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Transforming Growth Factor-Beta1 Gene Transfer is Associated with the Development of Regulatory Cells

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Adenovirus-mediated transfection of mouse cardiac allografts with active human transforming growth factor-beta 1 (TGF-β1) prolongs transplant survival provided that recipients are initially depleted of CD8+ T cells. To test if graft survival was prolonged by persistent TGF-β1 transgene expression, long-term transfected allografts were re-transplanted into naïve mice that were transiently depleted of CD8+ T cells. Retransplanted allografts were acutely rejected, indicating that TGF-β1 transgene expression did not suppress effector cell function. We next asked whether TGF-β1 gene transfer was associated with the development of regulatory cells. When splenocytes obtained from mice bearing long-term TGF-β1-transfected allografts were adoptively transferred into recipients of non-transfected cardiac allografts, prolonged allograft survival was observed, and increased levels of the regulatory T cell transcription factor Foxp3 were present. To further test for regulation, differentiated effector cells were obtained from mice that had rejected cardiac allografts and were adoptively transferred into mice bearing long-term TGF-β1 transfected cardiac allografts. The effector cells failed to mediate rejection in mice bearing TGF-β1-transfected allografts and we observed a significant increase in intra-graft Foxp3 expression. These findings indicate that TGF-β1 gene transfer allows for the development of regulatory cells that control graft-reactive T cell responses once therapeutic levels of the transgene product are no longer produced.

Key words: Adenoviruses, gene therapy, graft acceptance, heart, transforming growth factor-beta (TGF-β), T cells

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Introduction

Gene transfer of immunomodulatory agents into allografts represents a clinically applicable approach to prolong transplant survival (reviewed in 1,2). Since vascularized transplants are routinely perfused at the time of organ harvest, introduction of the transgene can be accomplished at this time. It has been suggested that local secretion of soluble transgene products after transfection results in high concentrations of the immunosuppressive agent within the micro-environment of the graft itself, thereby limiting the deleterious effects of systemic immunosuppression. The use of adenoviral vectors allows efficient transfection of quiescent cells (3,4), including the parenchymal cells of solid organ transplants. However, anti-adenoviral immune responses may result in inflammation and loss of transgene expression in some transfected tissues (5–7). We have demonstrated that although adenovirus-mediated gene transfer of mouse cardiac grafts may induce an anti-adenoviral immune response, this response ignores the transfected cardiac graft, and transgene expression persists without local inflammation (8). Hence, adenoviral vectors represent an effective means of gene delivery into cardiac transplants.

TGF-β has a broad range of anti-inflammatory properties (9), including the inhibition of Th1 responses (10), E-selectin expression by endothelial cells (11), and CTL development (12). TGF-β also inhibits B cell proliferation and induces apoptosis in B cells (13,14) and fully differentiated plasma cells (9). Finally, TGF-β inhibits IFNγ and inducible nitric oxide synthase (iNOS) production, and increases expression of IL-1 receptor antagonist by activated macrophages (9,15). Indeed, we and others have demonstrated that adenovirus-mediated transfection with TGF-β1 (Ad-TGF-β 1) prolongs cardiac allograft survival (16–18). In our study (18), transfection of cardiac allografts, with adenovirus encoding the active form of human TGF-β1, promoted long-term cardiac allograft survival provided that recipients were transiently depleted of CD8+ cells at the time of transplantation. Th1 priming in the spleen and alloantibody responses in recipients were ablated by TGF-β1 transfection of the allograft, demonstrating systemic effects on the immune response (18), yet precursor graft-reactive T cells were readily detectable. The mechanism by which TGFβ1 gene transfer promotes long-term graft survival and controls graft-reactive precursor cells is not known. For example, Ad-TGF-β1 transfection of grafts results in

expression of TGF-β1 mRNA for at least 60 days post-transplant (18), suggesting that persistent transgene expression may continuously control immune responses. Yet, the systemic nature of the immunosuppression suggests that mechanisms other than local TGF-β1 expression may be operative.

