

Heart Transplants in Interferon- γ , Interleukin 4, and Interleukin 10 Knockout Mice

Recipient Environment Alters Graft Rejection

Anne Räsänen-Sokolowski,* Patricia L. Mottram,[§] Troels Glysing-Jensen,* Abhay Satoskar,[‡] and Mary E. Russell^{||}

*Cardiovascular Biology Laboratory and [‡]Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts 02115; [§]University of Melbourne, Department of Surgery, Royal Melbourne Hospital, Melbourne 3050, Australia; and ^{||}Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Abstract

To study the role of cytokines in long-term cardiac allografts we have used recipient mice with targeted gene deletions ($-/-$) in IFN- γ , IL-4, or IL-10. In wild-type and IL-4 $-/-$ recipients immunosuppressed with a 30-d course of anti-CD4 and anti-CD8, graft survival was > 87 d. This time was significantly reduced in IFN- γ $-/-$ (62 ± 19 d, $P < 0.05$) and IL-10 $-/-$ recipients (55 ± 4 d, $P < 0.0001$). Histology showed mononuclear cell infiltration, patchy necrosis, fibrosis, and vascular thickening in all groups. Intra-graft transcript levels measured by ³²P-reverse transcriptase PCR showed different inflammatory patterns. IFN- γ $-/-$ recipients had higher IL-2 transcripts and selective alteration in macrophage activation that may have contributed to decreased graft survival. Decreased graft survival in IL-10 $-/-$ recipients was associated with increases in iNOS and IFN- γ -driven responses. Finally, in grafts from IL-4 $-/-$ recipients, there were increases in CD3 transcripts concurrent with TNF- α levels. This increase suggests that IL-4 may regulate T cell infiltration through TNF- α -mediated inflammatory cell recruitment. Concurrent evaluation of these three isolated cytokine deletions has shown that the recipient environment caused distinct graft modifications. (*J. Clin. Invest.* 1997. 100:2449–2456.) Key words: inflammatory activation • graft survival • targeted gene deficiency • cytokine regulation

Introduction

Th1-type cytokines (IFN- γ and IL-2) promote inflammatory responses to injury by activation of leukocytes, opsonization, and phagocytosis (1). In contrast, Th2-type cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13) are believed to exert antiinflammatory effects by their ability to suppress macrophage activation, provide B cell help, and downregulate Th1-type cytokine production (1). In transplantation, previous descriptive studies have shown various Th1/Th2-type responses. We observed a

mixed Th1/Th2-associated response with high IFN- γ (Th1), IL-4, and IL-10 (Th2) transcript levels in long-surviving allografts (2). Others have shown the presence of Th1-type cytokines in rejecting grafts, and expression of Th2-type cytokines with low levels of Th1-type cytokines in long-surviving grafts (3–8). Functional data supporting the concept that Th2 cells may promote graft acceptance has been more controversial (9). For example, acute cardiac rejection can occur at the same rate after transfusion of either polarized Th1 or Th2 cells into SCID recipients (9). The availability of gene knockout mice has now allowed exploration of the functional role of individual cytokines in transplant rejection. There are reports showing that acute rejection develops in heart allografts in nonimmunosuppressed IFN- γ $-/-$, IL-12 $-/-$ (which regulates Th1 differentiation), and IL-4 $-/-$ recipients (10–12).

The purpose of this study was to investigate the effects of IFN- γ , IL-4, or IL-10 gene deletion in immunosuppressed cardiac allografts. We used a heterotopic, vascularized heart transplant model with MHC class I and II mismatches between donor and wild-type or gene knockout recipients. A 30-d-course of T cell-depleting mAb was used to delay the onset of rejection and to produce long-surviving allografts. The three knockout recipient environments, devoid of immunoregulatory cytokines, IFN- γ , IL-4, and IL-10, were evaluated by comparing cardiac allograft survival, histologic outcome, and T cell and macrophage activation.

Methods

Murine cardiac transplantation. CBA/CaJ (H-2^k) or BALB/cByJ (H-2^d) donors depending on availability were used for heterotopic cardiac transplantation (13, 14) into C57BL (H-2^b) recipients that were either wild-type (CBA to C57BL/6J, $n = 11$; BALB/c to C57BL/6J, $n = 8$) or had targeted gene disruption in IFN- γ (C57BL/6J, $n = 10$) (15), IL-4 (C57BL/6J, $n = 10$) (16), or IL-10 (C57BL/10j, $n = 10$) (17). Iso-graft controls included IFN- γ $-/-$ ($n = 7$), IL-4 $-/-$ ($n = 6$), and IL-10 $-/-$ ($n = 5$). All mice were purchased from Jackson Laboratories (Bar Harbor, ME). The targeted gene disruption was confirmed using triple PCR assays that amplify a portion of the targeted cassette and a portion of the targeted exon as recommended by Jackson Laboratories (www.jax.org). To attenuate acute rejection, we treated recipients with mAb against CD4 (clone GK1.5, rat IgG2b; American Type Culture Collection, Rockville, MD) and CD8 (clone 2.43, rat IgG2b; American Type Culture Collection) at the dose of 500 μ g for each mAb per injection for days 1–4 after transplantation, and weekly thereafter for 30 d. We have previously shown $> 94\%$ depletion of CD4⁺ and CD8⁺ cells during this treatment (2). Previous time course analysis by flow cytometry has shown that 80% of the CD3⁺ and CD4⁺ cells repopulate 40 d after last mAb injection (18), whereas others have shown that the capacity to reject allografts returns with 28 d of ceasing mAb treatment (19).

Grafts were harvested when the palpation score was < 1 (13) or when the grafts had reached 100 d after transplantation. When one of these endpoints was achieved, hearts were harvested (ranging 37–133 d after transplantation). Transverse sections of grafts were processed

Address correspondence to Mary E. Russell, M.D., Cardiovascular Biology Laboratory, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115. Phone: 617-432-4996; FAX: 617-432-2980; E-mail: russell@cvtlab.harvard.edu

Received for publication 11 June 1997 and accepted in revised form 16 September 1997.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.
0021-9738/97/11/2449/08 \$2.00

Volume 100, Number 10, November 1997, 2449–2456

<http://www.jci.org>

for histologic evaluation, or snap-frozen for RNA extraction as previously described (2).

Histological analysis. The degree of acute rejection was evaluated and graded from paraffin sections using coded samples evaluated by two observers forming a consensus opinion. A modification of the International Society for Heart and Lung Transplantation (ISHLT) criteria was used (20). The scale was from 0 to 4, where 0 stands for no rejection, 1 for mild focal (A) or diffuse (B) perivascular and interstitial infiltration with no parenchymal necrosis, 2 for moderate, unifocal infiltration with/without focal myocyte injury, 3 for moderate multifocal (A) or diffuse (B) infiltration with myocyte injury, and 4 for severe rejection with aggressive, diffuse infiltration, edema, myocyte necrosis, hemorrhage, and vasculitis. To assess mean values for each group, 1B and 3B scores were calculated as 1.5 or 3.5, respectively.

