

# Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



## **Immune Sources of Transforming Growth Factor- $\beta$ 1 Reduce Transplant Arteriosclerosis : Insight Derived From a Knockout Mouse Model**

Jörg Koglin, Troels Glysing-Jensen, Anne Räisänen-Sokolowski and Mary E. Russell  
*Circ. Res.* 1998;83:652-660

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

Copyright © 1998 American Heart Association. All rights reserved. Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circres.ahajournals.org/cgi/content/full/83/6/652>

Subscriptions: Information about subscribing to Circulation Research is online at  
<http://circres.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:  
[journalpermissions@lww.com](mailto:journalpermissions@lww.com)

Reprints: Information about reprints can be found online at  
<http://www.lww.com/reprints>

# Immune Sources of Transforming Growth Factor- $\beta_1$ Reduce Transplant Arteriosclerosis

## Insight Derived From a Knockout Mouse Model

Jörg Koglin, Troels Glysing-Jensen, Anne Räisänen-Sokolowski, Mary E. Russell

**Abstract**—Activated CD4-positive T cells are essential in the early stages of arteriosclerotic lesion development after cardiac transplantation. Besides its parenchymal effects, transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) mediates immunosuppressive effects on proliferation and activation of CD4 cells. This study was designed to assess immune contributions of TGF- $\beta_1$  to arteriosclerosis by comparing the effect of TGF- $\beta_1$ -deficient and -competent infiltrating inflammatory cells on the development of intimal thickening in a heterotopic mouse transplant model (CBA to C57B6). Transplant arteriosclerosis was evaluated in cardiac grafts placed into knockout recipients heterozygous for TGF- $\beta_1$  (n=7) and was compared with those placed into wild-type recipients (n=11). At 55 days, allografts in TGF- $\beta_1$ -deficient recipients had increased concentric intimal thickening. Computer-assisted analysis of all elastin-positive vessels (n=173) showed significantly increased luminal occlusion ( $67.8 \pm 5.6\%$ ) in grafts from TGF- $\beta_1$ -deficient recipients compared with wild-type recipients ( $47.4 \pm 4.1\%$ ,  $P=0.003$ ). To determine whether TGF- $\beta_1$  deficiency altered CD4 activation patterns, we studied intragraft cytokine expression. Using  $^{32}\text{P}$ -reverse-transcriptase polymerase chain reaction assays, we show that TGF- $\beta_1$ -deficient recipients had an increased expression of the transcription factor STAT 4, interferon gamma, and interleukin-2 (Th1-type response) and unaltered or reduced expression of the transcription factor STAT 6, interleukin-4, and interleukin-10 (Th2-type response). Hence, when present, immune sources of TGF- $\beta_1$  attenuate transplant arteriosclerosis. This effect is associated with attenuation of Th1 forces. (*Circ Res.* 1998;83:652-660.)

**Key Words:** heart transplantation ■ growth factor ■ Th1 cell ■ cytokine ■ graft rejection

Transplant arteriosclerosis is an accelerated form of intimal thickening that develops after cardiac transplantation. Alloimmune responses are believed to initiate an inflammatory cascade culminating in neointimal smooth muscle cell proliferation, perivascular fibrosis, and expansion of extracellular matrix.<sup>1,2</sup> The importance of CD4-positive T cells in this cascade has been established using rodent models.<sup>3,4</sup> Depletion or inhibition of CD4 cells prevents or significantly reduces neointimal thickening. However, less is known about the specific molecular mechanisms through which these T-cell effects are conferred. One area of active investigation has focused on cytokine pathways because activated CD4 cells differentiate into distinct Th1 and Th2 subsets.<sup>5</sup> Th1 cells produce interferon gamma (IFN- $\gamma$ ), interleukin (IL)-2, and tumor necrosis factor- $\beta$  (TNF- $\beta$ ), whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13.<sup>6</sup> Recently, we<sup>7</sup> and others<sup>8</sup> have collected in vivo evidence that Th1 forces promote arteriosclerotic lesion development. Using recipient mice with targeted deletion of IFN- $\gamma$  in our mouse transplant model, we showed that allograft vessel thickening and neointimal smooth muscle cell expansion are reduced when IFN- $\gamma$ -mediated Th1 responses are absent.<sup>7</sup>

Transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) is a polypeptide cytokine produced by a large variety of different cell types that can modulate cellular differentiation, cell proliferation, and extracellular matrix formation.<sup>9,10</sup> Though not focus of the present study, much attention has focused on the fibrotic roles of TGF- $\beta_1$  in vascular remodeling. Increased expression of TGF- $\beta_1$  in tissue from primary arterial and secondary restenotic lesions suggested a role as a regulator of arteriosclerotic lesion development.<sup>11-14</sup> In vitro studies have shown that TGF- $\beta_1$  has actions that could promote as well as attenuate lesion development. For example, TGF- $\beta_1$  inhibits both migration and proliferation of vascular smooth muscle cells in vitro.<sup>15,16</sup> At the same time, TGF- $\beta_1$  promotes extracellular matrix expansion through stimulation of matrix formation<sup>17,18</sup> and suppression of matrix degradation.<sup>19</sup> TGF- $\beta_1$  promotes a prothrombotic state by stimulation of plasminogen activator inhibitor activity and protein synthesis in smooth muscle cells.<sup>20</sup>

Far less attention has focused on the immunosuppressive effects of TGF- $\beta_1$ . Early death due to multifocal inflammatory processes in mice with homozygous disruption of the TGF- $\beta_1$  argues that its dominant effect involves immune cell

Received September 11, 1997; accepted July 1, 1998.

From the Cardiovascular Biology Laboratory, Harvard School of Public Health (J.K., T.G.-J., A.R.-S., M.E.R.), Brigham and Women's Hospital (M.E.R.), and Harvard Medical School (M.E.R.), Boston, Mass.

Correspondence to Mary E. Russell, MD, Cardiovascular Biology Laboratory, Harvard School of Public Health, 677 Huntington Ave, Boston, MA 02115. E-mail russell@cvlab.harvard.edu

© 1998 American Heart Association, Inc.

responses.<sup>21-23</sup> The immunosuppressive role of TGF- $\beta_1$  is of great interest because it has been shown to inhibit Th1 responses. TGF- $\beta_1$  suppresses lymphocyte proliferation and inhibits activation of natural killer cells and T cells.<sup>24,25</sup> When added to CD4-positive lymphocytes, TGF- $\beta_1$  inhibits development of IFN- $\gamma$ -producing cells.<sup>26</sup> Furthermore, TGF- $\beta_1$  antagonizes the effects of IL-2 and TNF- $\beta$ .<sup>10</sup> Several lines of evidence suggest that TGF- $\beta_1$  acts as an inhibitor of immunological responses during cardiac allograft rejection. First, TGF- $\beta_1$  expression and activity are increased in tissue from rejecting rat allografts.<sup>27</sup> Second, immunosuppressive actions of cyclosporine A in humans are associated with increased expression of TGF- $\beta_1$ .<sup>28</sup> Third, treatment with recombinant TGF- $\beta_1$ <sup>25,29</sup> or TGF- $\beta_1$ -encoding vectors<sup>30</sup> results in reduced rejection and prolonged survival of the graft in different transplant models.

