and transferred to the sides on the recipient tail from which an equivalent amount of skin had been removed. The grafts were covered with a clear spray bandage (NEW-Skin, Dedtech Labs, Jackson, WY) and further protected with a light, loosely fitted transparent glass tube. Grafts were monitored visually daily for the first 2 weeks and twice a week thereafter. The grafts were scored as rejected when >90% was necrotic.

*Histology*

21 and 120 days after skin grafting, the accepted skin allografts from (2Cxdm2)_{F1} mice were harvested, fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Sections were stained with haematoxylin and eosin and examined under light microscopy. The accepted syngeneic skin grafts were treated in the same way and used as control.

*Isolating Graft infiltrating cells.*

Skin allografts were cut into small pieces, incubated in a collagenase/dispase solution with 0.2 unit/ml collagenase (SIGMA CHEMICAL CO.) and 0.2 unit/ml dispase (ICN BIOMEDICALS Inc.) in α-MEM. at 37°C for 30 minutes. Following incubation the
cells were gently pressed through a stainless steel mesh, and then filtered. Cell suspensions were washed twice with phosphate buffered saline and then either stained for surface markers or used in *in vitro* cytotoxicity and suppression assays.

**Cell surface marker staining.**

Spleen, lymph node or graft infiltrating cells were stained with FITC-conjugated 1B2 or anti-CD3 mAb, CyChrome-conjugated anti-CD8 mAb, and one of PE-conjugated anti-CD4 mAb, PE-conjugated anti-CD11b mAb, PE-conjugated anti-NK1.1 or PE-conjugated anti-γδ TCR mAb (all from Pharmingen). Data were acquired and analyzed on an EPICS® XL-MCL flow cytometry machine (COULTER CORPORATION, Miami, Florida). Statistical analysis was performed using the student’s T-test.

**Enrichment and purification of DN T cells**

Lymph node, spleen, or graft infiltrating cell suspensions were incubated for 30 minutes with RL172-4 (anti-CD4 depleting mAb) and 3.168 (anti-CD8 depleting mAb) at 4°C, washed, and incubated for 45 minutes with rabbit C’ (Cedarlane, Hornby, Ontario) at 37°C. The cells were washed 3 times, and used in suppression or cytotoxicity assays. The suspension contained <1% CD4+ and CD8+ T cells following depletion according to FACS analysis. To purify DN T cells, cell suspensions were additionally stained with biotin-labeled anti-CD3 mAb (Pharmingen, CA), washed with a 0.5% PBS/BSA, and labeled with MACS anti-biotin microbeads (Miltenyi Biotech, CA) for 15 minutes. The cells were washed, and the CD3+ T cells were purified on an LS column (Miltenyi Biotech, CA).

**Suppression assays.**
Cell suspensions from spleen, lymph nodes or graft infiltrating cells were collected, stained to determine the proportion of DN T cells, and used as putative suppressor cells. Naïve (2Cxdm2)F1 or (B6xdm2)F1 splenocytes were used as responders (1000 1B2⁺CD8⁺ cells/well or 10⁴ (B6xdm2)F1 CD8⁺ T cells/well), and were stimulated with irradiated (20Gy) (B6xBALB/c)F1 or SJL spleen cells (10⁵ cells/well) in the presence of 50U/ml recombinant interleukin (rIL)-2 and 30U/ml rIL-4. After 3 days, cells were labeled with 1 µCi/well of [³H] TdR, harvested 18 hours later and counted in a TopCount (Packard, Meriden, CT) scintillation counter. Suppression was calculated using the equation: % Suppression = 1-(E/R), where E is the cpm of each well, and R (responders) is the cpm of the responders alone.

Cytotoxicity assays.