Reverse transcriptase PCR (RT-PCR).¹ To measure relative differences in transcript levels between cardiac transplants, we used a semiquantitative ³²P-RT-PCR technique published in detail previously (2, 21, 22). Modifications included dilution of cDNA (1:1) with sterile water before PCR, and hot start was performed using AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, CA) instead of AmpliTaq DNA polymerase (23). The specificity of IL-4 and IFN- γ primers was increased by using the touchdown PCR technique (24), which included a gradual decrease in the annealing temperature from 70°C to 60°C over ten cycles, and an additional 22–28 cycles thereafter at an annealing temperature of 56°C. Accession numbers, primer sequences (5'→3'), annealing temperatures, and number of cycles for IL-2, iNOS, MCP-1, and AIF-1 were as published earlier (2) or as follows: IL-12p40 (M86671), AAA CAG TGA ACC TCA CCT GTG ACA C (sense); TTC ATC TGC AAG TTC TTG GGC G (antisense) (32 cycles, 56°C); IL-4 (M25892), CCA GCT AGT TGT CAT CCT GCT CTT C (sense); CAG TGA TGT GGA CTT GGA CTC ATT C (antisense) (38 cycles, 56°C, touchdown); IL-10 (M37897), TGC TAT GCT GCC TGC TCT TAC TGA C (sense); AAT CAC TCT TCA CCT GCT CCA CTG (antisense) (34 cycles, 58°C); IFN- γ (M28621), AGC TCT GAG ACA ATG AAC GCT ACA C (sense); ACC TGT GGG TTG TTG ACC TCA AAC (antisense) (32 cycles, 56°C, touchdown); CD3 ζ (L03353), AAA GTG TCT GTT CTC GCC TGC ATC (sense); ACC TTC TCT CTC TCT TCT GTT CCC G (antisense) (34 cycles, 56°C); TNF- α (M13049), AAA AGA TGG GGG GCT TCC AGA ACT C (sense); AGA TAG CAA ATC GGC TGA CGG TGT G (antisense) (30 cycles, 58°C). PCR amplification with the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) housekeeping gene was performed to assess variations in cDNA or total RNA loading between samples. The mean was obtained from triplicate analyses from the same sample. Corrected values were derived by dividing the measured ³²P value for the transcript of interest by the mean G3PDH value for the sample. To estimate activation level of T cells, T cell cytokines, IL-2, IL-4, and IFN- γ , transcript levels were normalized against CD3 ζ transcripts by taking the ratio of cytokine to CD3 ζ .

Immunohistology. The presence of cytokine gene products upregulated at the transcript level was demonstrated by immunostaining using the method of Andersson and Andersson (25). In brief, frozen sections ($n = 2$ –3/group) were fixed in fresh 4% paraformaldehyde and washed with 0.1% vol/wt saponin in PBS and 0.01% sodium azide. Endogenous peroxidase activity was blocked by incubating the slides in 1% H₂O₂ in PBS. Normal rabbit serum (10%) was used for blocking. Appropriate primary rat anti-mouse antibodies (anti-IL-2, clone S4B6; PharMingen, San Diego, CA), anti-IL-10 (clone JES5-16E3, PharMingen), anti-IFN- γ (clone R4-6A2, PharMingen) and TNF- α (clone XT-22, PharMingen) were added at an end con-

centration of 5–10 μ g/mL and incubated overnight at 4°C. Bound primary antibody was detected using a secondary antibody rabbit anti-rat IgG (Vector Laboratories, Inc., Burlington, CA) followed by application of avidin-biotin complex (Vector Laboratories) according to manufacturer's instructions. Horseradish peroxidase label was detected using an AEC kit (Vector Laboratories), and sections were counterstained with Mayer's hematoxylin. Negative controls included omission of primary antibody, use of an irrelevant antibody, and staining of tissue sections from corresponding knockout animals (IFN- γ and IL-10) that were all negative.

Statistical analysis. All data are given mean \pm SD per subgroup. The product limit (Kaplan-Meier) estimate of the cumulative survival was assessed with a Breslow-Gehan-Wilcoxon test to evaluate significant differences in graft survival (26). The PCR data was subjected to multiple analysis of variance without replication (StatView 4.5; Abacus Concepts, Inc., Berkeley, CA). If the multiple analysis of variance was significant, individual comparisons were made by the Student's t test, and the level of significance was corrected by the Bonferroni method (27, 28).

Results

Murine model of attenuated allograft rejection. Mouse strain combinations with both MHC class I and II mismatches were selected to simulate the high degree of mismatch seen in human cardiac transplants. A 30-d course of anti-CD4 and anti-CD8 mAb was used to attenuate acute rejection. There was no significant difference in graft survival (Fig. 1) in the two different wild-type strain combinations, i.e., BALB/c to C57BL/6J and CBA to C57BL/6J (99 ± 26 vs. 87 ± 32 d, respectively, $P = 0.298$). Here, suboptimal levels of immunosuppression allowed the development of interrelated processes that can lead to contractile failure in long-term allografts. There were no obvious histological differences in the responses seen in allografts that included mononuclear cell infiltration, myocyte necrosis, edema, fibrosis and vascular narrowing or occlusion (Fig. 2).

Graft survival in IFN- γ $-/-$ recipients. Graft survival times for CBA to C57BL/6J grafts after the 30-d course of anti-CD4 and anti-CD8 mAb, ranged from 51 to 133 d (mean, 87 ± 32 d, median, 71 d; $n = 11$) in wild-type recipients and from 37 to 101 d (mean 62 ± 19 d, median 54 d; $n = 10$) in IFN- γ $-/-$ recipients (Fig. 1 A). Thus, IFN- γ $-/-$ recipients had lower graft survival times compared with wild-type controls. Only 10% of the grafts in IFN- γ $-/-$ recipients survived > 100 d, compared with 36% of the wild-type controls ($P < 0.05$). All IFN- γ $-/-$ isografts ($n = 7$) survived indefinitely.

Graft survival in IL-4 $-/-$ and IL-10 $-/-$ recipients. Deletion of Th2-type cytokines was studied in BALB/c to C57BL strain combinations using either wild-type, IL-4 $-/-$, or IL-10 $-/-$ recipients. Survival (Fig. 1 B) of BALB/c grafts in wild-type controls ranged from 70 to 126 d (mean 99 ± 26 d, median 101 d, $n = 8$). Grafts in IL-4 $-/-$ recipients survived from 53 to 120 (mean 94 ± 24 d, median 94 d; $n = 10$, $P = \text{NS}$). In fact, mean survival time for grafts in IL-4 $-/-$ recipients was comparable to wild-type controls. In contrast, BALB/c hearts placed in IL-10 $-/-$ recipients showed significantly shorter graft survival ranging from 46 to 58 (mean 55 ± 4 d, median 57 d, $n = 10$, $P < 0.0001$). All isografts (IL-4 $-/-$, $n = 6$, IL-10 $-/-$, $n = 5$) survived indefinitely. Hence, donor hearts placed into recipients with targeted gene deletion in IL-10 but not in IL-4, had significantly decreased survival time. This result suggested that IL-10 is required for long-term survival of cardiac allografts in 30-d course immunosuppressed recipients.