Given that TGF- $\beta_1$  might have opposing effects in different cellular compartments within the same organ system, it has been difficult to assign a simple biological role for TGF- $\beta_1$  in arteriosclerotic lesion development. Our vascularized heterotopic mouse model of transplant arteriosclerosis offers the opportunity to study one aspect of this question. In this model, selective disruption of recipient sources permits the study of TGF- $\beta_1$  deficiency confined to infiltrating immune cells. By placing wild-type donor hearts into recipients with targeted deletion of the TGF- $\beta_1$  gene, one can study the effect of ongoing reductions in TGF- $\beta_1$  on arteriosclerotic lesion development. However, mice with heterozygous gene deletion (TGF- $\beta_1$  +/-) have to be used because homozygous gene disruption produces death within 3 weeks after birth due to multifocal inflammatory disease.<sup>22,23</sup> This combination of a wild-type donor and a knockout recipient creates a cardiac allograft microenvironment that is composed of a TGF- $\beta_1$ -intact parenchyma (donor-derived) and TGF- $\beta_1$ -deficient infiltrating inflammatory cells (recipient-derived). To study the effect of immune sources of TGF- $\beta_1$  on transplant arteriosclerosis, we compared vessel morphometry, vascular cell composition, and cytokine expression in grafts from TGF- $\beta_1$ -deficient recipients and wild-type recipients.

## Materials and Methods

### Mice

Male CBA/CaJ (H-2<sup>b</sup>) mice aged 6 to 8 weeks were used as heart donors. As organ recipients, male heterozygous TGF- $\beta_1$ -deficient TGF- $\beta_1$  +/- mice on a C57BL/6J (H-2<sup>b</sup>) background were compared with C57BL/6J (H-2<sup>b</sup>) wild-type mice. All mice were obtained from Jackson Laboratory, Bar Harbor, Me.

### Heterotopic Cardiac Transplantation

Vascularized heterotopic abdominal cardiac transplantation was performed as described by Corry et al,<sup>31</sup> and hearts were harvested as described previously.<sup>4</sup> Allografts from TGF- $\beta_1$  +/- recipients (n=7) were compared with those from allogeneic wild-type recipients (n=11). CD4 and CD8 antibodies (GK1.5 and 2.43; 2.0 mg IP, days 1 to 4 and then weekly to day 28) were used as T cell-depleting immunosuppressive therapy. This program of immunosuppression was used to delay the onset of rejection and produce grafts undergoing chronic rejection.<sup>7,32</sup> Graft function was evaluated daily by measuring the force of palpable heart beat. Grafts and native host hearts were harvested when the palpation score was  $\leq 1$  (on a scale from 0 to 4) or when the graft reached 55 days. After perfusion with PBS, cardiac allografts were harvested.

### Histological Analysis of Rejection

The degree of parenchymal rejection was evaluated in transverse paraffin sections of all grafts stained with hematoxylin and eosin. Allografts were graded for severity of rejection using a modified ISHLT grading system (scale 0 [no rejection] to 4 [severe rejection]).<sup>33,34</sup> Grading was performed by 2 independent observers in a blinded fashion. Scores are reported as mean value for all grafts in each recipient group.

### Morphometric Vessel Analysis

Vascular analysis was performed in paraffin sections stained with Verhoeff's elastin as previously described.<sup>7,32</sup> Microscopic images of all elastin-positive vessels were captured. The captured images were used to trace the lumen and the internal elastic (NIH 1.6 software). The intimal area was determined by subtracting the area of the lumen from the area enclosed by the internal elastic lamina. From these data, we derived the percentage of luminal occlusion. Vessels were classified by size as large subepicardial and smaller intramyocardial arteries and analyzed separately. In addition, we analyzed sections stained for  $\alpha$ -actin (counterstained with Verhoeff's elastin) to estimate the area of the smooth muscle cell-rich media. From these data, we derived intima/media ratios as a parameter corrected for vessel size.<sup>32</sup> Values are calculated as the mean from all captured vessels per heart and reported as mean  $\pm$  SEM for all grafts in each recipient group.

### Analysis of Vascular Composition

To evaluate the composition of arteriosclerotic lesions, we compared staining with Masson's trichrome, anti- $\alpha$ -smooth muscle cell actin (counterstained with Verhoeff's elastin, clone 1A4, 1:20 000, Sigma Chemical Co),<sup>7</sup> and anti-CD45 (clone 30F11.1, 1:1000, Pharmingen)<sup>35</sup> in representative paraffin sections from allografts transplanted into TGF- $\beta_1$  +/- recipients (n=5) and wild-type recipients (n=4). Lymphocytes, capable of differentiating toward Th1 or Th2, were detected in frozen sections from the same allografts using an antibody for CD4 (clone GK1.5, 1:100).

To estimate potential differences in perivascular fibrosis and neointimal smooth muscle cell expansion, we performed quantitative image analysis using NIH Image 1.6. Microscopic images of all vessels in this subset of grafts were captured separately. Because of inherent resolution limits of the video capture system used for image analysis, only a subset of vessels was studied. Larger vessels that had areas delineated by the internal elastic lamina of  $\geq 350 \mu\text{m}^2$  and that had  $\geq 40\%$  luminal occlusion were included.<sup>7</sup> For each vessel cross section, the size was defined by tracing the external elastic lamina. The neointima was defined by tracing the internal elastic lamina and the lumen. Perivascular fibrosis was quantified by measuring the perivascular area as the area encompassed by pixels of the color intensity of collagen-positive tissue (blue) and was normalized by the respective vessel area. To estimate the contribution of smooth muscle cells to the expanded neointima, the percent neointimal area stained specifically for  $\alpha$ -smooth muscle cell actin was detected by measuring the area encompassed by pixels of the color intensity of immunopositive cells. A mean from all captured vessels per heart was calculated and is reported as the mean value for all grafts in each recipient group.