Cell suspensions from spleen, lymph nodes or graft infiltrating cells were prepared as described above, and stimulated overnight in the presence of IL-2, IL-4 and irradiated (B6xBALB/c)F1 (L^d^+) and used as effectors. (B6xdm2)F1 splenocytes were stimulated with either irradiated (20Gy) (B6xBALB/c)F1 (anti-L^d^) or SJL (anti-H-2^s^) spleen cells. 4 days later, the activated L^d^-specific or H-2^s^ specific CD8⁺ T cells or BW5147 (H-2^k^) tumour cells were labeled with 10µCi/ml of [³H] TdR at 37°C overnight and used as targets (10⁴ cells/well). After co-culture of the effector cells and targets at 37°C for 18 hours in the presence of fresh irradiated allogeneic splenocytes, the cells were harvested and counted in a TopCount (Packard, Meriden, CT) scintillation counter. Specific cell lysis was calculated using the equation: % Specific killing = (S-E)/S x 100, where E (experimental) is cpm of retained DNA in the presence of effector cells, and S (spontaneous) is cpm of retained DNA in the absence of effector cells.
Results

*DLI is required for activation of recipient-derived antigen-specific DN regulatory T cells.*

We have previously demonstrated that pretransplant infusion of single class I locus L\textsuperscript{d} mismatched splenocytes leads to permanent survival of L\textsuperscript{d+} skin allografts and normal rejection of third-party skin allografts\textsuperscript{31,46}. We also demonstrated that in vitro generated DN T cell clones can specifically suppress and kill anti-donor T cells in vitro\textsuperscript{32}. It is not clear, however, whether DLI directly activates recipient-derived antigen-specific DN regulatory T cells, or protects donor-specific allografts from rejection by another mechanism. To address this issue, groups of mice were given DLI or left untreated, and then given skin allografts. One week after transplantation, the ability of peripheral DN T cells in the spleens and lymph nodes of recipient mice to suppress the proliferation of anti-donor CD8\textsuperscript{+} T cells were compared between non-DLI and DLI treated mice. We found that only the lymphocytes from DLI-treated animals were able to suppress the proliferation of anti-donor CD8\textsuperscript{+} T cells in a dose dependant manner, whereas no suppressive activity was observed by non-DLI-treated recipient DN T cells (Figures. 1a and 1b). Furthermore, peripheral DN T cells isolated from DLI-treated, but not untreated mice, were able to kill activated syngeneic 1B2\textsuperscript{+}CD8\textsuperscript{+} T cells dose-dependently (Figure 1c), and the killing mediated by DN T cells purified from DLI treated mice was antigen-specific as CD8\textsuperscript{+} T cells activated by third-party SJL alloantigens (H-2\textsuperscript{S}) were not killed (Figure 1d). These data confirm the results derived from DN T cell clones. More importantly, these findings clearly demonstrate that DLI is required for activation of recipient-derived DN antigen-specific immunoregulatory T cells, which provides a novel explanation for how DLI induces donor-specific transplantation tolerance.
DN T cells form the majority of graft-infiltrating cells in DLI-treated L^d+ skin allografts.

Although extensive studies have demonstrated that regulatory T cells can down regulate immune responses to self and alloantigens systemically, whether these regulatory cells can also inhibit immune responses and prevent tissue damage locally remains unclear. Since cellular infiltrates have been reported in non-rejected grafts as well as in tissues protected from autoimmune destruction^{26,53-57}, we hypothesized that DN regulatory T cells might be able to migrate to the skin allografts and execute their function locally. To test this novel hypothesis, (2Cxdm2)_{F1} mice were given a DLI from L^d-mismatched (B6xBALB/c)_{F1} mice and transplanted with both (B6xBALB/c)_{F1} and a syngeneic skin grafts one week later. At 21 and 120 days after transplantation both grafts were harvested and stained with Haematoxylin and Eosin. As shown in Figure 2a, a very dense mononuclear cell infiltration in accepted L^d-mismatched (B6xBALB/c)_{F1} skin grafts was found at 21 days after transplantation (middle panel). The number of graft infiltrating cells, although markedly reduced at 120 days (lower panel), was still significant compared to those seen in syngeneic skin grafts (upper panel).

To analyze the phenotype of graft infiltrating cells, cells were collected from DLI-treated donor-specific (B6xBALB/c)_{F1} skin allografts at 1, 2, 8 and 15 weeks after transplantation, and stained with a variety of mAbs, including 1B2, anti-CD4, anti-CD8, anti-CD11b, anti-NK1.1 and anti-γδ-TCR mAbs. As shown in Figure 2b, almost half of the graft infiltrating cells were comprised of 1B2^{+}DN T cells at 1 week following skin grafting, and the proportion of DN T cells was higher than other cell types at all time points. We further examined the subsets of donor-specific T cells in L^d+ skin grafts, and found that DN T cells comprised >70% of 1B2^{+} T cells. Approximately 5% of 1B2^{+} T cells were CD8^{+},
and less than 20% were CD4+ (representative data shown in Figure 2c). These data demonstrate that DN T cells form the majority of infiltrating cells in accepted donor-specific skin grafts of DLI treated recipients.