1. **Abbreviations used in this paper:** ISHLT, International Society for Heart and Lung Transplantation; RT-PCR, reverse transcription PCR.

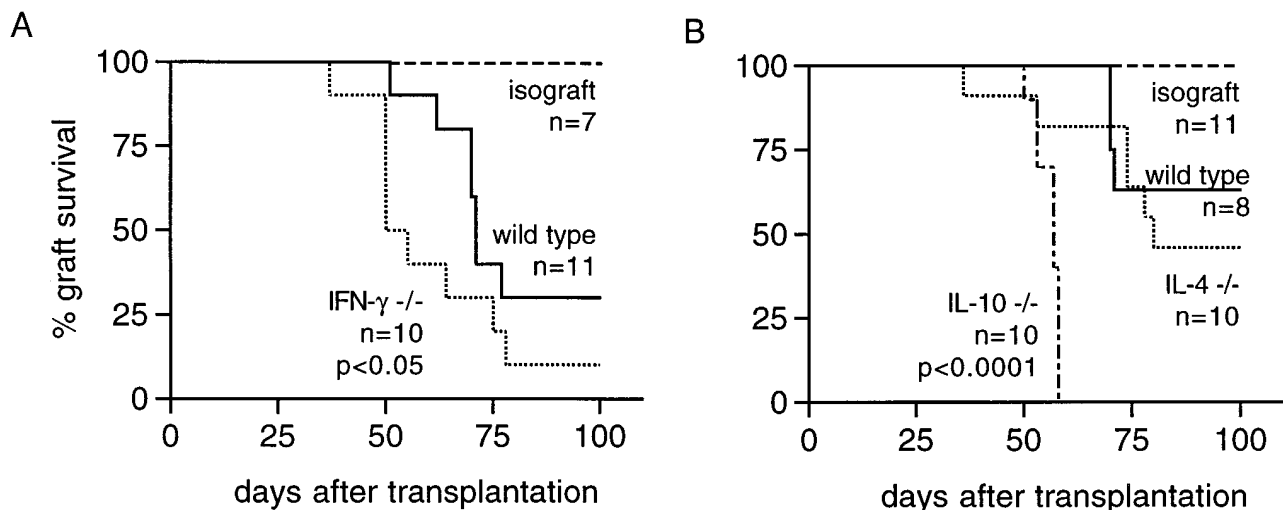


Figure 1. Cardiac graft survival in recipients with targeted deletions in IFN- γ , IL-4, and IL-10. *A* shows graft survival in the CBA to C57BL/6J model. There was a small but significant ($P < 0.05$) decrease in graft survival in IFN- γ $-/-$ when compared with wild-type mice. *B* shows graft survival in the BALB/c to C57BL/6J model. There was no difference in graft survival in IL-4 $-/-$ mice, but IL-10 $-/-$ recipients rejected cardiac allografts at an accelerated pace; $P < 0.0001$. There was no significant difference in graft survival in these two different strain combinations. All isografts survived indefinitely (> 100 d). Number of the animals (n) is shown for each group.

Histologic evaluation. Fig. 2 depicts representative histologic sections from cardiac transplants placed in various knockout or wild-type recipients. Allografts in all groups showed diffuse mononuclear cell infiltration, variable degrees of interstitial edema, patchy areas of necrosis, occasional hemorrhage, scattered fibrosis, and prominent arterial luminal oc-

clusion. Within a single graft, areas of severe destruction were visible next to mildly affected areas, demonstrating the patchy nature of the rejection process throughout the cardiac allograft. Isografts had no rejection, as expected for inbred mouse strains. ISHLT rejection scores for each graft are summarized in Table I. The severity of acute rejection in IL-10 $-/-$

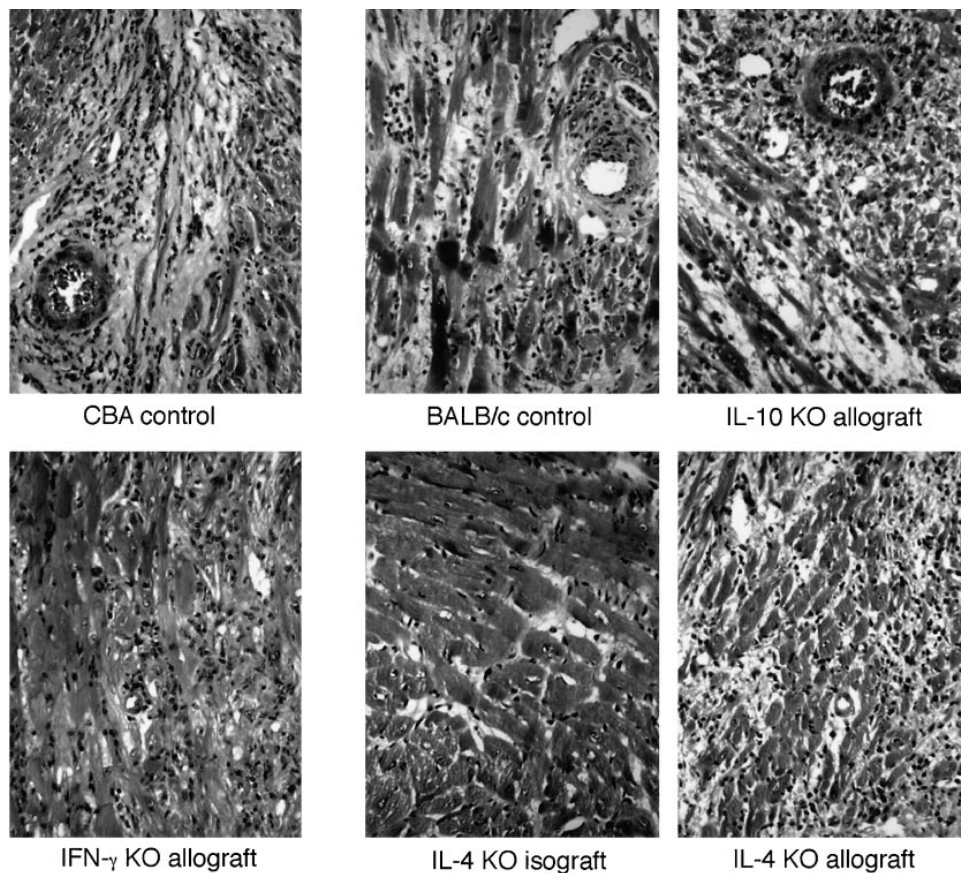


Figure 2. Histology of murine cardiac transplants. Allografts in all groups showed severe mononuclear cell infiltration, variable degrees of interstitial edema, patchy areas of necrosis, fibrosis (typical of resolving rejection), and prominent arterial luminal occlusion. Tissue destruction appeared to be worse in IL-10 knockout (*KO*) recipients, whereas the density of inflammatory cells seemed to be greater in IL-4 *KO* recipients when compared with BALB/c controls. All isografts (only IL-4 *KO* isograft shown) had preserved myocardial structure with few infiltrating cells and no vascular thickening (hematoxylin and eosin staining, original magnification 100 \times).