Our previous study (18) demonstrated the need for transient depletion of CD8+ T cells for TGF-β1 gene transfer to mediate these immunosuppressive effects. However, graft-reactive CD8+ T cell responses were kept in check once these cells re-populated the periphery. A growing body of evidence indicates that CD8+ T cells are more difficult to control than are their CD4+ counterparts (reviewed in 19). Furthermore, it is well documented that CD4+ T cells may acquire T regulatory (T_{reg}) function in the transplant setting (reviewed in 20). These findings are in keeping with the notion that regulatory cells develop with time in recipients of TGF-β1-transfected allografts. The current study examines the potential role of persistent transgene expression and regulatory cells in the control of graft-reactive T cell responses following TGF-β1 gene transfer. Our findings support a role for regulatory cells in promoting graft survival once therapeutic levels of transgene expression have subsided.

Materials and Methods

Mice

Female C57BL/6 (H-2^b), and BALB/c (H-2^d) mice between 6 and 12 weeks of age were obtained from Charles River Laboratories (Raleigh, NC). C3H/HeN (H-2^k) mice were obtained from Harlan (Indianapolis, IN).

TGF-β1 encoding adenovirus

Ad-TGF-β1 consists of the cDNA encoding the active form of human TGF-β1 under the control of an RSV promoter placed in a replication-defective E1- and E3- deleted serotype 5 adenovirus vector (21). The TGF-β1 gene was cloned into the Not 1 site of the proviral plasmid, pAdRSV. Recombinant adenoviral clones were generated as described (22) and were subjected to two rounds of plaque purification prior to large-scale expansion and purification over cesium chloride gradients. Stocks of Ad-TGFβ1 were produced for *in vivo* use in the Vector Core at the University of Michigan Medical Center. Each stock of Ad-TGF-β1 was assessed for the ability to encode bioactive TGF-β1 by transfecting human kidney 293 cells (MOI = 100) and quantifying active TGF-β1 in the supernatant using the CCL4 mink lung epithelial cell assay (23).

TGF-β1 transfection of vascularized cardiac transplants

Prior to *in situ* perfusion of the donor heart, the left superior vena cava and the distal aorta were ligated to retain the perfusate. The aorta was then held in position by suture to facilitate perfusion using a syringe with a 30-gauge needle. The donor heart was flushed with 400 μL of heparin (250 U/mL), then perfused with 200 μL of lactated Ringer's solution containing adenoviral vectors (5×10^8 pfu). Following perfusion, donor grafts were harvested and placed in iced Ringer's for approximately 1 h prior to transplantation. Transfection occurs during the 1-h period of cold storage, which mimics the cold ischemic time human organs experience during transport. The transfected grafts were anastomosed to the great vessels in the abdomen as described previously (24,25). Briefly, the donor aorta and pulmonary artery were anastomosed end-to-side with the recipient's abdominal aorta and inferior vena cava. In this model, the transplanted heart is perfused with the recipient mouse's blood and resumes contraction. Transplant function was monitored by daily abdominal palpation.

Re-transplantation of cardiac allografts

TGF-β1-transfected cardiac allografts were removed from primary allograft recipients at 50 days post-transplant and re-transplanted into secondary recipients as previously described (25). Briefly, the primary allograft was carefully dissected from surrounding adhesions using a fine-tip cautery, vessels attached to the primary allograft were ligated, and the heart was removed from the primary recipient and transplanted into the secondary recipient via end-to-side anastomoses of transplant-associated aorta and vena cava to recipient aorta and vena cava. The re-transplanted hearts were then perfused with the recipient mouse's blood and resumed contraction spontaneously. Graft function was monitored by daily abdominal palpation.