**DLI promotes migration of DN T cells to donor-specific skin allografts.**

Based on the findings that DLI leads to activation of antigen-specific DN regulatory T cells in the recipients (Figure 1), and that the DN T cells accumulate in accepted donor-specific skin allografts (Figure 2), we reasoned that DLI may induced donor-specific transplantation tolerance by promoting DN regulatory T cell migration to donor-specific skin allografts. Therefore, we compared the number of infiltrating DN T cells in DLI and non-DLI treated mice after transplantation. As shown in Figure 3, the number of DN T cells in Ld+ skin grafts of DLI treated mice was significantly higher than those in non-DLI treated mice (4-fold increase on day 3 (p=0.0006) and 20-fold increase on day 7 (p<0.0001)). To further determine whether DN T cells migrate to allografts in an antigen-specific fashion, we compared the number of graft-infiltrating DN T cells in Ld+ and third party SJL skin allografts. We found that although DN T cells infiltrated both Ld+ and third party skin grafts in DLI treated mice at 3 days post-transplantation, the number of DN T cells continued to increase in Ld+ skin grafts, but decreased in third party skin grafts. At 7 days after transplantation the number of DN T cells in Ld+ skin grafts was 8 times higher than that seen in 3rd-party skin grafts (Figure 3). These data demonstrate that pretransplant DLI promotes DN T cells to migrate to skin allografts, and to persist in donor-specific but not third party skin allografts.

**Inflicting 1B2+DN T cells from accepted skin grafts are able to specifically kill activated anti-donor T cells.**
Our findings that DLI activates peripheral antigen-specific DN regulatory T cells (Figure 1), and that DN T cells are the predominant cell type in DLI treated accepted L\textsuperscript{d+} skin allografts (Figure 2) suggest that DN T cells may migrate to the donor-specific allografts in order to down-regulate anti-donor T cell responses locally. To test this hypothesis, infiltrating cells from DLI-treated L\textsuperscript{d+} (B6xBALB/c)\textsubscript{F1} skin grafts were harvested, and their ability to suppress the proliferation of anti-L\textsuperscript{d} CD8\textsuperscript{+} T cells was assessed. As shown in Figure 4a, graft infiltrating cells are able to suppress the proliferation of anti-L\textsuperscript{d} CD8\textsuperscript{+} T cells in a dose dependent manner. To further study whether graft infiltrating 1B2\textsuperscript{+} DN T cells can specifically kill anti-L\textsuperscript{d} CD8\textsuperscript{+} T cells, 1B2\textsuperscript{+} DN T cells were collected from L\textsuperscript{d+}(B6xBALB/c)\textsubscript{F1} skin allografts of DLI treated mice 1 week after transplantation and used as effector cells. Both activated L\textsuperscript{d}-specific 1B2\textsuperscript{+}CD8\textsuperscript{+} T cells and third-party allogeneic (H-2\textsuperscript{k}) tumour cells were used as targets in a JAM cytotoxicity assay\textsuperscript{58}. Graft infiltrating 1B2\textsuperscript{+}DN T cells were able to more effectively kill activated anti-L\textsuperscript{d} T cells than third-party allogeneic tumour cells (Figure 4b). These data indicate that DN regulatory T cells from DLI-treated recipients are not only able to specifically migrate to L\textsuperscript{d+} skin grafts, but also to retain their antigen-specific regulatory function, suggesting that these graft infiltrating DN regulatory T cells may protect skin allografts from rejection by eliminating anti-graft T cells locally.

Validation of the effect of DLI on activation and migration of DN regulatory T cells in normal mice.