Table I. Acute Rejection Scores of Cardiac Allografts Placed in Knockout and Wild-type Recipients

Mouse strain	Acute rejection scores*	mean±SD
Control (CBA to C57)	4, 4, 3A, 4, 3A, 4, 1A, 3A, 2, 3A	3.1±1.0
IFN- γ -/- allograft	4, 3B, 3A, 3B, 4, 3B, 3B, 3A, 4	3.3±0.5, $P = 0.46^{\ddagger}$
IFN- γ -/- isograft	0, 0, 0, 0, 0, 0, 0	0±0, $P < 0.0001$
Control (BALB/c to C57)	3A, 2, 2, 4, 3A, 3B, 4, 2, 4	3.0±0.9
IL-4 -/- allograft	3A, 4, 4, 4, 3B, 4, 4, 2, 3A	3.5±0.7, $P = 0.13$
IL-4 -/- isograft	0, 0, 0, 0, 0, 0	0±0, $P < 0.0001$
IL-10 -/- allograft	4, 3A, 3B, 4, 4, 3B, 3B, 4, 4	3.8±0.3, $P = 0.021$
IL-10 -/- isograft	0, 0, 0, 0, 0	0±0, $P < 0.0001$

*ISHLT rejection scores from 0 to 4 where 0 stands for no rejection, 1 for mild focal (A) or diffuse (B) perivascular and interstitial infiltration with no parenchymal necrosis, 2 for moderate, unifocal infiltration with/without focal myocyte injury, 3 for moderate multifocal (A) or diffuse (B) infiltration with myocyte injury, and 4 for severe rejection with aggressive, diffuse infiltration, edema, myocyte necrosis, hemorrhage and vasculitis. To derive mean values in each group, 1B and 3B scores were calculated as 1.5 and 3.5, respectively. ‡ Statistical significance using MANOVA with Bonferroni correction compared with wild-type control where $P < 0.0167$ is considered significant.

recipients appeared to be worse when compared with wild-type controls. Grafts from IL-10 -/- recipients had more myocyte destruction with interstitial widening, and hemorrhage consistent with more accelerated parenchymal rejection. There were, however, no significant differences in acute rejection scores (3.8±0.3 versus 3.0±0.9, respectively; $P = 0.021$). In the setting of IL-4 deletion, the density of infiltrating cells within the grafts appeared higher, but ISHLT scores were similar in IL-4 -/- and wild-type recipients (3.5±0.7 vs. 3.0±0.9, $P = 0.13$). The modified ISHLT scale typically used on endomyocardial biopsies is probably insensitive to differences in the degree of infiltration in the whole graft sections. Taken together, isolated deficiencies in the cytokines did not prevent development of graft failure, but qualitatively altered some of the histologic manifestations of rejection. Examination of graft histology at earlier time points is likely to disclose more prominent differences associated with the role of these cytokines in early alloimmune injury.

Inflammatory activation patterns in IFN- γ -/- recipients.

To compare inflammatory activation patterns, we measured mean corrected transcript levels of cytokines and monokines in a series of grafts using semiquantitative 32 P-RT-PCR assays. Immunoperoxidase staining was used to confirm the presence of the gene product. Analyses of CBA-transplanted hearts comparing wild-type with IFN- γ -/- C57BL/6J recipients are shown in Fig. 3. T cell infiltration, estimated using CD3 ζ transcript levels, was similar in both IFN- γ -/- and wild-type recipients. As expected, mean transcript levels for IFN- γ were high in wild-type allografts, and barely detectable in IFN- γ -/- recipients. In contrast, IFN- γ -/- recipients demonstrated significant increases in IL-2 transcript levels when compared with both isografts or wild-type controls ($P < 0.008$). Representative immunostaining confirmed the presence of the IL-2 gene product in grafts from IFN- γ -/- recipients. IL-2 antigen was typically found within the thin cytoplasmic rims of small mononuclear cells with T cell morphology as shown in Fig. 4 A. In the IFN- γ -/- group, there was a large variation in IL-12 and IL-4 transcripts, as illustrated by scattergram of individual animals without significant difference. IL-10 transcripts were similar in both IFN- γ -/- and wild-type recipients. IFN- γ -/- isografts had lower transcript levels of IL-4 and IL-10 ($P < 0.02$) than did either allograft group.

Analysis of the monokine transcript levels showed three

different patterns in the face of IFN- γ deletion: decreased, increased, and unaltered expression. The two IFN- γ inducible monokines, AIF-1 (29) and iNOS, were significantly lower in IFN- γ -/- recipients than in wild-type animals ($P < 0.0001$ for both). MCP-1, which is regulated by multiple pathways (30), was significantly higher in IFN- γ -/- recipients ($P < 0.003$), while TNF- α produced by both T cells and macrophages showed an unaltered transcript pattern when compared with wild-type, similar to that seen for CD3 ζ .

Inflammatory activation patterns in IL-4 -/- and IL-10 -/- recipients. To study whether macrophage activation was attenuated in the setting of Th2-type cytokine deficiencies, we compared intragraft mRNA levels for BALB/c transplants placed in wild-type C57BL/6J controls with IL-4 -/- and IL-10 -/- recipients (Fig. 5). Three patterns became apparent: levels for IL-12, AIF-1, and MCP-1 were similar in either knockout recipient group when compared with the wild-type group. In IL-10 -/- recipients, iNOS transcript levels were significantly higher when compared with the other two groups ($P < 0.002$), while levels for TNF- α , known to be downregulated by IL-4, were significantly higher in IL-4 -/- recipients. The presence of the TNF- α gene product was confirmed both by immunostaining and by the antigen localized within the surrounding cytoplasm of larger mononuclear cells with monocyte/macrophage morphology (Fig. 4 B).

CD3 ζ transcript levels were significantly higher in IL-4 -/- animals when compared with wild-type controls and IL-10 ($P < 0.004$), suggesting an increase in T cell infiltration (Fig. 6). Intragraft IL-10 transcript levels were significantly higher in IL-4 -/- recipients when compared with the wild-type, and were barely detectable in IL-10 -/- recipients. Immunostaining (Fig. 4 C) confirmed the presence of the IL-10 protein in IL-4 -/- recipients. IL-10 localized predominantly to large mononuclear cells with abundant cytoplasm typical of monocyte/macrophages. IFN- γ transcripts were variable, but appeared higher in both knockout groups. Only the increase in the IL-10 -/- group, however, reached significance ($P < 0.02$).