### Cytokine Expression: Gene Transcripts

Reverse-transcriptase polymerase chain reaction (PCR) to measure relative transcript levels was performed as published previously.<sup>32,34</sup> Briefly, total RNA was extracted from ventricular sections with RNazol B (Tel-Test Inc). First-strand cDNA synthesis was completed for all samples at the same time to improve comparability (cDNA kit, GIBCO BRL). Transcript levels were analyzed from a cDNA panel prepared from allografts (placed in TGF- $\beta_1$  +/- recipients [n=6] and wild-type recipients [n=7]) and native host hearts from TGF- $\beta_1$  +/- (n=7) and wild-type (n=7) recipients. PCR primers were designed with the use of MacVector 5.0 (Oxford Molecular Scientific) and synthesized on an Oligo 1000 DNA synthesizer (Beckman). Primer sequences, sequence accession num-

**TABLE 1. Primer Sequences, Sequence Accession Numbers, Annealing Temperatures, and Cycle Numbers**

	Sequence
TGF- $\beta_1$ (M13177), 55°C/28 cycles	
Sense	TAA TGG TGG ACC GCA ACA ACG C
Antisense	TCC CAG ACA GAA GTT GGC ATG GTA G
STAT 4 (U06923), 55°C/30 cycles	
Sense	ACT CAT TTG TGG TCG AGC GAC AGC
Antisense	AAT TGC CAG CTC ATC ACT TCC CAG G
STAT 6 (L47650), 55°C/28 cycles	
Sense	TGC AAC GGC TCT ATG TTG ACT TTC C
Antisense	TTG GAC CAG GAC CAT TGA GAG GAG
IFN- $\gamma$ (M28621), 60°C/29 cycles	
Sense	AGC GGC TGA CTG AAC TCA GAT TGT AG
Antisense	GTC ACA GTT TTC AGC TGT ATA GGG
IL-2 (K02292), 60°C/34 cycles	
Sense	TGA TGG ACC TAC AGG AGC TCC TGA G
Antisense	GAG TCA AAT CCA GAA CAT GCC GCA G
IL-4 (M25892), 60°C/36 cycles	
Sense	ATG GGT CTC AAC CCC CAG CTA GT
Antisense	GCT CTT TAG GCT TTC CAG GAA GTC
IL-10 (M37897), 58°C/34 cycles	
Sense	TGC TAT GCT GCC TGC TCT TAC TGA C
Antisense	AAT CAC TCT TCA CCT GCT CCA CTG
GAPDH (M32599), 50°C/20 cycles	
Sense	TGA AGG TCG GTG TGA ACG GAT TTG GC
Antisense	CAT GTA GGC CAT GAG GTC CAC CAC

Sequence accession numbers are shown in parentheses.

bers, annealing temperatures, and cycle numbers were as shown in Table 1.

For each primer pair, conditions were optimized to generate a single specific band. The identity of the PCR product was confirmed by restriction analysis. Triplicate samples were amplified using 0.625 U AmpliTaq gold DNA polymerase (Perkin Elmer) in a total volume of 25  $\mu$ L. After initial activation of the specific polymerase at 95°C for 9 minutes, the thermal cycling parameters were denaturation at 94°C for 30 seconds, annealing at a primer-optimized temperature for 20 seconds, and extension at 72°C for 60 seconds (increased by 2 seconds per cycle) followed by a final extension of 7 minutes at the end of all cycles. [<sup>32</sup>P]dCTP (150 000 cpm per reaction) was included for quantitative PCR studies. The amount of incorporated [<sup>32</sup>P]dCTP in amplified product bands from dried agarose gels was measured by volume integration (Molecular Dynamics). Corrected levels of the specific product were derived by dividing the amplified product value by the mean value for the control gene GAPDH in the respective sample.

### Cytokine Expression: Gene Products

To confirm the presence of cytokine gene products, which had been shown to be regulated at the transcript level, we performed immunostainings in frozen sections as published previously.<sup>34</sup> The antibodies we used were anti-IFN- $\gamma$  (clone R4-6A2, 1:100, Pharmingen), anti-IL-2 (clone S4B6, 1:100, Pharmingen), and anti-IL-10 (clone JES5-16E3, 1:100, Pharmingen).

### Statistical Analysis

For comparison between TGF- $\beta_1$  +/- and wild-type groups, unpaired *t* tests were used. For comparison of >2 groups, ANOVA was used. If the ANOVA determined significance, the Bonferroni/Dunn

procedure was used for post hoc testing. Group data are expressed as mean  $\pm$  SEM. A value of *P* < 0.05 was considered significant.

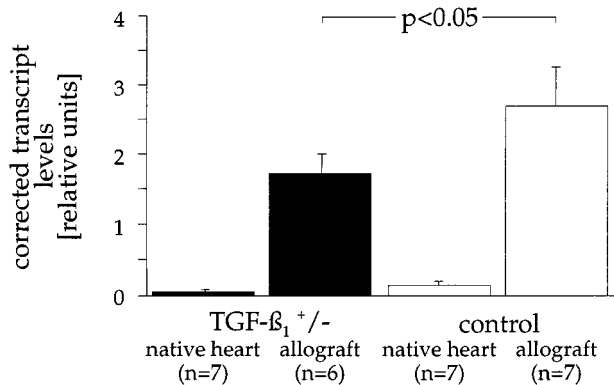
## Results

### TGF- $\beta_1$ Expression

Corrected transcript levels for TGF- $\beta_1$  were higher in allografts compared with native hearts (Figure 1). As expected, grafts placed in TGF- $\beta_1$  +/- recipients had lower corrected transcript levels ( $1.75 \pm 0.31$  relative units) compared with grafts in wild-type recipients ( $2.72 \pm 0.59$  relative units, *P* = 0.04). Infiltrating, inflammatory cells of the respective recipients are most likely responsible for this difference, since donor hearts in both TGF- $\beta_1$  +/- and wild-type recipients were homozygous for TGF- $\beta_1$ . Native hearts had corrected transcript levels of  $0.08 \pm 0.03$  relative units in TGF- $\beta_1$  +/- recipients and  $0.17 \pm 0.06$  relative units in wild-type recipients. Thus, use of TGF- $\beta_1$  +/- recipients produced reductions of  $\approx 35\%$  in graft TGF- $\beta_1$  levels. Given that TGF- $\beta_1$  itself has been shown to stimulate TGF- $\beta_1$  expression, paracrine effects may have partially masked differences produced by heterozygous gene deletion.

### Histological Analysis of Allografts

All allografts placed in wild-type and TGF- $\beta_1$ -deficient recipients survived  $\geq 55$  days. By histological analysis, both wild-type and TGF- $\beta_1$ -deficient recipients produced allo-

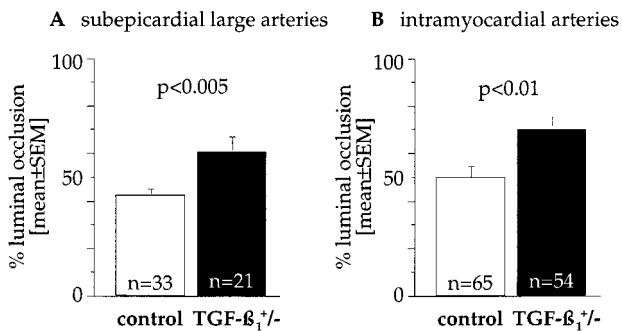


**Figure 1.** TGF- $\beta_1$  mRNA expression is decreased in allografts placed into heterozygous TGF- $\beta_1$   $+/-$  recipients compared with those placed into wild-type control animals. Reverse-transcriptase [ $^{32}$ P]PCR amplification normalized against GAPDH was compared in allografts and native host hearts of TGF- $\beta_1$   $+/-$  and wild-type recipients. Each bar represents the mean  $\pm$  SEM from all cDNAs in each subgroup performed in triplicate.

grafts that undergo chronic rejection with diffuse inflammatory infiltrates, patches of myocyte necrosis, advanced interstitial fibrosis, and scattered interstitial edema and hemorrhage. A modified ISHLT scoring system was used to estimate parenchymal rejection, and mean histological grading scores were similar in allografts from TGF- $\beta_1$ -deficient recipients ( $3.3 \pm 0.3$ ) compared with allografts from wild-type recipients ( $2.9 \pm 0.4$ ,  $P=0.4$ ).

