

Cardiac Allografts from IL-4 Knockout Recipients: Assessment of Transplant Arteriosclerosis and Peripheral Tolerance¹

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To study the role of IL-4 in tolerance induction and transplant arteriosclerosis, BALB/c hearts were transplanted into C57BL/6J wild-type or IL-4 knockout (IL-4^{-/-}) recipients. A 30-day course of anti-CD4/8 mAb was used to induce long term graft survival. Primary graft survival was 50% (5 of 10) in IL-4^{-/-} recipients comparable to 63% (5 of 8) in wild-type recipients. Mice with allografts surviving >80 days were tested for tolerance by challenge with a second donor or third party (CBA) heart. Secondary donor-strain heart grafts survived >30 days, but showed histologic evidence of ongoing alloimmune response. Third party hearts rejected rapidly. Although immunostaining and ³²P RT-PCR assays showed no differences in the mononuclear cell infiltration and T cell activation between IL-4^{-/-} and wild-type tolerant recipients, some monokines (IL-12, TNF- α , and allograft inflammatory factor-1) were up-regulated in grafts from IL-4^{-/-} recipients. Computer-assisted analysis of elastin-stained vessels revealed that the severity of vascular thickening (percentage of luminal occlusion, mean \pm SD, $n = 329$) was similar in grafts from IL-4^{-/-} (63.7 \pm 16.9%) and wild-type (69.5 \pm 17.6%) recipients. Thus, IL-4 deficiency did not alter primary or secondary graft survival, infiltration, or vascular thickening. The selective alterations in monokine expression suggests that alternative pathways are activated and may compensate in IL-4^{-/-} mice. *The Journal of Immunology*, 1998, 161: 602–609.

Interleukin-4, which is produced by T cells, basophils, mast cells, and NK cells (1), was originally identified as a B cell growth factor. It is now known to play a central role in stimulating Th2 differentiation and inhibiting Th1-type responses (2). In contrast, Th1 activities are stimulated by IFN- γ and IL-12 and are down-regulated by IL-4, IL-10, and TGF- β (3). In the transplant field, descriptive studies have shown a predominance of Th1 cytokines in grafts undergoing acute rejection. In grafts surviving long term, decreases in the Th1 cytokines (IL-2 and IFN- γ) with concomitant increases in the Th2 products, IL-4, and IL-10 have been reported (4–6). These findings have led to the hypothesis that Th1-type responses are required for acute rejection, while Th2 type responses maintain graft acceptance and are possibly involved in the progression of chronic rejection and transplant arteriosclerosis (7).

However, the hypothesis that IL-4 may mediate peripheral tolerance in recipients of long surviving allografts following transient T cell depletion has been controversial (6, 8–10). Studies have shown IL-4 expression in rejecting grafts (11, 12) and mixed Th1/Th2-type responses, including IFN- γ , IL-2, and IL-4 expression, in the allografts that survived long term (13). Functional studies using mAb or recombinant protein treatments have further contributed to the perplexity. Anti-IL-4

mAb administration blocked neonatal tolerance induction, supporting the hypothesis (14). In contrast, administration of soluble IL-4R, to neutralize IL-4 function in vivo, unexpectedly prolonged graft survival in mice instead of decreasing it (15). Similar prolonged allograft survival was seen in IL-4R transgenic mice (15). Administration of rIL-4 did not significantly alter heart or skin graft survival (16), and transfusion of IL-4-producing Th2 cells into SCID recipients caused rapid graft rejection (17). Hence, further investigations are required to clarify the role of IL-4 in peripheral tolerance induction and maintenance.

IL-4 has also been implicated as a mediator of chronic rejection. This form of rejection produces a more insidious form of graft decline that occurs later in the transplant course. The histologic hallmarks are progressive parenchyma fibrosis and transplant arteriosclerosis (18). Some studies in rodent models have shown that IL-4 expression is higher in allografts that develop prominent vascular thickening (7, 13, 19). To date there are no functional studies that establish a causative role for IL-4 in the process of myofibrotic expansion of the neointima, the hallmark of chronic rejection.