In vivo depletion of CD8+ T cells

C57BL/6 mice were injected i.p. with 1 mg of anti-CD8 mAb (hybridoma 2.43, purified by Ligocyte Pharmaceuticals, Inc., Bozeman, MT) 1 day prior to transplantation with BALB/c cardiac allografts. An additional 1 mg was injected the day of transplantation, and on day +5, following transplantation. This protocol results in transient depletion of CD8+ cells, which re-populate the periphery approximately 30 days post-transplant (18).

Adoptive transfer of splenocytes

To test for the presence of regulatory cells, C57BL/6 recipients of BALB/c TGF-β1-transfected allografts were sacrificed at day 50 post-transplant, and the spleens were harvested. For isolation of differentiated effector splenocytes, spleens were harvested from unmodified C57BL/6 recipients of BALB/c cardiac allografts at 10 days post-transplant, once the recipients had actively rejected their transplants. Spleens were processed through wire mesh, and splenocytes were filtered through a 30-μm pore size nylon mesh and re-suspended in sterile PBS. For adoptive transfer, 5×10^7 splenocytes in 200 μL were injected via the tail vein into recipients at the indicated times.

Qualitative assessment of Foxp3 mRNA levels

RNA was isolated by phenol-chloroform extraction, and cDNA generated by a standard reverse transcription protocol. Foxp3 primers were designed using Primer Premier 5.0 software (Biosoft International, Palo Alto, CA) and were sense 5′ CCAAGGTGAGCGAGTGTC and anti-sense 5′ AAGGCAGAGTCAGGAGAAGT. Control GAPDH primers were designed using Primer 2 software (Scientific and Educational Software, State Line, PA) and were sense 5′ CTGGTGCTGAGTATGTCGTG and anti-sense 5′ CAGTCTTCTGAGTGGCAGTG. Real-time PCR was performed on a Cepheid Smart Cycler© System (Cepheid, Sunnyvale, CA) and primer binding to DNA was detected by SYBER Green I™ dye (Roche, Indianapolis, IN). Relative quantification of Foxp3 expression was obtained by subtracting the cycle threshold (Ct), or the point above baseline at which fluorescence is detected, of GAPDH from the Ct of Foxp3. The resulting difference in the cycle number is the exponent of the base 2 representing the fold difference between Foxp3 and GAPDH in cardiac cDNA samples.

Statistical analysis

Results were analyzed using a Fischer's PLSD test and p-values ≤ 0.05 were considered statistically different.

Results

Prolonged TGF-β1 transgene expression does not prevent rejection of re-transplanted cardiac allografts

We have previously reported that TGF-β1 transgene expression persists for at least 60 days in this system (18). While transgene expression decreases with time (8), it is not known whether transgene expression persists at therapeutic levels. Therefore, we determined whether persistent transgene expression in mice bearing long-term TGF-β1-transfected cardiac allografts contributed to transplant survival. To test this possibility, BALB/c TGF-β1-transfected cardiac allografts were removed from their original C57BL/6 recipients at day 50 post-transplant and re-transplanted into secondary C57BL/6 recipients that were transiently depleted of CD8+ cells. Allograft function was monitored in these secondary recipients. Re-transplantation of BALB/c TGF-β1-transfected hearts into secondary recipients resulted in acute rejection (Figure 1). However, rejection of these re-transplanted TGF-β 1-transfected allografts was slightly delayed when compared to BALB/c cardiac grafts that were transplanted into BALB/c primary recipients and then re-transplanted into CD8-depleted C57BL/6 secondary recipients. This suggests that persistent low-level TGF-β1 transgene expression may contribute to allograft survival, although this level of transgene expression is inadequate to completely suppress the rejection response. As a control for the success of the re-transplantation technique, syngeneic C57BL/6 hearts were transplanted into primary C57BL/6 hosts and then re-transplanted on day 50 into secondary C57BL/6 hosts. These re-transplanted syngeneic grafts continued to function normally until the termination of the experiment. Together, our results indicate that expression of TGF-β1 in the re-transplanted hearts had fallen below the therapeutic threshold required for immunosuppression, and that these long-term TGF-β1-transfected cardiac allografts retain their immunogenicity.