### Quantitative Morphometric Analysis of Intimal Thickening

Elastin-stained sections were used to evaluate the frequency and severity of arteriosclerotic lesion development. Although all vessels in allografts from both groups had some degree of intimal thickening, the severity of lesion development was increased in allografts from TGF- $\beta_1$ -deficient recipients. With the use of computer-assisted morphometric analysis, a total of 176 vessels were analyzed in subgroups according to the vessel size. As shown in Figure 2, both subepicardial large arteries and small intramyocardial arteries had significant increases in luminal occlusion in allografts from TGF- $\beta_1$



**Figure 2.** Luminal occlusion of both large subepicardial (A) and small intramyocardial (B) arteries is increased in allografts placed into heterozygous TGF- $\beta_1$   $+/-$  recipients ( $n=7$ ) compared with those placed into C57B6 wild-type controls ( $n=11$ ). Percent luminal occlusion from all captured vessels per heart is reported as mean  $\pm$  SEM for all grafts in each recipient group.

$+/-$  recipients compared with allografts from wild-type controls. In large subepicardial arteries, the mean luminal occlusion was increased by  $\approx 40\%$  from  $42.3 \pm 2.2\%$  in wild-type controls to  $60.7 \pm 5.4\%$  in TGF- $\beta_1$   $+/-$  recipients ( $P=0.003$ ). In small intramyocardial arteries, the mean luminal occlusion showed a comparable increase from  $50.0 \pm 5.1\%$  in wild-type controls to  $70.6 \pm 5.7\%$  in TGF- $\beta_1$   $+/-$  recipients ( $P=0.008$ ).

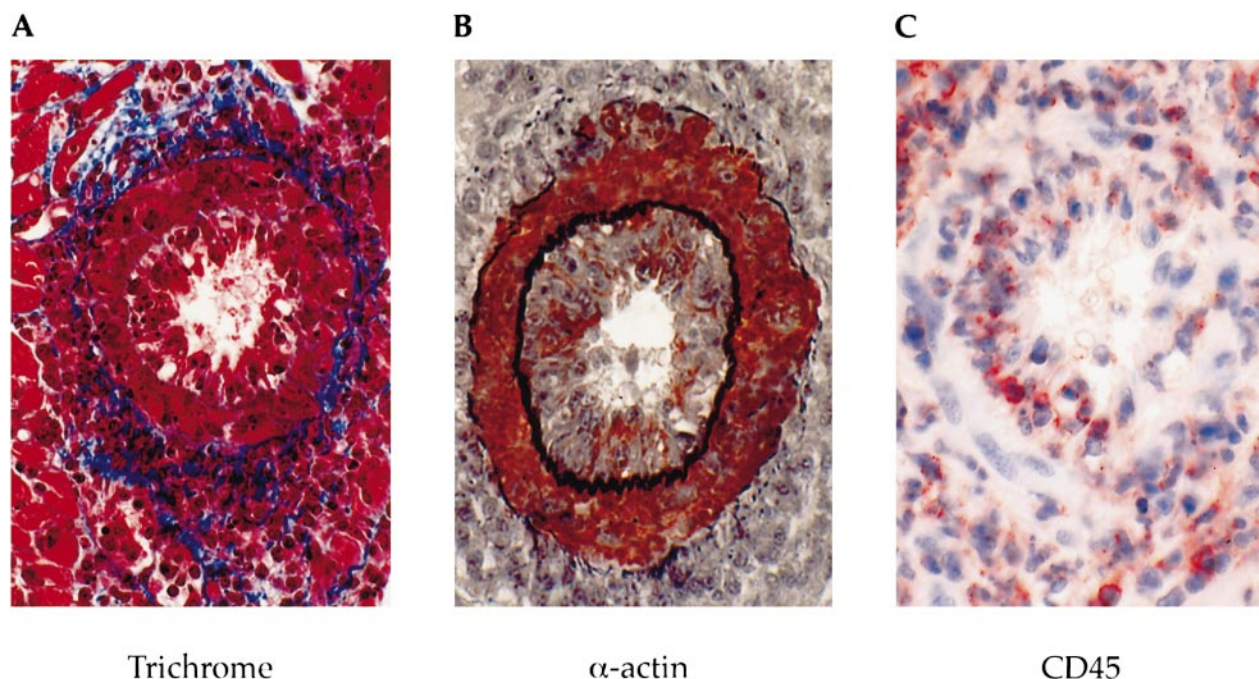
To demonstrate that the increase in luminal occlusion was independent of vessel shrinkage, we measured the intimal and medial areas in 40 vessels from a subset of grafts stained for  $\alpha$ -actin. The mean intimal area had a 40% increase from  $1505 \pm 502 \mu\text{m}^2$  in wild-type controls to  $2175 \pm 458 \mu\text{m}^2$  in TGF- $\beta_1$   $+/-$  recipients. The mean medial areas were comparable in grafts from both groups (wild-type,  $2812 \pm 764 \mu\text{m}^2$ ; TGF- $\beta_1$   $+/-$ ,  $3161 \pm 651 \mu\text{m}^2$ ). This resulted in a significant increase in the mean intima/media ratio in grafts placed into TGF- $\beta_1$ -deficient recipients ( $0.73 \pm 0.06$ ) compared with the ratio in wild-type recipients ( $0.50 \pm 0.04$ ,  $P < 0.05$ ). Hence, the intimal area increased independently of the medial area. This suggests that the increase in percent luminal occlusion was due to neointimal expansion rather than vessel shrinkage.

### Analysis of Vascular Composition

The vascular composition was analyzed using a series of staining protocols to assess the contribution of inflammatory cells, perivascular fibrosis, and neointimal smooth muscle cells in arteriosclerotic lesions in representative grafts from each group. For all 3 stains, vascular lesions in grafts placed into TGF- $\beta_1$   $+/-$  and wild-type recipients were similar in appearance. Figure 3 shows typical histological sections from an allograft vessel in a TGF- $\beta_1$ -deficient recipient.

CD45-positive cells were found scattered throughout the perivascular and myocardial spaces (Figure 3C). Mononuclear cells staining positive for the leukocyte marker, CD45, accounted for  $\approx 50\%$  of the cells in the expanded neointima. To detect lymphocyte subsets capable of Th1/Th2 differentiation, we performed immunostaining in frozen sections from cardiac allografts in both recipient groups for CD4. Infiltrating CD4-positive mononuclear cells were shown to be scattered throughout the myocardial and perivascular spaces, accounting for roughly one third of the CD45+ cells positive for CD4 (data not shown). There was no obvious difference in grafts from TGF- $\beta_1$   $+/-$  and wild-type recipients in the amount, intensity, or distribution of CD45-positive and CD4-positive cells.