Given the high degree of T cell infiltration suggested by the increase in CD3 ζ transcripts, we estimated cytokine production per T cell by normalizing against CD3 levels. This showed that IFN- γ transcripts were 4.3 times higher in IL-10 -/- than in wild-type recipients ($P < 0.002$). The presence of IFN- γ -positive mononuclear cells throughout the cardiac tissue in

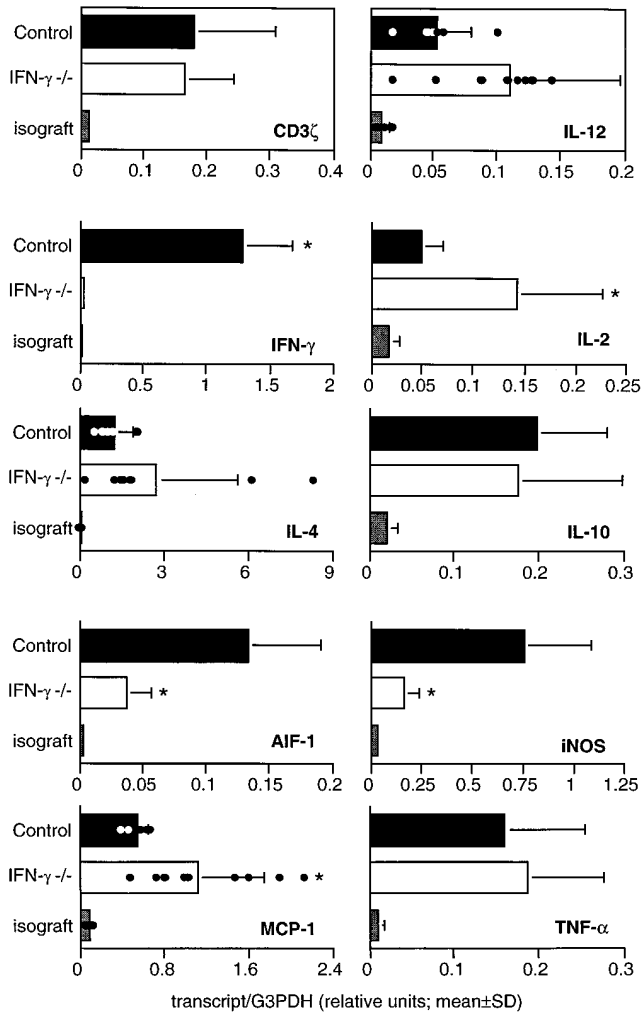


Figure 3. Transcript analysis in IFN- γ -/- recipients. Mean IL-2 transcript level was significantly higher in IFN- γ -/- recipients when compared with wild-type or isografts ($*P < 0.008$). In contrast, IFN- γ -inducible factors, iNOS and AIF-1, were lower ($*P < 0.0001$), but MCP-1 was higher ($*P < 0.003$) in IFN- γ -/- when compared with wild-type recipients. Scattergram on the bars shows determination of individual animals in each group, indicating large heterogeneity. Number of the animals (cDNAs) in each group: control, 6; IFN- γ -/- allografts, 9; IFN- γ -/- isografts, 6. Each bar represents mean of the group \pm SD.

IL-10 -/- recipients was confirmed by immunostainings (Fig. 4 D). Similar patterns were seen for IL-2 and IL-4, but did not reach significance. This result suggested that the level of T cell activation was higher in the IL-10 -/- than in wild-type recipients. In the IL-4 -/- group, CD3 ζ -corrected levels for IFN- γ and IL-2 transcripts were comparable to wild-type levels. Thus, grafts in the IL-4 -/- group had increased T cell infiltration, but these cells were less activated than those in the grafts from IL-10 -/- recipients.

Discussion

By concurrently evaluating isolated cytokine deletions of IFN- γ , IL-4, or IL-10, we show that the recipient environment in long-surviving cardiac transplants caused distinct graft modifica-

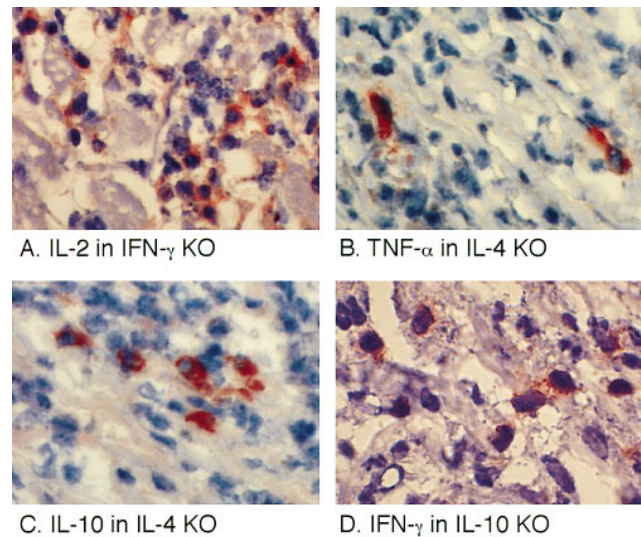


Figure 4. Immunostaining confirmed the presence of IL-2, TNF- α , IL-10, and IFN- γ gene product in mononuclear cells infiltrating the cardiac allografts. (A) IL-2 positive cells in IFN- γ -/- (KO) recipient. (B) TNF- α positive cells in an IL-4 -/- recipient. (C) IL-10 positive cells in an IL-4 -/- recipient. (D) IFN- γ positive cells in an IL-10 -/- recipient (original: A, 125 \times ; B-D, 188 \times).

tions. Our major findings were that deletion of IL-10, or unexpectedly, IFN- γ , but not deletion of IL-4, significantly impaired allograft survival in this murine model where immunosuppression is used to attenuate rejection. Deletion of any of these cytokines changed responses to the allograft in a way that indicated that there were compensatory pathways activated in the absence of these cytokines.

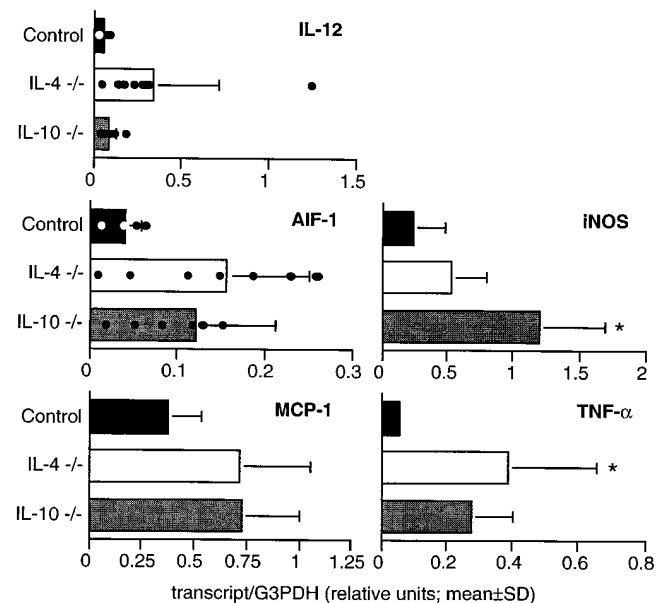


Figure 5. Macrophage activation markers in IL-4 -/-, IL-10 -/-, and wild-type recipients. Mean transcripts for TNF- α were significantly ($*P = 0.006$) higher in IL-4 -/- recipients. In contrast, IL-10 -/- mice had higher levels of iNOS ($*P = 0.0002$). Number of the animals (cDNAs) in each group: control, 5; IL-4 -/-, 8; IL-10 -/-, 7. Each bar represents mean \pm SD for all grafts in each group.