TGF-β1 transfection is associated with the development of regulatory cells

The fact that persistent transgene expression did not prevent rejection of re-transplanted allografts suggested that other mechanisms were operative in preventing graft-reactive T cell responses. To test for potential regulatory cell activity in this setting, splenocytes were obtained from mice bearing TGF-β1-transfected allografts at day 50 post-transplant and adoptively transferred into C57BL/6 mice transiently depleted of CD8+ cells. These mice were transplanted with unmodified BALB/c cardiac allografts one day after the adoptive transfer of cells. Transfer of splenocytes from mice bearing long-term TGF-β1-transfected allografts resulted in prolonged survival of unmodified BALB/c cardiac allografts (Allo TGF-β; Figure 2), indicating that regulatory cells were present in the transferred splenocytes. In addition, splenocytes transferred from mice bearing long-term syngeneic allografts expressing TGF-β1 did not prolong allograft survival in CD8-depleted recipients (Syn TGF-β; Figure 2), indicating that regulatory cells were induced only in the presence of alloantigen and TGF-β1 transfection. To assess regulatory cell specificity, splenocytes from mice bearing long-term TGF-β1-transfected BALB/c allografts were transferred into C57BL/6 mice that were

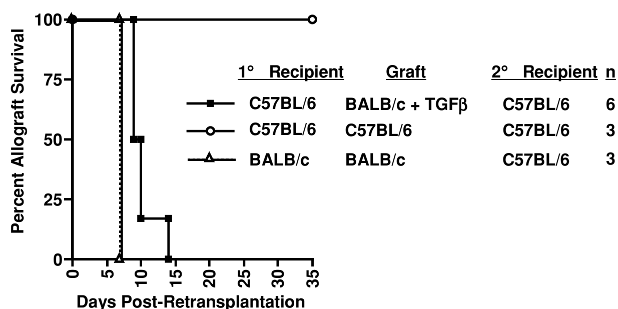

Figure 1: Transfection of cardiac allografts with TGF-β1 does not confer survival after re-transplantation. TGF-β1-transfected cardiac allografts were removed from C57BL/6 primary allograft recipients at day 50 post-transplant and re-transplanted into C57BL/6 secondary allograft recipients that were transiently depleted of CD8+ T cells. Time of rejection was compared to BALB/c syngeneic grafts removed from BALB/c primary recipients and re-transplanted into C57BL/6 secondary recipients. As controls for the success of the re-transplantation procedure BALB/c syngeneic transplants were removed from BALB/c primary recipients at day 50 post-transplant and re-transplanted into BALB/c secondary recipients. Graft function was monitored by daily palpation, and the time the transplant stopped beating was recorded as the day of rejection.

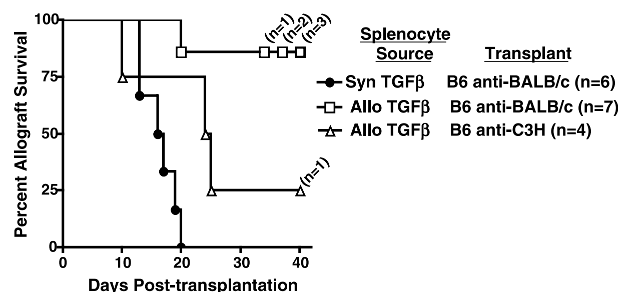

Figure 2: Adoptive transfer of splenocytes from mice bearing long-term TGF-β1-transfected allografts prolongs survival of unmodified cardiac allografts. Splenocytes were harvested at day 50 post-transplant from C57BL/6 mice bearing TGF-β1-transfected BALB/c cardiac allografts (Allo TGF-β). Control splenocytes were harvested at day 50 post-transplant from C57BL/6 mice bearing TGF-β1-transfected syngeneic allografts (Syn TGF-β). Naïve mice were injected i.p. with 1 mg anti-CD8 mAb on days -2 and -1 relative to splenocyte adoptive transfer (5×10^7 cells i.v.) and then were transplanted with unmodified BALB/c (B6 anti-BALB/c) or third-party C3H (B6 anti-C3H) cardiac allografts the day after adoptive transfer. Graft function was monitored by daily palpation and the time the transplant stopped beating was recorded as the day of rejection.