The above data have shown in (2Cxdm2)\textsubscript{F1} transgenic mice that L\textsuperscript{d} mismatched pretransplant DLI activates recipient DN T cells which can migrate to donor-specific skin allografts and kill anti-donor T cells. Since (2Cxdm2)\textsubscript{F1} transgenic mice have a higher than
average proportion of DN T cells in the periphery, it is important to verify these findings in normal mice. First, we addressed the question whether DLI can also activate antigen-specific DN T cells in the periphery of non-transgenic mice. To this end, (B6xdm2)F1 mice were given DLI from (B6xBALB/c)F1 mice. As dm2 is a BALB/c L^d loss mutant, in this setting the only mismatch between donor and recipient is L^d, which mimics the transgenic model used in the above studies. Using DLI treated (B6xdm2)F1 mice, we previously demonstrated that (B6xBALB/c)F1 grafts survive indefinitely, but third party SJL skin grafts are rejected. In order to determine whether DLI leads to activation of peripheral DN regulatory T cells in non-transgenic mice, DN T cells were purified from the spleen of DLI treated (B6xdm2)F1 mice 7 days after transplantation and assessed for their ability to kill anti-donor CD8^+ T cells. As seen in (2Cxdm2)F1 transgenic mice, purified splenic DN T cells from DLI-treated mice were able to kill anti-L^d but not anti-H-2^s CD8^+ T cells (Figure 5a).

Next, we studied whether DLI promotes the migration of DN T cells to donor-specific skin allografts. (B6xdm2)F1 mice were given an L^d mismatched DLI from (B6xBALB/c)F1 mice followed by donor-specific skin grafting 7 days later. As controls, a group of (B6xdm2)F1 mice were transplanted with (B6xBALB/c)F1 skin grafts without pretransplant DLI. One week after transplantation, graft-infiltrating cells were harvested from skin allografts of both DLI-treated and non- DLI treated mice, and stained using anti-CD3, anti-CD4 and anti-CD8 mAbs. The percentages of graft infiltrating CD4^+ , CD8^+ and DN T cells in DLI-treated mice were compared with that of the non- DLI-treated mice. As shown in Figure 5b, nearly 40% of the graft infiltrating T cells were DN T cells in DLI-treated L^d+ skin grafts whereas only 6.5% of DN T cells were found in non- DLI-treated
skin grafts. Furthermore, the percentage of CD8\(^+\) T cells in DLI-treated skin grafts was lower than that seen in non-DLI-treated L\(^d\) skin grafts (Figure 5c). These data are consistent with the findings from (2Cxdm2)\(_{F1}\) transgenic mice and demonstrate that DLI promotes the migration of DN T cells into skin allografts in non-transgenic (B6xdm2)\(_{F1}\) mice.

The finding that the skin allografts from DLI-treated mice have a higher number of DN T cells and lower number of CD8\(^+\) T cells indicates the possibility that the graft infiltrating DN T cells may be able to suppress anti-donor CD8\(^+\) T cells. To determine the function of graft infiltrating DN T cells, these cells were isolated from DLI-treated L\(^d+\) (B6xBALB/c)\(_{F1}\) skin grafts, and tested for their ability to suppress anti-L\(^d\) and third party anti-SJL CD8\(^+\) T cells. As shown in Figure 5d, graft infiltrating DN T cells are able to suppress the proliferation of anti-L\(^d\) (donor-specific), but not anti-H-2\(^s\) (third-party) CD8\(^+\) T cells \textit{in vitro}. Together, these data corroborate the previous findings in (2Cxdm2)\(_{F1}\) transgenic mice and demonstrate that pretransplant DLI leads to activation of antigen-specific DN regulatory T cells in normal mice, which are able to infiltrate skin allografts and specifically suppress anti-donor CD8\(^+\) T cells following transplantation.

**Discussion**

It has been previously established that pretransplant DLI can enhance donor-specific allograft survival in rodents\(^1,2,4-7\), primates\(^59,60\) and man\(^9\). However, the mechanism by which DLI promote donor-specific allograft survival remains elusive. Various types of regulatory T cells, including CD4\(^+\), CD8\(^+\) and DN T cells have been shown to be involved in DLI-induced tolerance\(^4,27-32\). Roelen et al were able to prolong graft survival by adoptively transferring CD4\(^+\) regulatory T cells that were induced by donor specific transfusion\(^29\). Using a donor-specific transfusion and heart allograft transplantation model,
Douillard et al showed that a subset of CD8+ T cells (Vβ18-Dβ1-Jβ2.7) may function as regulatory T cells30, as administering an anti-TCR specific DNA vaccination to eliminate Vβ18-Dβ1-Jβ2.7 CD8+ T cells abolished heart allograft tolerance28. Similarly, data from Iwakoshi et al imply that CD4+ regulatory T cells are activated in mice treated with a donor specific transfusion together with anti-CD154 mAb since depleting CD4+ T cells abolished long term skin graft survival27. Here we extend our previous studies using in vitro generated DN T cell clones32, and demonstrate that DN regulatory T cells from DLI-treated recipients can specifically down-regulate anti-donor immune responses.