Recently, the availability of mice with targeted gene deletion of IL-4 (20, 21) has allowed direct study of the effects of this cytokine in vivo. The mice are phenotypically normal, without aberration in B or T cell development. However, after challenge, lymphocytes from IL-4^{-/-} mice produce aberrant Th2 responses to *Nippostrongylus brasiliensis* infection or anti-CD3 cross-linking. IL-4^{-/-} mice are particularly useful in studies of tolerance and transplant arteriosclerosis, since both these conditions require long term survival. The first reports using IL-4^{-/-} mice as recipients in transplantation did not reveal differences in the rate of islet or cardiac rejection (22). Lakkis et al. (23) extended this observation by showing that IL-4 was not required for long term survival of murine cardiac allografts that received CTLA4 Ig induction therapy. We have recently confirmed that long term survival can also be induced without IL-4 after a 30-day course of anti-CD4/8 (24). However, no studies to date have examined the functional role of

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IL-4 in peripheral tolerance or in the development of transplant arteriosclerosis (chronic rejection).

The purpose of this study was to test the interrelated hypotheses that IL-4 is required for peripheral tolerance induction and development of vascular thickening associated with chronic rejection. To study these questions, we compared vascularized cardiac allografts placed in IL-4^{-/-} recipients with controls placed in wild-type recipients focusing on the subgroups in which long term graft survival was achieved after anti-CD4 and anti-CD8 mAb induction therapy (24). Parameters of peripheral tolerance induction, vascular thickening, and leukocyte infiltration and activation were compared in allografts from tolerant IL-4^{-/-} and wild-type recipients.

Materials and Methods

Mice

CBA/CaJ (H-2^k) and BALB/c (H-2^d) were used as donors of heart allografts. Recipients were C57BL/6J (H-2^b; The Jackson Laboratory, Bar Harbor, ME) with and without IL-4 gene deletion (21). Isografts were performed from IL-4^{-/-} donor to IL-4^{-/-} recipient. The targeted gene deletion was backcrossed 12 times onto C57BL/6J, and its presence was confirmed in our laboratory using multiplex PCR assays that amplified a portion of the neomycin cassette and a portion of the targeted exon (www.jax.org).

Murine cardiac transplantation

Vascularized abdominal heterotopic heart grafts from 8- to 12-wk-old male mice were transplanted into age-matched male recipients ($n = 33$) as previously described (25, 26). Twenty-four of these transplants had been completed as part of the larger body of work comparing graft survival and inflammatory activation among three cytokine knockout recipients (24). In the earlier study we found heterogeneity in onset of late rejection in the IL-4^{-/-} group. The purpose of this study was to characterize further a subset of long term surviving grafts. Transplant function was evaluated by regular palpation graded on a scale from 4 (functioning well) to 0 (not beating) (25).

mAb treatment and assessment of peripheral tolerance

C57BL/6J recipients were either left untreated or were given mAb against CD4 (clone GK1.5, rat IgG2b, American Type Culture Collection, Rockville, MD) and anti-CD8 (clone 2.43, rat IgG2b, American Type Culture Collection) for 1 to 4, 7, 21, and 28 days after transplantation to attenuate acute rejection. Treated recipients received 500 μ g of each mAb/mouse i.p. on the indicated days (24). Mice with transplants surviving >80 days were tested for donor Ag-specific tolerance with a second heart attached to the jugular and carotid vessels in the neck of the recipient either from donor-strain (BALB/c) or third party (CBA/CaJ (H-2^k)) mice (27). CD4 cells typically recover to within normal range by 3 to 4 wk after cessation of mAb treatment (6, 28). We performed second transplants >80 days after transplantation (>50 days after last mAb injection) when mice are capable of rejecting a third party heart transplant that is used to challenge the recipient. Although placement of second cardiac grafts is technically more difficult, this form of challenge has proved more reliable than skin grafts in demonstrating tolerance (29). Primary and secondary allografts from tested recipients were harvested, and histologic samples were taken when second donor hearts had survived >30 days or for third party hearts when they rejected (palpation score, <1).