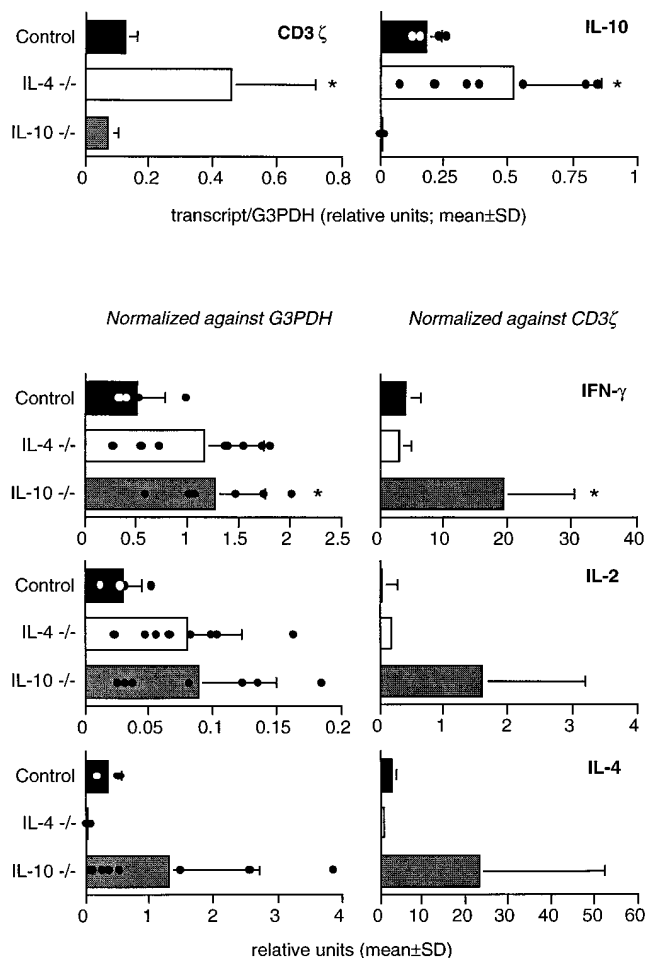


Figure 6. Transcript analysis of inflammatory factors in IL-4 $-/-$ and IL-10 $-/-$ recipients. CD3 ζ ($*P < 0.004$) and IL-10 ($*P < 0.01$) transcript levels (*top*) corrected with G3PDH were significantly higher in IL-4 $-/-$ recipients when compared with wild-type, while IFN- γ levels were higher in IL-10 knockout recipients (*left*, $*P < 0.02$). When corrected with CD3 ζ transcripts (*right*), the increase in T cell cytokines in IL-4 $-/-$ recipients returned to the level of wild-types, reflecting greater density of T cells in the allograft. Number of the animals (cDNAs) in each group: control, 5; IL-4 $-/-$, 8; IL-10 $-/-$, 7. Each bar represents mean \pm SD for all grafts in each group.

First, IL-10 is not required for rejection as demonstrated by the decline in graft function and prominent histologic changes in the grafts from IL-10 $-/-$ recipients. Secondly, the accelerated rejection seen in the absence of IL-10 suggests that this cytokine may inhibit the immune responses that lead to graft failure, or even have a role in graft acceptance. To date, the role of IL-10 in transplantation has been controversial. The effects of systemic administration of IL-10 on graft survival after murine cardiac transplantation were inconclusive (31) or showed that IL-10 had a detrimental effect (32, 33). Similarly, graft survival was not changed after intraperitoneal administration of IL-10 (32). Perhaps beneficial effects of IL-10 are only conferred if local concentrations in the graft environment are high. Along these lines, retroviral gene transduction of viral IL-10 prolonged graft survival from 12 to 39 d when injected directly into nonvascularized neonatal cardiac allografts

(34). This result is consistent with our finding that IL-10 may have a protective effect in allografts as deletion of IL-10 accelerated rejection. Direct transfer of viral IL-10 into the transplanted organ may have produced intragraft levels of IL-10 superior to those from intraperitoneal absorption or systemic delivery. Alternatively, the viral form of IL-10 may have superior immunosuppressive effects, given that retroviral introduction of murine IL-10 into neonatal grafts had no beneficial effect. Further studies reconstituting the various forms of IL-10 in the graft environment may resolve the important controversy of whether IL-10 has a protective role in transplantation.

The mechanism underlying the accelerated rejection we saw with IL-10 $-/-$ recipients requires further exploration. One factor may be that the T cells that infiltrated the graft have a higher state or degree of activation. After T cell cytokine transcript levels were corrected for the level of CD3 transcripts, the elevation in IFN- γ transcripts was greater in IL-10 $-/-$ recipients. Analysis of selected factors has indicated some of the other potential mechanisms. For example, iNOS, an IFN- γ -inducible factor, was significantly higher in IL-10 $-/-$ recipients. Nitric oxide-driven injury, associated with enhanced IFN- γ -mediated responses, may provide one possible explanation for the accelerated graft failure seen in IL-10 $-/-$ recipients (35). Taken together, our studies are the first to use mice with targeted gene deletion of IL-10 as a way of demonstrating that IL-10 attenuates the response leading to allograft rejection.

IL-4-driven Th2-like responses have been hypothesized to regulate long-term graft acceptance (36, 37). While it is clear from our studies that IL-4 is not required for rejection, our findings have not completely addressed whether IL-4 actually promotes graft acceptance. One might expect that if IL-4 did promote graft survival, then in the absence of IL-4, graft survival should be shorter. Contrary to this expectation, we found that allografts placed in IL-4 $-/-$ recipients had similar graft survival to wild-type recipients. The survival past 100 d in our grafts in IL-4 $-/-$ recipients indicates that long-term graft acceptance can occur without IL-4. Our data extends the observation of Lakkis et al., that heart allografts in nonimmunosuppressed or murine CTLA4Ig-treated IL-4 knockout recipients show similar survival times compared to wild-type controls (12). One possible explanation for the comparable survival times is that other Th2-associated cytokines like IL-5, IL-6, IL-10, or IL-13 may compensate for the lack of IL-4 in knockout animals. Indeed, we found significant upregulation of IL-10 in the face of IL-4 deficiency.

The observation that TNF- α transcripts were high in the absence of IL-4 provides *in vivo* support for the *in vitro* study of Szalay et al. (38), demonstrating that IL-4 inhibits TNF- α production. The concurrent increases in TNF- α and CD3 transcripts in the IL-4 $-/-$ recipients appear to be related, since TNF- α has been shown to promote inflammatory cell recruitment into tissues through selectins (39). When T cell cytokine levels (IL-2 and IFN- γ) were normalized against the increase in CD3, they were similar to those of the wild-type group. Thus, elevated cytokine levels after G3PDH correction in the IL-4 $-/-$ recipients may have reflected the degree of T cell infiltration rather than increased activation of the infiltrating cells. This result raises the possibility that IL-4 may suppress T cell infiltration.

In our studies, cardiac allografts in IFN- γ $-/-$ recipients receiving a 30-d course of immunosuppression, had a small but

significant decrease in graft survival. This result is contrary to what might be predicted by the hypothesis that IFN- γ mediates the injury, culminating in acute rejection. Our observations, however, are consistent with two recent reports showing accelerated cardiac graft failure in untreated or CTLA4Ig-treated IFN- γ knockout recipients (10, 33). Taken together, these results show that cardiac rejection can occur independently of IFN- γ , and that IFN- γ may actually inhibit pathways that lead to graft failure.