Trichrome staining highlighting collagen deposition (blue fibers) was seen predominantly in the adventitial spaces of most arteries, with sparse positive staining in the expanded neointima (Figure 3A). The perivascular fibrosis surrounding vessels had similar appearances in grafts from TGF- $\beta_1$   $+/-$  recipients and those from wild-type controls. Staining for  $\alpha$ -smooth muscle cell actin identifies vessels undergoing arteriosclerosis by highlighting the media and the presence of sclerotic cells in the expanded neointima (Figure 3B). As would be expected in our model at this time point, the media is intact, and the expanded neointima has smooth muscle cells interspersed with mononuclear cells. In spite of the increased



**Figure 3.** Vascular composition. Photomicrographs showing the serial cross section of a representative 55-day allograft vessel from a TGF- $\beta_1$  +/- recipient stained with Masson's trichrome (collagen), anti- $\alpha$ -actin (smooth muscle cell), and anti-CD45 (mononuclear cells) (original magnification  $\times 100$ ). Neointimal formation is accompanied by perivascular collagen accumulation (A). The expanded neointima is composed of  $\alpha$ -actin-positive smooth muscle cells (B) interspersed with CD45-positive mononuclear cells (C).

neointima, there were no striking differences in grafts from TGF- $\beta_1$  +/- and wild-type recipients.

To identify potential quantitative differences, we used image analysis to measure positivity in all vessels ( $n=40$ ) in this representative subset of grafts stained with Masson's trichrome and anti- $\alpha$ -actin. As shown in Table 2, image analysis showed similar degrees of perivascular collagen accumulation. The neointimal area that stained positive for  $\alpha$ -actin was also comparable in grafts from TGF- $\beta_1$  +/- and wild-type recipients. Hence, TGF- $\beta_1$  deficiency in the recipient did not alter perivascular fibrosis and the proportional contribution of neointimal smooth muscle cells to the expanded neointima.

### Cytokine Activation Patterns in Allografts of TGF- $\beta_1$ -Deficient and Control Recipients

To assess differences in Th1-type responses, we compared mRNA expression for IFN- $\gamma$  and IL-2 as signature cytokines as well as for STAT 4, the specific transcription factor for differentiation of Th1 cells.<sup>5</sup> As shown in Figure 4A, corrected transcript levels were significantly increased in allo-

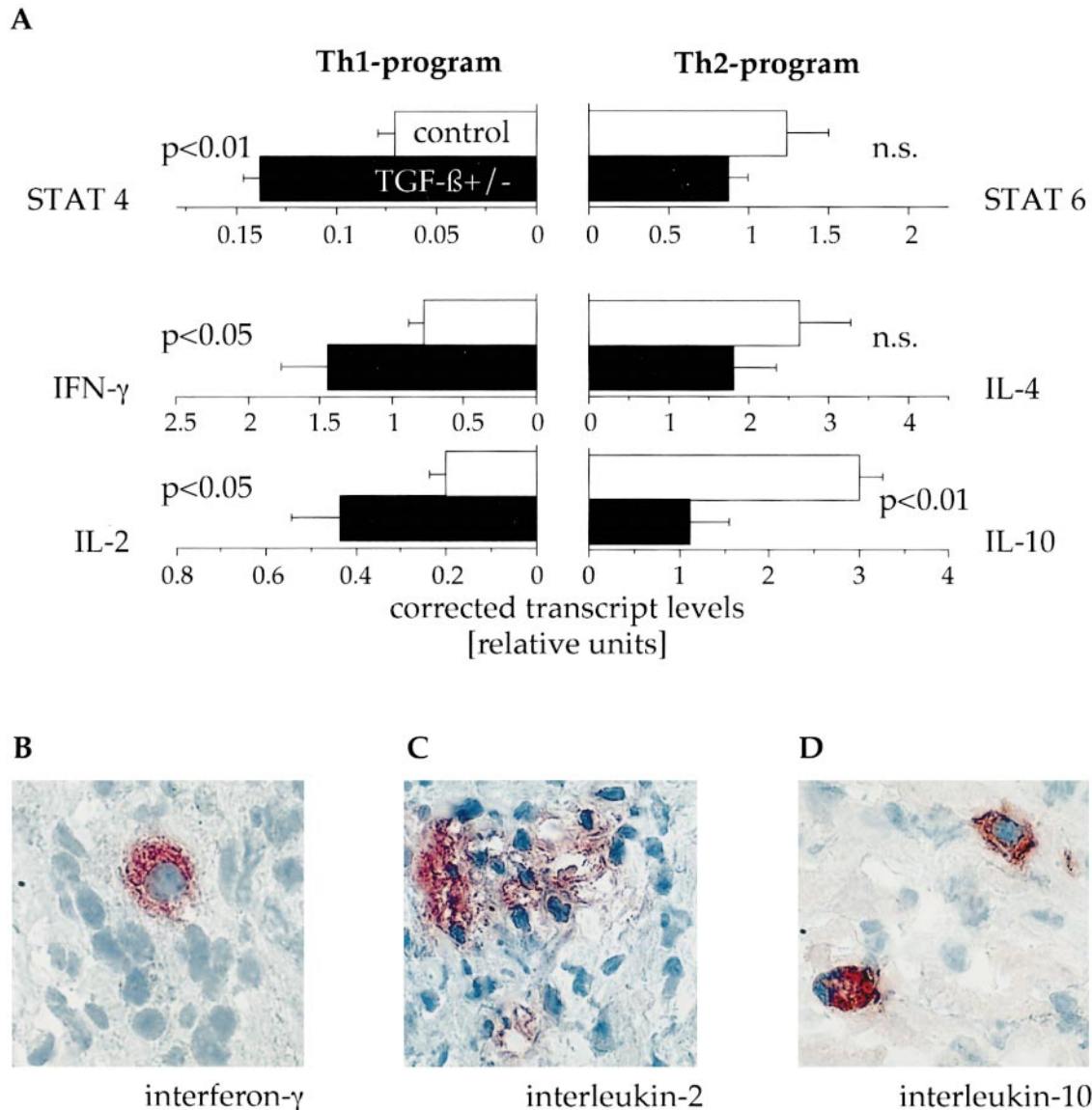
grafts from TGF- $\beta_1$  +/- recipients compared with wild-type recipients (STAT 4,  $0.14 \pm 0.01$  versus  $0.07 \pm 0.01$  relative units, respectively [ $P=0.002$ ]; IFN- $\gamma$ ,  $1.47 \pm 0.33$  versus  $0.79 \pm 0.10$  relative units, respectively [ $P=0.006$ ]; and IL-2,  $0.44 \pm 0.11$  versus  $0.21 \pm 0.04$  relative units, respectively [ $P=0.005$ ]). The presence of the IFN- $\gamma$  and IL-2 gene product was confirmed by immunostaining in grafts from TGF- $\beta_1$  +/- and wild-type recipients. The IFN- $\gamma$  antigen was typically found within the thin cytoplasmic rim of mononuclear cells diffusely distributed throughout the perivascular myocardium (Figure 4B). IL-2 antigen localized intracellularly and extracellularly in disseminated clusters of mononuclear cells (Figure 4C).