Immunohistochemistry

To detect cell surface Ags and cytokines, transverse cryostat (4- μ m) sections from the basal portion of the heart were embedded in OCT medium (Tissue Tek, Miles, Elkhart, IN) and quickly frozen in isopentane cooled below -90° C. Sections were fixed with 4% paraformaldehyde and treated with saponin to increase cell membrane permeability before addition of primary Ab (30). The Abs used for cell typing were KT3 (CD3), affinity purified from ascites from nude rats at the Department of Surgery, Royal Melbourne Hospital (Melbourne, Australia). The Abs used for cytokine analysis included XMG1-2 (IFN- γ) obtained from Dr. Charmaine Simeonovic (Australian National University, Canberra, Australian Capital Territory); and 125.A8 (IL-2R), F4/80 (macrophages), and B220 (B cells) obtained from Dr. Thomas Mandel (The Walter and Eliza Hall Institute, Australia). These were used as supernatant from cell cultures. S4B6 (IL-2) was purchased from PharMingen (San Diego, CA), and ammonium sulfate-precipitated 11B11 (IL-4) was obtained from Dr. Christina Cheers (Uni-

versity of Melbourne, Melbourne, Australia). Binding of mAbs was detected by the four-layer peroxidase-antiperoxidase method described previously (31). Labeled cells within 10 high power fields/section/mouse were counted and expressed as mean cells per high powered ($\times 400$) field of view \pm SD. Control sections, which showed some background staining, were treated with primary Ab omitted.

RT-PCR

To measure relative differences in transcript levels between long term surviving cardiac transplants from IL-4^{-/-} and wild-type recipients, we used a semiquantitative ³²P RT-PCR technique described in detail previously (13, 24, 32). PCR amplification for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH)³ was performed to assess potential variations in cDNA or total RNA loading between samples. The mean G3PDH obtained from at least three analyses was assessed to normalize other transcript level measurements obtained from the same cDNA sample. Corrected values were derived by dividing the measured amount of incorporated ³²P for the transcript of interest by the mean G3PDH value for the same cDNA sample.

Vascular analysis

Paraffin sections were treated with Verhoeff elastin stain to visualize the internal elastic lamina (33). The severity of vascular thickening (percentage of luminal occlusion) as well as the frequency (percentage of diseased vessels) were analyzed in two sections from each graft that met the criteria for experimental tolerance. Microscopic images of each elastin-stained vessel (total of 329, mean of 12.7 vessels/graft) were captured, and the percentage of luminal occlusion was tabulated by tracing the internal elastic lamina and the lumen with the ScionImage 1.59 software (National Institutes of Health, Baltimore, MD). The frequency of the disease in the graft was defined as the percentage of diseased (>0% luminal occlusion) vessels. The mean \pm SD for all grafts in each transplant subgroup are reported.

Statistical analysis of RT-PCR and morphometric data

Results are given as the mean per subgroup \pm SD, which was derived from the mean per graft. The data were subjected to multiple analysis of variance without replication (StatView 4.1, Abacus Concepts, Berkeley, CA). If the multiple analysis of variance was significant, individual comparisons were made by Student's *t* test, and the level of significance was corrected by Bonferroni's method. To evaluate significance in tolerance induction, Fisher's exact test was used.