Despite the demonstration of the involvement of regulatory T cells in DLI-induced transplantation tolerance, it is not clear how DLI promotes allograft survival through regulatory T cells. In this paper we demonstrate in both transgenic and normal mice that pretransplant DLI can activate recipient-derived DN regulatory T cells, and promote the migration of these regulatory T cells to donor-specific skin allografts. We also demonstrate that both peripheral and graft infiltrating DN T cells from DLI treated animals can specifically kill anti-donor CD8+ T cells (Figures 1c-d and 5a), whereas an equivalent number of DN T cells from non-DLI treated mice had no suppressive activity (Figure 1a-b). These data provide, for the first time, direct evidence that DLI can enhance donor-specific skin graft survival through promoting activation and migration of antigen-specific DN regulatory T cells, which in turn can down-regulate anti-donor responses both systemically and locally.

We have previously demonstrated that DLI can lead to permanent acceptance of donor-specific but not third party skin allografts31,46. Although similar numbers of DN T cells were observed in both donor specific Ld+ (B6xBALB/c)F1 and third party SJL skin
grafts 3 days after transplantation, at day 7 the number of DN T cells substantially increased in (B6xBALB/c)F1 skin grafts, and markedly decreased in SJL skin grafts (Figure 3). These data indicate that although the migration of DN T cells to third party skin grafts at 3 days post transplantation may be the result of non-specific inflammatory responses, DN T cells are only able to accumulate in donor-specific skin grafts. Moreover, cytotoxicity assays using DN T cells from DLI treated transgenic and normal mice demonstrate that DN T cell mediated killing is antigen specific (Figures 1d, 4b, and 5a). Together, these findings help to explain why donor-specific grafts are protected whereas third party skin grafts are rejected.

The finding that DN regulatory T cells migrate to and compose the majority of graft infiltrating cells is intriguing. Graft-infiltrating cells in general are considered detrimental to graft survival, and infiltrating cytotoxic T cells in grafts have been correlated with graft rejection61,62. The presence of graft infiltrating lymphocytes is one of several criteria for the diagnosis of graft rejection50. On the other hand, several reports have indicated the presence of infiltrating lymphocytes in well functioning grafts26,30,53-56,63, and recent studies have implicated subsets of graft-infiltrating lymphocytes as regulatory cells30,55. In particular, CD8+ regulatory T cells have been found in DLI-treated cardiac allografts28,30 and in kidney allografts following the oral administration of allogeneic splenocytes55. In this paper, we demonstrated a 20-fold increase in the number of graft infiltrating DN regulatory T cells in DLI-treated transgenic mice (Figure 3) and a 6-fold increase in normal mice (Figure 5b and 5c) following skin transplantation compared with that in non-DLI treated controls. Furthermore, graft infiltrating DN T cells isolated directly from skin grafts of DLI-treated mice can suppress and specifically kill anti-donor T cells (Figure 5a
and 5d) in both transgenic and non-transgenic mice. These data demonstrate that not only peripheral but also graft-infiltrating DN T cells from DLI treated mice have a regulatory function. Our findings support a novel concept that graft-infiltrating T cells can be beneficial to graft survival, and indicate the importance of determining the identity of infiltrating cells in the diagnosis of graft rejection.

In our previous studies we have shown that DN regulatory T cells have the capacity to kill activated syngeneic CD8\(^+\) T cells that carry the same TCR through Fas/FasL interactions\(^{32}\). The data presented in this paper, together with our previous findings supports the following model to explain how DLI induces donor-specific skin graft protection through DN regulatory T cells. DLI leads to the activation of recipient DN regulatory T cells that carry TCRs recognizing alloantigens expressed on the donor lymphocytes. Following transplantation, the activated DN regulatory T cells migrate to the skin grafts, along with anti-donor T cells. Anti-donor CD8\(^+\) T cells are then killed by DN regulatory T cells through Fas/FasL interactions. Since DN regulatory T cells are unable to kill T cells that carry a different TCR, third party skin grafts are not protected. This model is consistent with other reports showing that intra-graft FasL expression can confer immune privilege\(^{64}\), and a report suggesting that Fas/FasL is necessary for DLI-induced tolerance in H-Y mice\(^{65}\).