depleted of CD8+ cells and transplanted with third-party C3H allografts (Allo TGF-β → B6 anti-C3H). Survival of these third-party allografts was slightly, but not statistically prolonged, possibly reflecting a cross-reactivity between BALB/c and C3H alloantigens (26). Indeed, histologic assessment of third-party grafts that experienced delayed rejection revealed a less intense infiltrate and more preserved cardiac myocytes when compared to rejected control grafts (Syn TGF-β → B6 anti-BALB/c) (data not shown).

Regulatory cells associated with TGF-β1 gene transfer prevent rejection mediated by effector cells

Since regulatory cells associated with TGF-β1 gene transfer were capable of suppressing the initial immune response to a transplanted cardiac allograft, we next asked whether these cells could control rejection responses mediated by differentiated effector cells. To this end, we isolated splenocytes from unmodified C57BL/6 mice that had rejected BALB/c cardiac allografts, and adoptively transferred these splenocytes into recipients of TGF-β1-transfected allografts at day 50 post-transplant. Adoptive transfer of effector cells failed to induce rejection of TGF-β1-transfected allografts, and these grafts continued to function until the termination of the experiment (Figure 3). As a positive control for effector cell function, these cells were injected into SCID recipients of BALB/c allografts, where they induced acute rejection by day 10 post-adoptive transfer (Figure 3). It should be noted that the transferred effector cells included CD8+, as well as CD4+ effector T cells, indicating that TGF-β1 gene transfer was effective against CD8+ cells in this context.

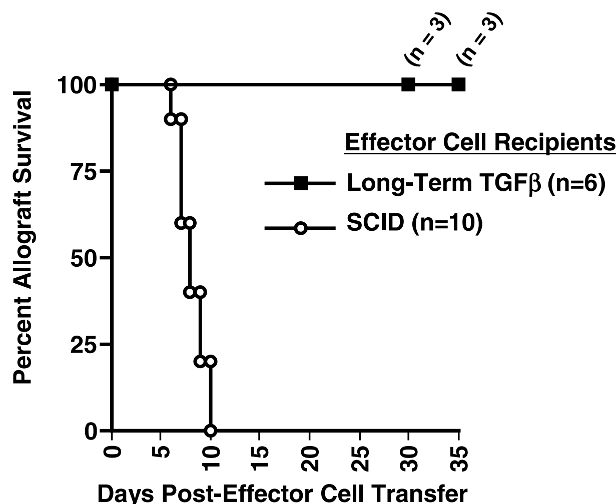

Figure 3: Differentiated effector cells are incapable of mediating graft rejection in mice bearing day-50 TGF-β1-transfected allografts. C57BL/6 recipients of TGF-β1-transfected allografts were transiently depleted of CD8+ T cells. At day 50 post-transplant, recipients were injected with 5×10^7 effector splenocytes isolated on day 10 from C57BL/6 recipients that had acutely rejected BALB/c cardiac allografts. As controls, C57BL/6 SCID recipients of BALB/c allografts were also injected with effector cells. Graft function was monitored by daily palpation and the day of rejection was recorded at the time the transplant stopped beating. Results are recorded as the day post-effector cell transfer that transplant function ceased.