The accelerated response to alloimmune stimulation parallels the unexpected early onset of collagen-induced arthritis in IFN- γ R $-/-$ mice (40, 41). The mechanisms underlying the protective effects of IFN- γ after these distinct types of immune injury deserve exploration. One possibility is that IFN- γ regulates the cytokine cascade that contributes to the pathogenesis of diseases. Evidence for regulation includes the increase in IL-2 expression in the face of IFN- γ or IFN- γ receptor deficiency. For example, IL-2 protein levels in serum were higher in IFN- γ R $-/-$ when compared with those of wild-type mice in the collagen-induced arthritis model (41). Similarly, in our analysis of grafts with attenuated rejection, intragraft IL-2 gene transcript levels are increased in recipients with IFN- γ deficiency. This pattern was also present in untreated grafts placed in IFN- $-/-$ recipients using qualitative PCR analysis (10). Taken together, it appears that IFN- γ actions may be multifactorial with some injury-promoting and -protecting responses.

Here we also show that IFN- γ selectively alters macrophage activation *in vivo*. There were three different transcript patterns produced by recipients with IFN- γ gene deletion. IFN- γ -mediated monokine activation was reduced for AIF-1 and iNOS. In contrast, MCP-1 was increased. For the other monokines that we examined (IL-10, TNF- α , IL-12), there were no apparent alterations. The increase in IL-2 transcripts and persistent but selective monokine expression may indicate potential alternative pathways mediating the alloimmune response that culminates in graft rejection in the absence of IFN- γ . The observed alterations in these macrophage effectors may have distinct functional consequences on the various processes involved in rejection. For example, the impaired graft survival may arise because a protective influence from a macrophage effector has been lost, while IFN- γ -activated macrophages may contribute to later phases of rejection leading to graft failure in our model. Hence, IFN- γ may regulate the balance in macrophage-activating and effector forces in the cardiac allograft.

This study of individual targeted gene deletions for three cytokines commonly found in long-surviving allografts emphasizes the complex interrelated role of cytokines in the inflammatory network (42, 43). For example, our results from grafts undergoing attenuated rejection in IL-4 $-/-$ and IFN- γ $-/-$ recipients showed that compensatory pathways most likely play a key role in controlling rejection. Identifying these pathways will be crucial in developing combination therapy. In contrast, the results from IL-10 $-/-$ recipients showed that it is possible to identify factors with primary, nonredundant roles. Thus, efforts to augment IL-10 levels within the graft may be of therapeutic value. Our studies using these knockout models provide a new way to dissect the functional contributions of individual cytokines to the various aspects of the alloimmune response. Such studies may help us to identify how to disrupt selectively the cytokine cascade to attenuate transplant rejection.

Acknowledgments

The authors thank Ms. Dorothy Zhang for embedding the paraffin blocks.

This work was supported in part by Bristol-Myers-Squibb Pharmaceuticals (Princeton, NJ), National Institutes of Health grant R29HL54897 and the Milton Fund, Harvard University, Cambridge, MA. A. Räisänen-Sokolowski received support from Finska Läkaresällskapet, Finnish Cultural Foundation, Helsinki, and the Aarno Koskelo Foundation, Espoo, Finland. P.L. Mottram received support from the University of Melbourne, National Health and Medical Research Council, and National Heart Foundation, Canberra, Australia.

References

1. Abbas, A., K. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature*. 383:787-793.
2. Räisänen-Sokolowski, A., T. Glysing-Jensen, P. Mottram, and M. Russell. 1997. Sustained anti-CD4/CD8 blocks inflammatory activation and intimal thickening in mouse heart allografts. *Arterioscl. Thromb. Vasc. Biol.* 17:2115-2122.
3. Dallman, M., C. Larsen, and P. Morris. 1991. Cytokine gene transcription in vascularised organ grafts: analysis using semiquantitative polymerase chain reaction. *J. Exp. Med.* 174:493-496.
4. Takeuchi, T., R. Lowry, and B. Konieczny. 1992. Heart allografts in murine systems: the differential activation of TH2-like effector cells in peripheral tolerance. *Transplantation (Baltimore)*. 53:1281-1294.
5. Sayegh, M.H., E. Akalin, W.W. Hancock, M.E. Russell, C.B. Carpenter, P.S. Linsley, and L.A. Turka. 1995. CD28-B7 blockade after alloantigenic challenge *in vivo* inhibits Th1 cytokines but spares Th2. *J. Exp. Med.* 181(5):1869-1874.
6. Binder, J., M. Lehmann, E. Graser, W. Hancock, B. Watschinger, K. Onodera, M. Sayegh, H.-D. Volk, and J. Kupiec-Weglinski. 1996. The effects of nondepleting CD4 targeted therapy in presensitized rat recipient of cardiac allografts. *Transplantation (Baltimore)*. 61:804-811.
7. Hancock, W.W., M.H. Sayegh, X.-G. Zheng, R. Peach, P.S. Linsley, and L.A. Turka. 1996. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. *Proc. Natl. Acad. Sci. USA*. 93:13967-13972.
8. Onodera, K., W. Hancock, E. Graser, M. Lehmann, M. Sayegh, T. Strom, H.-D. Volk, and J. Kupiec-Weglinski. 1997. Type 2 helper T cell-type cytokines and the development of "infectious" tolerance in rat cardiac allograft recipients. *J. Immunol.* 158:1572-1581.
9. VanBuskirk, A.M., M.E. Wakely, and C.G. Orosz. 1996. Transfusion of polarized TH2-like cell populations into SCID mouse cardiac allograft recipients results in acute allograft rejection. *Transplantation (Baltimore)*. 62(2):229-238.
10. Saleem, S., B. Konieczny, R. Lowry, F. Baddoura, and F. Lakkis. 1996. Acute rejection of vascularized heart allografts in the absence of IFN- γ . *Transplantation (Baltimore)*. 62:1908-1911.
11. Picotti, J., S. Chan, R. Goodman, J. Magram, E. Eichwald, and D. Bishop. 1996. IL-12 antagonism induces T helper 2 responses, yet exacerbates cardiac allograft rejection. *J. Immunol.* 157:1951-1957.
12. Lakkis, F., B. Konieczny, S. Saleem, F. Baddoura, P. Linsley, D. Alexander, R. Lowry, T. Pearson, and C. Larsen. 1997. Blocking the CD28-B7 T cell costimulation pathway induced long term cardiac allograft acceptance in the absence of IL-4. *J. Immunol.* 158:2443-2448.
13. Corry, R.J., H.J. Winn, and P.S. Russell. 1973. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation (Baltimore)*. 16(4):343-350.
14. Mottram, P.L., J.A. Smith, A. Mason, A. Mirisklavos, L.J. Dumble, and G.J. Clunie. 1988. Electrocardiographic monitoring of cardiac transplants in mice. *Cardiovasc. Res.* 22(5):315-321.
15. Dalton, D.K., S. Pitts-Meek, S. Keshav, I.S. Figari, A. Bradley, and T.A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science*. 259 (5102):1739-1742.
16. Kühn, R., K. Rajewsky, and W. Müller. 1991. Generation and analysis of interleukin-4 deficient mice. *Science*. 254:707-710.
17. Kühn, R., J. Löhler, D. Rennick, K. Rajewsky, and W. Müller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 75:263-274.
18. Mottram, P.L., W.R. Han, L.J. Purcell, I.F. McKenzie, and W.W. Hancock. 1995. Increased expression of IL-4 and IL-10 and decreased expression of IL-2 and interferon-gamma in long-surviving mouse heart allografts after brief CD4-monoclonal antibody therapy. *Transplantation (Baltimore)*. 59(4):559-565.
19. Pearson, T., J. Madsen, C. Larsen, P. Morris, and K. Wood. 1992. Induction of transplantation tolerance in adults using donor antigen and anti-CD4 monoclonal antibody. *Transplantation (Baltimore)*. 54:475-483.