While the results presented in this paper focus on the beneficial effect of DLI through DN regulatory T cells, it is possible that other cells may participate in tolerance induction, for example by supplying cytokines to help DN regulatory T cells. We have previously demonstrated that DN regulatory T cells require both antigen stimulation and cytokines in order to expand and function \textit{in vitro}\(^{32}\). Furthermore, we have demonstrated
that DLI also leads to an increase in the concentration of IL-4 in the sera of treated animals. Since DN T cells do not produce IL-4, it is likely that this cytokine, and perhaps others, are produced by accessory cells. It is therefore possible that in addition to activating regulatory T cells, DLI may also stimulate accessory cells and/or the production of cytokines that are required for DN regulatory T cell activation and function.

In conclusion, we demonstrate in this paper that DLI leads to the activation of antigen-specific DN regulatory T cells in the periphery of recipient animals. These activated DN regulatory T cells can migrate to donor-specific skin allografts following transplantation. Both peripheral and graft-infiltrating DN regulatory T cells can kill anti-donor CD8\(^+\) T cells in an antigen specific manner. These findings demonstrate that DLI activated DN T cells can execute their function both systemically and locally. Our data also suggests that graft-infiltrating T cells can be beneficial, highlighting the importance of determining the identity of graft-infiltrating cells in the diagnoses of graft rejection.

Acknowledgements

We thank H. Eilson for the 1B2 hybridoma, and D.Y. Loh for his permission of using 2C transgenic mouse.

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Figure Legends

**Figure 1.** *DN T cells from DLI-treated mice can suppress anti-donor CD8⁺ T cells.* (a and b) *(2Cxdm2) F₁* mice were given DLI (■) or left untreated (▼), and then transplanted as described in the methods. At 1 week after transplantation, splenocytes (a) or lymph node cells (b) were collected from recipient mice and the proportion of DN T cells was determined by flow cytometry. Varying numbers of lymphocytes (up to 7.0x10⁴ total cells) containing the indicated numbers of DN T cells were used as putative suppressor cells, and cultured with 1000 naïve 1B2⁺CD8⁺ T cell responder cells and irradiated (B6xBALB/c)⁺F₁ splenocytes. 3 days later, 1μCi of [³H] TdR was added to each well, the plate was incubated overnight, and harvested 18 hours later. Data shown is the proliferation in cpm for 3 replicates in 2 independent experiments. (c) Naïve 1B2⁺CD8⁺ T cells were stimulated for 4 days with irradiated (B6xBALB/c)⁺F₁ cells, labeled overnight with 10 µCi of [³H] TdR, and used as target cells. DLI-treated (■) and untreated (▼) lymph node cells were used at various numbers as effector cells. Data shown is % specific killing for 3 replicates in 2 independent experiments. (d) DN T cells were purified from the lymph nodes of DLI-treated mice 3 days after transplantation as described in the methods, and used as effector cells. Activated anti-Ld⁺ (■) or anti-H-2s (▼) CD8⁺ T cells were used as targets at ratios as indicated. The data shown is the mean % specific killing for 3 replicates in 2 independent experiments.

**Figure 2.** *DN T cells infiltrate donor-specific skin allografts and form the dominant subset of graft-infiltrating T cells.* (a) *(2Cxdm2) F₁* mice were given a DLI followed by transplantation of both (B6xBalb/c)⁺F₁ allogeneic and (B6xdm2)⁺F₁ syngeneic skin grafts. At
21 (top and middle panels) and 120 days (bottom panel) after transplantation, the accepted skin grafts were harvested and stained with haematoxylin and eosin. In each instance, an overview picture of the skin graft site is shown in the left panel (X 50), and a close-up of the deep dermis is shown on the right (X 400). Top panel: syngeneic graft at 21 days post-transplant. The epidermis and dermis are normal, and dermal appendages can be seen (left). In the deep dermis, there is no cellular infiltrate, and the blood vessels and collagen fibers are normal. Middle panel: allograft at 21 days post-transplant. The epidermis shows acanthosis, hyperkeratosis and focal keratotic plugging. The dermis is dedmatous and there is a very heavy cellular infiltrate in the mid and deep dermis (left). The infiltrate in deep dermis is predominantly lymphocytic in type (right). Bottom panel: allograft at 120 days post-transplant. The skin structure is normal aside from the deep dermis where there is a mild cellular infiltrate and fibrosis (left). The deep dermis shows increase in collagen fibers and is less vascular than normal skin. The infiltrate cells are much less numerous than that was seen at 21 days, and are predominantly lymphocytes.