Regulatory cells are present in cardiac allografts after cell transfer

Recent studies have shown that TGF-β-dependent regulatory cells can be identified in graft tissue in mice tolerating allografts (27). To test for the presence of regulatory cells in heart allografts in our recipient mice, we used quantitative real-time RT PCR to determine the relative intra-graft expression of Foxp3, which has been identified as a transcription factor essential for the development and function of CD4+/CD25+ T_{reg} (28,29). Levels of Foxp3 expression varied in long-term TGF-β1-transfected allografts, although the average relative expression in TGF-β1-transfected allografts was greater than the expression in syngeneic allografts also expressing TGF-β1 (Figure 4, comparison 1). More important, grafts in mice that received splenocytes from recipients of TGF-β1-transfected allografts (Allo TGF-β trans; Figure 4) showed significantly greater levels of Foxp3 expression ($p = 0.03$) than rejected grafts from recipients that received splenocytes from mice bearing TGF-β1-transfected syngeneic allografts (Syn TGF-β trans, Figure 4, comparison 2), suggesting that the transferred regulatory cells trafficked to the allograft. We also assessed the presence of regulatory cells in the TGF-β1-transfected grafts of mice that received differentiated effector cells. Grafts from these recipients also showed a significant increase in Foxp3 expression when compared with grafts from SCID recipients of adoptively transferred effector cells ($p = 0.01$; Figure 4, comparison 3). Together, these results suggest that TGF-β1 gene transfer associated regulatory cells are capable of migrating to cardiac allografts and preventing allograft rejection.

Discussion

We have previously reported that adenovirus-mediated transfection of cardiac allografts with the active form of TGF-β1 is effective in preventing graft-reactive T cell priming and promoting graft acceptance provided that CD8+ T cells are transiently depleted (18). However, precursor graft-reactive cells re-appear as CD8+ cells re-populate the periphery, but these cells are maintained in a quiescent state. The current study evaluated mechanisms involved in preventing the activation of these graft-reactive precursor cells. At least two possibilities come to mind: first, it is possible that persistent TGF-β1 transgene expression continues to suppress the activation of quiescent precursor cells. Indeed, we have found that adenoviral-mediated transfection of cardiac transplants results in transgene expression for at least 60 days (8,18), albeit at lower levels than that observed during the early post-transplant

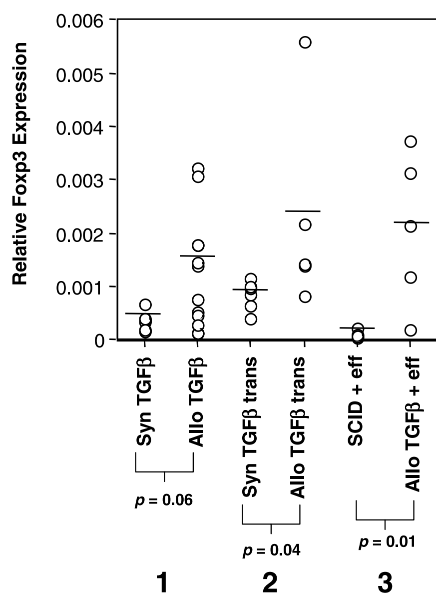

Figure 4: Relative intra-graft expression of Foxp3 is increased by transfection with TGF-β1. Total RNA was isolated from cardiac grafts under the following conditions: Comparison 1: Cardiac grafts were harvested on day 50 from C57BL/6 mice transiently depleted of CD8+ T cells that received TGF-β1 transfected C57BL/6 grafts (syn TGF-β), or TGF-β1-transfected BALB/c grafts (allo TGF-β). Comparison 2: Grafts were harvested from CD8-depleted recipients of unmodified BALB/c allografts that received splenocytes from long-term recipients of C57BL/6 TGF-β1-transfected allografts (syn TGF-β trans) or from long-term recipients of BALB/c TGF-β1-transfected allografts (allo TGF-β trans). Grafts were harvested at the time of transplant rejection (syn TGF-β trans, see Figure 2) or at day 35 post-transplant (allo TGF-β trans). Comparison 3: Grafts were harvested from SCID recipients of TGF-β1-transfected BALB/c grafts that received differentiated effector cells (SCID + eff) or from long-term C57BL/6 recipients of TGF-β1-transfected BALB/c allografts that received differentiated effector cells (allo TGF-β + eff). Grafts were harvested at the time of transplant rejection (SCID + eff, see Figure 3) or at day 35 post-effector cell transfer (allo TGF-β + eff). Relative levels of Foxp3 expression were quantified as described in materials and methods. The average relative expression of Foxp3 for each experimental group is indicated by horizontal lines, and the p-values between groups are reported.