Th2-type responses were assessed by comparison of mRNA expression for the signature cytokines IL-4 and IL-10 and the transcription factor STAT 6. As shown in Figure 4A, differences between allografts from TGF- $\beta_1$  +/- and wild-type recipients for STAT 6 and IL-4 (STAT 6,  $0.89 \pm 0.13$  versus  $1.25 \pm 0.27$  relative units, respectively [ $P=0.12$ ]; IL-4,  $1.82 \pm 0.54$  versus  $2.67 \pm 0.63$  relative units, respectively [ $P=0.14$ ]) did not reach significance. However, IL-10 expression was significantly reduced in allografts of knockout versus control recipients (IL-10,  $1.14 \pm 1.08$  versus  $3.03 \pm 0.71$  relative units, respectively [ $P<0.001$ ]). The presence of the IL-10 gene product was confirmed by immunostaining. Similar to the IFN- $\gamma$  staining pattern, IL-10 antigen was also localized to the thin cytoplasmic rim of mononuclear cells, which were distributed throughout the perivascular myocardium (Figure 4D). Taken together, there was increased expression in the Th1 program associated with decreased IL-10 transcript levels.

**TABLE 2. Comparison of Perivascular Fibrosis (Perivascular Collagen-Positive Area/Vessel Area) and Neointimal SMC Expansion (Percent Neointimal Area Stained  $\alpha$ -Actin Positive) in Allograft Vessels From TGF- $\beta_1$ -Deficient ( $n=19$ ) and Wild-type ( $n=21$ ) Recipients**

	Wild-type	TGF- $\beta_1$ +/-	<i>P</i>
Perivascular fibrosis	$0.52 \pm 0.13$	$0.42 \pm 0.06$	NS
Neointimal SMC expansion	$24 \pm 2\%$	$22 \pm 2\%$	NS

SMC indicates smooth muscle cell. Values are mean  $\pm$  SEM.



**Figure 4.** Th1/Th2 cytokine activation patterns. A, Corrected mRNA transcript levels of the Th1 transcription factor STAT 4 and the signature cytokines IFN- $\gamma$  and IL-2 were increased in allografts from TGF- $\beta_1$  +/- recipients (solid bars, n=6) compared with those from wild-type control animals (open bars, n=7), whereas the Th2 transcription factor STAT 6 and the signature cytokines IL-4 and IL-10 were unchanged or decreased in TGF- $\beta_1$  +/- recipients. Reverse-transcriptase [<sup>32</sup>P]PCR amplification was normalized against GAPDH and presented as corrected levels in relative units. Values are mean  $\pm$  SEM. B to D, IFN- $\gamma$  (B), IL-2 (C), and IL-10 (D) expression by mononuclear cells infiltrating the perivascular and myocardial spaces of allografts from both groups was confirmed by immunostaining. Photomicrography of representative sections in allografts from wild-type recipients is shown (original magnification  $\times 200$ ).

## Discussion

### Immune Sources of TGF- $\beta_1$ Reduce Intimal Thickening

The present study was designed to assess immunological contributions of TGF- $\beta_1$  on arteriosclerosis by comparing the effect of TGF- $\beta_1$ -deficient and -competent infiltrating inflammatory cells on the development of intimal thickening. By using recipient mice with targeted gene deletion, we produced ongoing TGF- $\beta_1$  deficiency confined to infiltrating immune cells. We demonstrate that reduction of TGF- $\beta_1$  levels results in exacerbated arteriosclerosis in wild-type donor hearts independent of parenchymal changes. Hence, we conclude that immune sources of TGF- $\beta_1$ , when present at

biological levels, mediate inhibitory effects on intimal thickening in our model.

To determine whether TGF- $\beta_1$  deficiency altered CD4 activation patterns, we studied intragraft cytokine expression. We found that increased intimal thickening in TGF- $\beta_1$ -deficient recipients correlated with increased expression of Th1 cytokines. We speculate that TGF- $\beta_1$ , when present, mediates its immunosuppressive effects on arteriosclerotic lesion development, at least in part, by attenuating Th1 forces.

### TGF- $\beta_1$ as Regulator of the Th1/Th2 Balance

We found that in the setting of chronic cardiac rejection, TGF- $\beta_1$  deficiency resulted in increased expression of Th1

signature cytokines IFN- $\gamma$  and IL-2 as well as of STAT 4, the transcription factor responsible for Th1 differentiation. This suggests that, when present, TGF- $\beta_1$  serves as a negative regulator of Th1 cell differentiation. These studies in mice with targeted deletion of TGF- $\beta_1$  confirm other reports characterizing its immune properties.<sup>10,21,26</sup> In vitro, TGF- $\beta_1$  has been shown to inhibit the development of both Th1 and Th2 cytokine-producing T cells.<sup>36–38</sup> In vivo, the data seem to vary dependent on the pathological stimulus. In response to *Candida albicans*, TGF- $\beta_1$  is obligatory for Th1 differentiation.<sup>39</sup> In contrast, Th1-mediated delayed-type hypersensitivity is inhibited by oral administration of TGF- $\beta_1$ , as manifested by inhibition of IL-2 and IFN- $\gamma$  and induction of IL-10.<sup>40</sup> TGF- $\beta_1$ -producing T cells suppress experimental autoimmune encephalomyelitis, another Th1 cell-driven process, by downregulating Th1 cells.<sup>41</sup> Taken together, these data suggest that one of the TGF- $\beta_1$  immune actions is to modulate CD4 effector pathways, as evidenced by the attenuation of Th1-type responses in vivo. The precise mechanism is unclear. However, one possibility is that TGF- $\beta_1$  acts as a transcriptional regulator.<sup>42</sup>

### Transplant Arteriosclerosis and the Th1/Th2 Paradigm

The presence of both Th1 and Th2 cytokines in grafts undergoing transplant arteriosclerosis has raised questions about the specific contributions of these distinct cytokines to intimal thickening.<sup>43</sup> Some progress has been made establishing functional roles for individual Th1 and Th2 cytokine programs in the various stages of transplant arteriosclerosis. Recently, we have used a mouse model involving major histocompatibility complex class I and II mismatched donors and recipients to study the Th1 cytokine IFN- $\gamma$ .<sup>7</sup> By using recipients with targeted deletion of IFN- $\gamma$ , we have shown that the frequency and severity of intimal thickening is reduced compared with the corresponding values in wild-type recipients. Analysis of vessel composition in grafts from IFN- $\gamma$ -deficient recipients also showed a decrease in neointimal smooth muscle cells, indicating that, when present, IFN- $\gamma$  promotes myointimal expansion.