(b) Graft infiltrating cells were collected from the accepted (B6xBALB/c)\(_{F1}\) skin grafts 1, 2, 8 and 15 weeks after transplantation. The cells were analyzed by flow cytometry for CD11b\(^+\), NK1.1\(^+\), \(\gamma\delta\)-TCR\(^+\), CD8\(^+\), CD4\(^+\) and 1B2\(^+\)DN T cells. The results shown are the proportions of positive graft infiltrating cells, each point represents data collected from at least 3 mice.

(c) Graft infiltrating cells were collected from the DLI-treated (B6xBALB/c)\(_{F1}\) skin grafts of (2Cxdm2)\(_{F1}\) mice 1 week after transplantation. The cells were analyzed by flow cytometry for expression of 1B2, CD4 and CD8. The histogram is gated on the 1B2\(^+\) T cell population and the percentages of the CD4\(^+\), CD8\(^+\) and DN T cells are explicitly indicated.
**Figure 3.** DN T cells accumulate in donor specific but not third party skin grafts. Graft infiltrating cells from Ld+ (dark bars) and third party (light bars) allografts of DLI-treated and untreated mice were collected on days 3 and 7 after transplantation and analyzed as described in Figure 2c. The number of 1B2+DN T cells in skin grafts of DLI treated and untreated recipients was determined by multiplying the number of total graft infiltrating cells by the proportion of 1B2+DN T cells determined by flow cytometry. Results are pooled from at least 4 skin grafts.

**Figure 4.** Graft infiltrating DN T cells can suppress and kill anti-donor CD8+ T cells. (a) Graft infiltrating cells were collected from 14 Ld+ skin grafts of DLI-treated mice, stained to determine the proportion of DN T cells, and used as suppressor cells. Naïve 1B2+CD8+ T cells were used as responders. Data shown is the mean proliferation in cpm for 3 replicates. (b) Graft infiltrating cells were collected as in (a) and were depleted for CD4+ and CD8+ T cells as described in the methods. The enriched DN T cells were stimulated overnight and used as effector cells in a cytotoxicity assay. Activated 1B2+CD8+ (anti-L^d, *) and BW5147 (third party, ■) cells were used as targets. Data shown is the % specific killing of target cells for 3 replicates.

**Figure 5.** DLI activates functional DN T cells in non-transgenic mice. (a) (B6xdm2)F1 mice were given DLI and transplanted as described in the methods. At 1 week after transplantation spleen cells were harvested from 4 mice, and DN T cells were purified as described in the methods. Activated anti-L^d (dark bars) or anti-H-2s (light bars) CD8+ T cells were used as targets at effector target ratios as indicated. Data shown is % specific
killing for 3 replicates. (b and c) (B6xdm2)F1 mice were given DLI (b) or left untreated (c) and transplanted as previously described. One week after transplantation graft-infiltrating cells were harvested and stained using anti-CD3, anti-CD4 and anti-CD8 mAbs. The data shown is gated on CD3+ T cells, and is pooled from 4 mice. (d) Graft-infiltrating T cells were purified from the Ld+ graft of DLI treated (B6xdm2)F1 mice 1 week after skin grafting. DN T cells were purified and used as putative suppressor cells at 5000 cells/well. Naïve (B6xdm2)F1 splenocytes cells were used as responder cells, and were stimulated with either (B6xBALB/c)F1 (Ld+) or SJL (H-2s) irradiated splenocytes as indicated. The data shows % inhibition of proliferation of CD8+ responder cells, and is pooled from 4 mice.
Figure 1
Figure 2
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Figure 4
Figure 5
Donor-lymphocyte infusion induces tolerance by activating systemic and graft-infiltrating double negative regulatory T cells

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