Results

Peripheral tolerance assessment in IL-4^{-/-} and wild-type recipients

Peripheral tolerance assessments comparing IL-4^{-/-} and wild-type mice on the inbred C57BL/6 background are shown in Table I. Untreated IL-4^{-/-} and wild-type recipients rejected BALB/c allografts in 8 to 9 days, confirming that IL-4 is not required for acute rejection. In cardiac allografts from wild-type recipients treated with anti-CD4/CD8, five of eight (63%) hearts survived long term with a mean survival time of 99 \pm 26 days as reported previously (24). Three mice accepted donor (BALB/c) second hearts for >30 days, while two mice challenged with third party hearts (CBA) rejected them in 11 and 16 days. Thus, treatment with anti-CD4/CD8 produced acceptance of a second donor-strain allograft, but rejection of third party graft consistent with donor-specific peripheral tolerance defined by conventional criteria in the tolerance field (34, 35).

Similar graft survival was seen in the IL-4^{-/-} recipient group treated with CD4/CD8, with 5 of 10 (50%) of transplants surviving long term. Mean survival of the primary heart grafts (94 \pm 24 days) was not significantly different from the wild-type group as reported previously (24). The presence of peripheral tolerance was demonstrated by challenge with a second donor (BALB/c, $n = 3$) or third party (CBA, $n = 2$) heart. All three second donor-strain hearts were accepted for >30 days, while third party hearts were

³ Abbreviations used in this paper: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; AIF-1, allograft inflammatory factor-1.

Table I. Cardiac allograft survival and tolerance assessment in wild-type and IL-4^{-/-} recipients

Group	Wild-Type Recipient	IL-4 ^{-/-} Recipient
Untreated	9, 8, 8, 8	8, 8, 8, 8, 8
Anti-CD4 and anti-CD8 ^a	70, 70, 71, >100, >101, >126, >126, >126	53, 74, 74, 78, 80, >107, >115, >115, >119, >120
Primary graft acceptance	5/8 (63%)	5/10 (50%)
Challenge		
Donor strain (BALB/c)	>30, >30, >30 (100% accepted)	>30, >30, >30 (100% accepted)
Third party (CBA)	11, 14 (100% rejected)	16, 18 (100% rejected)

^a Survival data reported in previous communication (Ref. 24).

rejected at 14 and 18 days after challenge. In addition, allografts from IL-4^{-/-} and wild-type recipients showed comparable palpation scores at the time of harvest, reflecting similar graft function. Thus, graft survival after treatment with anti-CD4/8 was not interrupted by the deletion of functional IL-4 in the recipient environment.

Evaluation of inflammatory activation in graft parenchymal tissue

Immunoperoxidase staining was compared in primary cardiac allografts with survival >110 days from the IL-4^{-/-} and wild-type recipients. In both groups, allograft tissue showed areas of mononuclear cell infiltration involving the parenchymal and perivascular regions. The infiltrate in these hearts consisted of both T cells (CD3 positive, Fig. 1a) and macrophages (F4/80 positive, Fig. 1b), with a few scattered B cells (B220) and IL-2R-positive cells (not shown). As summarized in Table II, counting the number of cells per field of view confirmed that the levels of CD3-, F4/80-, B220-, and IL-2R-positive cells were equivalent in hearts from both IL-4^{-/-} and wild-type groups. Rejecting grafts, which failed at <80 days with palpation scores of 0 at harvest, showed the expected higher expression of CD3 and a trend toward higher macrophage (F4/80) expression compared with long term surviving grafts. Again, similar immunoperoxidase staining was seen in allografts from IL-4^{-/-} and wild-type recipients. As expected, isografts, had significantly lower numbers of infiltrating cells. In addition, primary and secondary allografts in these two groups of mice showed similar patterns of mononuclear cell infiltration. Interestingly, in both primary and secondary allografts from IL-4^{-/-} and wild-type recipients, the inflammatory cell infiltration was not associated with impaired ventricular function. The mean palpation score (on the scale 0–4) for primary BALB/c hearts in the IL-4^{-/-} group was 1.8 ± 0.8, which was similar to that in the wild-type group (2.0 ± 0.9). Secondary BALB/c hearts were also beating well (2.3 ± 1.2 in IL-4^{-/-} and 2.3 ± 0.9 in wild-type group). These are crude demonstrations of preserved heart beat.