Roles for Th1 cytokines have also been demonstrated in other cardiac allograft models involving less severe immunogenetic mismatches. With the use of more qualitative analysis, both targeted gene deletion of IFN- $\gamma$  or treatment of the recipient with anti-IFN- $\gamma$  reduced intimal thickening.<sup>8</sup> Furthermore, this proarteriosclerotic effect of IFN- $\gamma$  has also been demonstrated in other forms of lesion development, such as atherosclerosis.<sup>44</sup> By crossing IFN- $\gamma$  receptor knockout mice with apolipoprotein E knockout mice, Gupta et al<sup>44</sup> produced reductions in vascular lesion size, lipid accumulation, and lesion cellularity. Taken together, IFN- $\gamma$ , a Th1 signature cytokine, plays a pivotal role in intimal thickening. The next step will be to determine whether IFN- $\gamma$  mediates these proarteriosclerotic effects through activation of T-cell and macrophage effector pathways that concentrate the inflammatory and fibrotic responses within the vasculature.

It is unlikely that cytokine modulation of arteriosclerosis involves a single cytokine in isolation. There is complex networking between individual Th1 and Th2 effector cyto-

kines and the programs. Each subset produces cytokines that serve as its own autocrine growth factor.<sup>5</sup> At the same time, each Th1 or Th2 subset is capable of producing cytokines that inhibit each other's differentiation.<sup>5</sup> The fact that targeted gene deletion of IFN- $\gamma$  reduces but does not completely abolish lesion development in our model<sup>7</sup> suggests that other Th1 responses, such as expression of IL-2, may make independent contributions. In addition, we and others have just begun to explore how individual Th2-type responses, classified as leukocyte suppressive, may contribute to the regulation of intimal thickening. This TGF- $\beta_1$  model can be used to determine whether targeting of molecular circuits controlling Th1/Th2 differentiation rather than individual Th1/Th2 effectors might be of therapeutic value.

### TGF- $\beta_1$ and Vascular Modeling

Beyond immunological effects, which are the focus of the present study, TGF- $\beta_1$  mediates other regulatory roles in arteriosclerosis.<sup>11–14</sup> TGF- $\beta_1$  is often considered proarteriosclerotic because it promotes extracellular matrix accumulation, activation of plasminogen activator inhibitor-1, and cell-matrix and cell-cell adhesion.<sup>17–20,45</sup> However, TGF- $\beta_1$  also has antiarteriosclerotic effects in vascular smooth muscle cells. In cell culture, TGF- $\beta_1$  has been shown to inhibit both migration and proliferation of vascular smooth muscle cells.<sup>15,16</sup> Activation of the latent form of TGF- $\beta_1$  has been shown to be blocked by plasminogen activator inhibitor-1 and lipoprotein(a).<sup>15,46</sup> Hence, elevated levels of these factors in the microenvironment may contribute to neointimal smooth muscle expansion by reducing the antiproliferative effect of active TGF- $\beta_1$  on neointimal smooth muscle cell expansion. Recently, evidence for such a role has been provided by a population study by Grainger et al<sup>47</sup> describing reduced serum levels for TGF- $\beta$  in patients with advanced arteriosclerosis.

Although of interest, the present study did not specifically assess the potential contributions of TGF- $\beta_1$  associated with the donor vascular smooth muscle cells. The mutant mice used in this study are characterized by selective disruption of the TGF- $\beta_1$  isoform and have no phenotypic overlap with mice with disruption in other mammalian isoforms of TGF- $\beta$  (TGF- $\beta_2$ <sup>48</sup> and TGF- $\beta_3$ <sup>49,50</sup>). Hence, we have selectively studied TGF- $\beta_1$  independent of the other TGF- $\beta$  isoforms. Using the present donor/recipient combination as a model to produce selective TGF- $\beta_1$  deficiency confined to immune sources, we were able to dissect out parenchymal effects from potential immunological effects. Because recipient deficiency of TGF- $\beta_1$  did not change the neointimal smooth muscle cell contribution, our findings suggest that the observed antiarteriosclerotic effects of TGF- $\beta_1$  are independent of direct vascular smooth muscle cell effects.

### Conclusions

We show that immune sources of TGF- $\beta_1$  contribute to vascular occlusion in this transplant arteriosclerosis model. By using TGF- $\beta_1$  +/- recipients, we show that TGF- $\beta_1$ , when present, has an inhibitory effect on intimal thickening. Our findings confirm that TGF- $\beta_1$  modulates proximal aspects of the immune response by regulating the Th1/Th2 balance in the setting of transplant arteriosclerosis. Beyond

transplantation, immunosuppressive effects of TGF- $\beta_1$  may also regulate inflammatory responses in other arteriosclerotic conditions.

### Acknowledgments

This study was supported by grants from the American Heart Association (grant-in-aid 975014ON) and the Milton Foundation. Dr Koglin was supported by a fellowship grant from the Deutsche Forschungsgemeinschaft, Bonn, Germany. We thank Dr Guy Reed for critical review and engaging discussions.