20. Billingham, M.E., R.B. Nathaniel, N.R.B. Cary, M.E. Hammond, J. Kemnitz, C. Marboe, H.A. McCallister, D.C. Snovar, G.L. Winters, and A. Zerbe. 1990. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: heart rejection study group. *J. Heart Transplant.* 9:587-593.
21. Russell, M.E., A.F. Wallace, W.W. Hancock, M.H. Sayegh, D.H. Adams, N.E.S. Sibinga, L.R. Wyner, and M.J. Karnovsky. 1995. Upregulation of cytokines associated with macrophage activation in the Lewis to F344 rat chronic cardiac rejection model. *Transplantation (Baltimore)*. 59(4):572-578.
22. Russell, M.E., W.W. Hancock, E. Akalin, A.F. Wallace, T. Glysing-Jensen, T. Willett, and M.H. Sayegh. 1996. Chronic cardiac rejection in the Lewis to F344 rat model: blockade of CD28-B7 costimulation by CTLA4lg modulates T cell and macrophage activation and attenuates arteriosclerosis. *J. Clin. Invest.* 97:833-838.
23. Birch, D., L. Kolmodin, W. Laird, N. McKinney, J. Wong, K. Young, G. Zangenberg, and M. Zoccoli. 1996. Simplified hot start PCR. *Nature*. 381:445-446.
24. Roux, K. 1995. Optimization and troubleshooting in PCR. *PCR Methods Appl.* 4(5):S185-S194.
25. Andersson, U., and J. Andersson. 1994. Immunolabeling of cytokine-producing cells in tissues and in cell suspension. In *Cytokine-producing Cells*. D. Fradelizie, and D. Emelie, editors. INSERM, Paris. 32-49.
26. Brown, M., L. Engelman, J. Frane, M. Hill, R. Jennrich, and J. Toporek. 1983. BMDP Statistical Software: 1983 Printing with Additions. University of California Press, Berkeley, CA. 733 pp.
27. Russell, M.E., D.H. Adams, L.R. Wyner, Y. Yamashita, N.J. Halnon, and M.J. Karnovsky. 1993. Early and persistent induction of monocyte chemoattractant protein 1 in rat cardiac allografts. *Proc. Natl. Acad. Sci. USA*. 90(13):6086-6090.
28. Russell, M.E., A.F. Wallace, L.R. Wyner, J.B. Newell, and M.J. Karnovsky. 1995. Upregulation and modulation of inducible nitric oxide synthesis in rat cardiac allografts with chronic rejection and transplant arteriosclerosis. *Circulation*. 92:457-464.
29. Utans, U., R.J. Arceci, Y. Yamashita, and M.E. Russell. 1995. Cloning and characterization of allograft inflammatory factor-1: a novel macrophage factor identified in rat cardiac allografts with chronic rejection. *J. Clin. Invest.* 95:2954-2962.
30. Rollins, B.J. 1996. Monocyte chemoattractant protein 1: a potential regulator of monocyte recruitment in inflammatory disease. *Mol. Med. Today*. 2(5):198-204.
31. Lowry, R.P., B. Konieczny, D. Alexander, C. Larsen, T. Pearson, S. Smith, and S. Narula. 1995. Interleukin-10 eliminates anti-CD3 monoclonal antibody-induced mortality and prolongs heart allograft survival in inbred mice. *Transplant. Proc.* 27(1):392-394.
32. Qian, S., W. Li, F. Fu, L. Lu, J. Fung, and A. Thomson. 1996. Systemic administration of cellular interleukin-10 can exacerbate cardiac allograft rejection in mice. *Transplantation (Baltimore)*. 62:1709-1714.
33. Konieczny, B., E. Elwood, C. Larsen, T. Pearson, P. Linsley, and F. Lakkis. May 1997. Absence of IFN-gamma reduces murine cardiac and skin allograft survival following costimulation blockade. *American Society of Transplant Physicians Annual Meeting, Chicago*. 7(Abstr.):86.
34. Qin, L., K.D. Chavin, Y. Ding, H. Tahara, J.P. Favaro, J.E. Woodward, T. Suzuki, P.D. Robbins, M.T. Lotze, and J.S. Bromberg. 1996. Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival. *J. Immunol.* 156(6):2316-2323.
35. Dusting, G. 1995. Nitric oxide in cardiovascular disorders. *J. Vasc. Res.* 32:143-161.
36. Lowry, R., and T. Takeuchi. 1994. The Th1, Th2 paradigm and transplantation tolerance. R.G. Landes Company, Austin, TX. 134 pp.
37. Mosmann, T., and S. Sad. 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today*. 17:138-147.
38. Szalay, G., C. Ladel, C. Blum, and S. Kaufmann. 1996. IL-4 neutralization or TNF- α treatment ameliorate disease by an intracellular pathogen in IFN- γ receptor deficient mice. *J. Immunol.* 157:4746-4750.
39. Hickey, M., P. Reinhardt, L. Ostrovsky, W. Jones, M. Jutila, D. Payne, J. Elliot, and P. Kubers. 1997. Tumor necrosis factor- α induces leukocyte recruitment by different mechanisms in vivo and in vitro. *J. Immunol.* 158:3391-3400.
40. Manourny-Schwartz, B., G. Chiochia, N. Bessis, O. Abehsira-Amar, F. Bateux, S. Muller, S. Huang, M. Boisser, and C. Fournier. 1997. High susceptibility to collagen-induced arthritis in mice lacking IFN- γ receptors. *J. Immunol.* 158:5501-5506.
41. Vermeire, K., H. Heremans, M. Vandeputte, S. Huang, A. Billiau, and P. Matthys. 1997. Accelerated collagen-induced arthritis in IFN- γ receptor-deficient mice. *J. Immunol.* 158:5507-5513.
42. Nickerson, P., J. Steiger, X. Zheng, A. Steele, W. Steurer, P. Roy-Chaudhury, and T. Strom. 1997. Manipulation of cytokine networks in transplantation: false hope or realistic opportunity for tolerance? *Transplantation (Baltimore)*. 63:489-494.
43. Orosz, C.G. 1996. Networked alloimmunity: a brief reexamination of some basic concepts in transplantation immunobiology. *J. Heart Lung Transplant.* 15:1063-1068.