period (8). To test whether the level of TGF-β1 transgene expression retained the ability to prevent the rejection response, we re-transplanted accepted TGF-β1-transfected cardiac allografts into naïve mice that were transiently depleted of CD8+ cells (Figure 1). These grafts were acutely rejected, indicating that the level of TGF-β1 expression had fallen below the threshold required to subvert the rejection response mounted by the secondary allograft recipients. It is possible that the re-transplantation procedure and associated ischemia may have induced pro-inflammatory cytokines such as TNFα, which Bromberg's group has shown can inhibit viral promoter driven transgene expression (30). However, it should be noted that the initial transplant procedure would also subject the graft to similar inflammatory processes, yet the level of TGF-β1 transgene expression is sufficient to inhibit the rejection response in the primary allograft recipient. This indicates that TGF-β1 transgene expression falls below a therapeutic threshold with time following transplantation. Rejection of these re-transplanted allografts also illustrates that these accepted grafts maintain their immunogenicity, thereby further supporting the notion that acceptance of TGF-β1-transfected allografts is an active process.

Since persistent TGF-β1 transgene expression did not appear to be the primary factor responsible for long-term allograft acceptance, a second possibility is that active regulation participates in graft acceptance. Indeed, several studies have demonstrated that TGF-β plays an important role in the induction of T_{reg} (27,31,32). To test the effect of TGF-β1 secreted by the graft on systemic alloimmune responses, we isolated splenocytes from mice bearing long-term TGF-β1-transfected grafts and adoptively transferred these cells into mice that were transiently depleted of CD8+ cells and received unmodified allografts. Cells from recipients of TGF-β1-transfected allografts, but not TGF-β1-transfected syngeneic grafts, prevented acute rejection (Figure 2). These results indicate that the presence of both alloantigen and TGF-β1 were required to induce regulation. Interestingly, this adoptive transfer approach revealed a possible third-party cross-reactivity of these regulatory cells in that they promoted a degree of protection when transferred into third-party allograft recipients. The presence of these regulatory cells was further illustrated by the experiments depicted in Figure 3, where differentiated effector cells transferred into mice bearing TGF-β1-transfected allografts could not induce rejection. Together, these results demonstrate that a population of regulatory cells develops when TGF-β1 is secreted within the allograft.

To verify that regulatory cells were functioning in our system, we assessed the levels of Foxp3 expressed within the allograft (Figure 4). Foxp3 has been identified as the transcription factor present in regulatory, but not activated CD4+/CD25+ T cells (28), and is currently considered the most reliable marker for T_{reg}. Therefore, increased Foxp3 expression within the graft would reflect the presence of T_{reg} that have trafficked to the allograft where they may mediate regulatory function. We have noted that levels of Foxp3 expression in the spleen are not informative, as naïve, non-transfected mice have detectable levels of Foxp3 expression due to the presence of endogenous T_{reg} (data not shown). We first determined the relative expression of Foxp3 in long-term allogeneic TGF-β1-transfected grafts versus syngeneic TGF-β1-transfected grafts (Figure 4, comparison 1). Variable levels of Foxp3 were expressed in accepted TGF-β1-transfected allografts with 5 of 12 expressing higher levels than TGF-β1-transfected syngeneic grafts. It should be noted that only low levels of Foxp3 were expressed in TGF-β1-transfected syngeneic