### References

- Salomon RN, Hughes CC, Schoen FJ, Payne DD, Pober JS, Libby P. Human coronary transplantation-associated arteriosclerosis: evidence for a chronic immune reaction to activated graft endothelial cells. *Am J Pathol.* 1991;138:791–798.
- Billingham ME. Histopathology of graft coronary disease. *J Heart Lung Transplant.* 1992;11(pt 2):S38–S44.
- Shi C, Lee WS, He Q, Zhang D, Fletcher DL Jr, Newell JB, Haber E. Immunologic basis of transplant-associated arteriosclerosis. *Proc Natl Acad Sci U S A.* 1996;93:4051–4056.
- Räisänen-Sokolowski A, Glysing-Jensen T, Mottram PL, Russell ME. Sustained anti-CD4/CD8 treatment blocks inflammatory activation and intimal thickening in mouse heart allografts. *Arterioscler Thromb Vasc Biol.* 1997;17:2115–2122.
- Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature.* 1996;383:787–793.
- Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today.* 1996;17:138–146.
- Räisänen-Sokolowski A, Glysing-Jensen T, Koglin J, Russell ME. Reduced transplant arteriosclerosis in murine cardiac allografts placed in interferon-gamma knockout recipients. *Am J Pathol.* 1998;152:359–365.
- Nagano H, Mitchell RN, Taylor MK, Hasegawa S, Tilney NL, Libby P. Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J Clin Invest.* 1997;100:550–557.
- Border WA, Ruoslahti E. Transforming growth factor-beta in disease: the dark side of tissue repair. *J Clin Invest.* 1992;90:1–7.
- Roberts AB, Sporn MB. The transforming growth factors- $\beta$ s. In: Sporn MB, Roberts AB, eds. *Peptide Growth Factors and Their Receptors*, I. Heidelberg, Germany: Springer-Verlag; 1990;95:419–472.
- Majesky MW, Lindner V, Twardzik DR, Schwartz SM, Reidy MA. Production of transforming growth factor beta 1 during repair of arterial injury. *J Clin Invest.* 1991;88:904–910.
- Nikol S, Isner JM, Pickering JG, Kearney M, Leclerc G, Weir L. Expression of transforming growth factor-beta 1 is increased in human vascular restenosis lesions. *J Clin Invest.* 1992;90:1582–1592.
- Reidy MA, Fingerle J, Lindner V. Factors controlling the development of arterial lesions after injury. *Circulation.* 1992;86(suppl III):III-43–III-46.
- Shi Y, O'Brien JE Jr, Fard A, Zalewski A. Transforming growth factor-beta 1 expression and myofibroblast formation during arterial repair. *Arterioscler Thromb Vasc Biol.* 1996;16:1298–1305.
- Grainger DJ, Kirschenlohr HL, Metcalfe JC, Weissberg PL, Wade DP, Lawn RM. Proliferation of human smooth muscle cells promoted by lipoprotein(a). *Science.* 1993;260:1655–1658.
- Kojima S, Harpel PC, Rifkin DB. Lipoprotein (a) inhibits the generation of transforming growth factor beta: an endogenous inhibitor of smooth muscle cell migration. *J Cell Biol.* 1991;113:1439–1445.
- Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, et al. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci U S A.* 1986;83:4167–4171.
- Ignatz RA, Massague J. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem.* 1986;261:4337–4345.
- Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJ, Angel P, Heath JK. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J.* 1987;6:1899–1904.
- Reilly CF, McFall RC. Platelet-derived growth factor and transforming growth factor-beta regulate plasminogen activator inhibitor-1 synthesis in vascular smooth muscle cells. *J Biol Chem.* 1991;266:9419–9427.
- Christ M, McCartney-Francis NL, Kulkarni AB, Ward JM, Mizel DE, Mackall CL, Gress RE, Hines KL, Tian H, Karlsson S, et al. Immune dysregulation in TGF-beta 1-deficient mice. *J Immunol.* 1994;153:1936–1946.
- Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A.* 1993;90:770–774.
- Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature.* 1992;359:693–699.
- Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, Sporn MB, Fauci AS. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med.* 1986;163:1037–1050.
- Wallick SC, Figari IS, Morris RE, Levinson AD, Palladino MA. Immunoregulatory role of transforming growth factor beta (TGF-beta) in development of killer cells: comparison of active and latent TGF-beta 1. *J Exp Med.* 1990;172:1777–1784.
- Letterio JJ, Roberts AB. TGF-beta: a critical modulator of immune cell function. *Clin Immunol Immunopathol.* 1997;84:244–250.
- Waltenberger J, Wanders A, Fellstrom B, Miyazono K, Heldin CH, Funa K. Induction of transforming growth factor-beta during cardiac allograft rejection. *J Immunol.* 1993;151:1147–1157.
- Shin GT, Khanna A, Ding R, Sharma VK, Lagman M, Li B, Suthanthiran M. In vivo expression of transforming growth factor- $\beta_1$  in humans. *Transplantation.* 1998;65:313–318.
- Carel JC, Schreiber RD, Falqui L, Lacy PE. Transforming growth factor beta decreases the immunogenicity of rat islet xenografts (rat to mouse) and prevents rejection in association with treatment of the recipient with a monoclonal antibody to interferon gamma. *Proc Natl Acad Sci U S A.* 1990;87:1591–1595.
- Qin L, Ding Y, Bromberg JS. Gene transfer of transforming growth factor-beta 1 prolongs murine cardiac allograft survival by inhibiting cell-mediated immunity. *Hum Gene Ther.* 1996;7:1981–1988.
- Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice: the role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation.* 1973;16:343–350.
- Koglin J, Glysing-Jensen T, Mudgett JS, Russell ME. Exacerbated transplant arteriosclerosis in NOS2-deficient mice. *Circulation.* 1998;97:2059–2065.
- Billingham ME, Cary NR, Hammond ME, Kemnitz J, Marboe C, McCallister HA, Snover DC, Winters GL, Zerbe A. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group: the International Society for Heart Transplantation. *J Heart Transplant.* 1990;9:587–593.
- Räisänen-Sokolowski A, Mottram PL, Glysing-Jensen T, Sato A, Russell ME. Heart transplants in interferon-gamma, interleukin 4, and interleukin 10 knockout mice: recipient environment alters graft rejection. *J Clin Invest.* 1997;100:2449–2456.
- Shi C, Russell ME, Bianchi C, Newell JB, Haber E. Murine model of accelerated transplant arteriosclerosis. *Circ Res.* 1994;75:199–207.
- Sad S, Mosmann TR. Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. *J Immunol.* 1994;153:3514–3522.
- Fargeas C, Wu CY, Nakajima T, Cox D, Nutman T, Delespesse G. Differential effect of transforming growth factor beta on the synthesis of Th1- and Th2-like lymphokines by human T lymphocytes. *Eur J Immunol.* 1992;22:2173–2176.
- Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science.* 1997;277:2005–2007.
- Spaccapelo R, Romani L, Tonnetti L, Cenci E, Mencacci A, Del Sero G, Tognellini R, Reed SG, Puccetti P, Bistoni F. TGF-beta is important in determining the in vivo patterns of susceptibility or resistance in mice infected with *Candida albicans*. *J Immunol.* 1995;155:1349–1360.
- Ianaro A, Xu D, O'Donnell CA, Di Rosa M, Liew FY. Expression of TGF-beta in attenuated *Salmonella typhimurium*: oral administration leads to the reduction of inflammation, IL-2 and IFN-gamma, but enhancement of IL-10, in carrageenin-induced oedema in mice. *Immunology.* 1995;84:8–15.
- Chen JK, Hoshi H, McKeehan WL. Transforming growth factor type beta specifically stimulates synthesis of proteoglycan in human adult

- arterial smooth muscle cells. *Proc Natl Acad Sci U S A*. 1987;84:5287–5291.
42. Brand T, Schneider MD. Transforming growth factor- $\beta$  signal transduction. *Circ Res*. 1996;78:173–179.
43. Strom TB, Roy-Chaudhury P, Manfro R, Zheng XX, Nickerson PW, Wood K, Bushell A. The Th1/Th2 paradigm and the allograft response. *Curr Opin Immunol*. 1996;8:688–693.
44. Gupta S, Pablo AM, Jiang X, Wang N, Tall AR, Schindler C. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest*. 1997;99:2752–2761.
45. Igotz RA, Heino J, Massague J. Regulation of cell adhesion receptors by transforming growth factor-beta: regulation of vitronectin receptor and LFA-1. *J Biol Chem*. 1989;264:389–392.
46. Grainger DJ, Kemp PR, Liu AC, Lawn RM, Metcalfe JC. Activation of transforming growth factor-beta is inhibited in transgenic apolipoprotein(a) mice. *Nature*. 1994;370:460–462.
47. Grainger DJ, Kemp PR, Metcalfe JC, Liu AC, Lawn RM, Williams NR, Grace AA, Schofield PM, Chauhan A. The serum concentration of active transforming growth factor-beta is severely depressed in advanced atherosclerosis [comments]. *Nat Med*. 1995;1:74–79.
48. Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development*. 1997;124:2659–2670.
49. Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet*. 1995;11:415–421.
50. Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MW, Doetschman T. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet*. 1995;11:409–414.