grafts, reflecting the inability of splenocytes obtained from these mice to adoptively transfer immunosuppression (Figure 2). The variability in the levels of Foxp3 within accepted allografts may reflect trafficking of T_{reg} between the graft and lymphoid tissues. Hence, regulatory cells may traffic to the graft on an as-needed basis. This possibility is supported by the observation that adoptive transfer of splenocytes from mice bearing long-term TGF-β1-transfected allografts, which promoted graft survival (Figure 2), coincided with increased Foxp3 expression within functioning grafts in splenocyte recipients (Figure 4, comparison 2). Furthermore, adoptive transfer of effector cells into mice bearing long-term TGF-β1-transfected allografts failed to induce rejection (Figure 3), a result which was reflected by increased Foxp3 expression within the graft (Figure 4, comparison 3). The trafficking capacity of T_{reg} has been recently demonstrated by Fu et al. (33), who reported that T_{reg} expressing the peripheral lymph node homing receptor, CD62L, migrate to lymph nodes and are more effective at suppressing immune responses than are T_{reg} that express low levels of CD62L. Our adoptive transfer studies (Figure 4) provide further evidence for regulatory cell migration to allografts.

Prolonged survival of TGF-β1-transfected allografts and the generation of regulatory cells in this system requires that the allograft recipient be transiently depleted of CD8+ T cells (18). In addition, the ability of splenocytes obtained from mice bearing long-term TGF-β1-transfected allografts to adoptively transfer suppression is dependent upon initial depletion of recipient CD8 cells (data not shown). Other studies have shown that T_{reg} are capable of suppressing CD8+ T cells both *in vitro* (34) and *in vivo* in transplant models (35,36). It should be noted that these studies employed transgenic TCR CD8+ T cells that respond to a single peptide, rather than the polyclonal CD8+ T cell population that is mobilized in our system. Using this cardiac allograft model, we (37) and others (38) have reported that the development of regulatory cells following anti-CD40L therapy does not require transient depletion of CD8+ T cells. This may be due to the sensitivity of CD4+ versus CD8+ T cells to TGF-β and other immunosuppressive agents (reviewed in 19). Hence, induction of regulatory cells following TGF-β1 gene transfer appears to have differential requirements when compared to other transplant settings (35–38). For example, production of IFNγ by CD4+ T cells is more dependent upon IL-12 (39), Stat 4 (40), and the Th1 transcription factor T-bet (41) than is production by their CD8+ counterparts. Since TGF-β inhibits T-bet expression (42), which is required for IFNγ production by CD4+ but not CD8+ T cells (41), it makes biologic sense that CD8+ T cells would be less susceptible than CD4+ T cells to TGF-β1 gene transfer. It should be noted that CD8+ T cells have an additional transcription factor, Eomesodermin, which facilitates T-bet-independent IFNγ production (43). Interestingly, adoptive transfer of differentiated effector splenocytes (which contain activated CD8+ cells) did not result in rejection of TGF-β1-transfected grafts (Figure 3). In this instance, Foxp3 expression was increased in the TGF-β1-transfected graft (Figure 4) and regulatory cells appeared to be capable of controlling the primed CD8+ response. This may reflect the fact that Foxp3-expressing regulatory cells have already developed in long-term recipients of TGF-β1-transfected allografts and are present in sufficient numbers to control the CD8+ T cell response.

In summary, this study has shown that adenoviral-mediated gene transfer of TGF-β1 is associated with the development of regulatory cells which traffic to the graft and are capable of controlling precursor as well as differentiated graft-reactive T cells. We suggest that these regulatory cells develop with time under the cover of transient CD8+ T cell depletion and intra-graft TGF-β1 expression, which wanes with time. To our knowledge, this is the first report of an immunosuppressive cytokine gene therapy regimen that facilitates the development of regulatory cells which continue to control the rejection response once therapeutic levels of the transgene product have subsided.

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