Immunostaining for T cell cytokines revealed that infiltrating mononuclear cells were activated in the long term surviving hearts, although less so than in rejecting allografts (Table II). Cytokines were generally expressed in areas of focal infiltrate in perivascular or parenchymal regions. Figure 1, d and f, shows cytokine expression in serial sections of a representative long term surviving cardiac allograft in a wild-type recipient. IL-2 (Fig. 1d) was strongly expressed in isolated clusters of small mononuclear cells. IL-4 staining was present in areas of focal infiltrate with diffuse positivity. Clear staining of IL-4 could be seen around individual cells in parenchymal regions in hearts from wild-type recipients (Fig. 1e). No IL-4 staining above background (Fig. 1c) levels was seen in hearts from the IL-4^{-/-} recipients. IFN-γ was also seen associated with clusters of small and large mononuclear cells in perivascular areas, with some mononuclear cells being strongly

positive in hearts from both IL-4^{-/-} and wild-type recipients (Fig. 1f). Hence, clear positive cytokine expression was seen in allografts from both IL-4^{-/-} and wild-type recipients.

To identify and quantitate potential alterations in the alloimmune response associated with IL-4 deficiency, we used semiquantitative ³²P RT-PCR assays to measure relative differences in intragraft gene transcripts levels (*n* = 5 in each group; Fig. 2). Selected monokine expression patterns were studied, as IL-4 typically suppresses macrophage responses (36). In the transplanted hearts from IL-4^{-/-} recipients IL-12, allograft inflammatory factor-1 (AIF-1) and TNF-α transcript levels were significantly higher compared with those in wild-type controls. However, monocyte chemoattractive protein-1 and inducible nitric oxide synthase levels were not altered by the absence of recipient sources of IL-4 (Fig. 2), indicating that IL-4 may selectively regulate monokine expression.

In contrast to the monokines, the cytokine transcript levels in primary transplanted hearts from IL-4^{-/-} and wild-type recipients were not significantly different (Fig. 2). T cell activation, as measured by Th1-type cytokine transcripts and IFN-γ and IL-2 transcripts were comparable in both groups. Transcripts for IL-4 were not above background levels in IL-4^{-/-} recipients, as would be expected in mice with a IL-4 gene deletion. The Th2-type cytokine, IL-10, produced by both T cells and monocytes, was not significantly altered in IL-4^{-/-} recipients compared with wild-type recipients. Detection of cytokines by immunoperoxidase staining confirmed the presence or the absence of the protein implied by detection of mRNA in the RT-PCR results. Taken together, these results imply that allografts from IL-4^{-/-} recipients show decreased macrophage suppression, but have T cell activation levels comparable to those in wild-type recipients.

Vascular characteristics of cardiac allografts

Elastin staining of long term surviving cardiac allografts from IL-4^{-/-} and wild-type recipients showed diffuse, prominent, obliterative vascular lesions. There was concentric vascular thickening diffusely involving both small and large vessels. The expanded neointima involved interposition of mononuclear cells and occasional myointimal cells (Fig. 3A). Quantitative comparisons were made by tabulating the severity of vascular thickening (Fig. 3B). Computer-assisted morphometric analysis of 329 elastin-positive vessels showed that the mean percent luminal occlusion was 63.7 ± 16.9% from grafts in IL-4^{-/-} recipients (*n* = 5) compared with 69.5 ± 17.6% in wild-type recipients (*n* = 5, *p* = NS). As expected, IL-4^{-/-} isografts had significantly less luminal occlusion (8.7 ± 5.7%, *n* = 6, *p* < 0.0001 compared with both allograft groups). The frequency of the disease measured as the percentage of diseased vessels (luminal occlusion, >0%) in the grafts from IL-4^{-/-} recipients was 92.3 ± 12.5% (*n* = 56 vessel cross-sections) and thus comparable to 99.2 ± 1.9% (*n* = 63) in wild-type recipients (*p* = NS). In isografts, only 24.5 ± 7.5% of the vessels

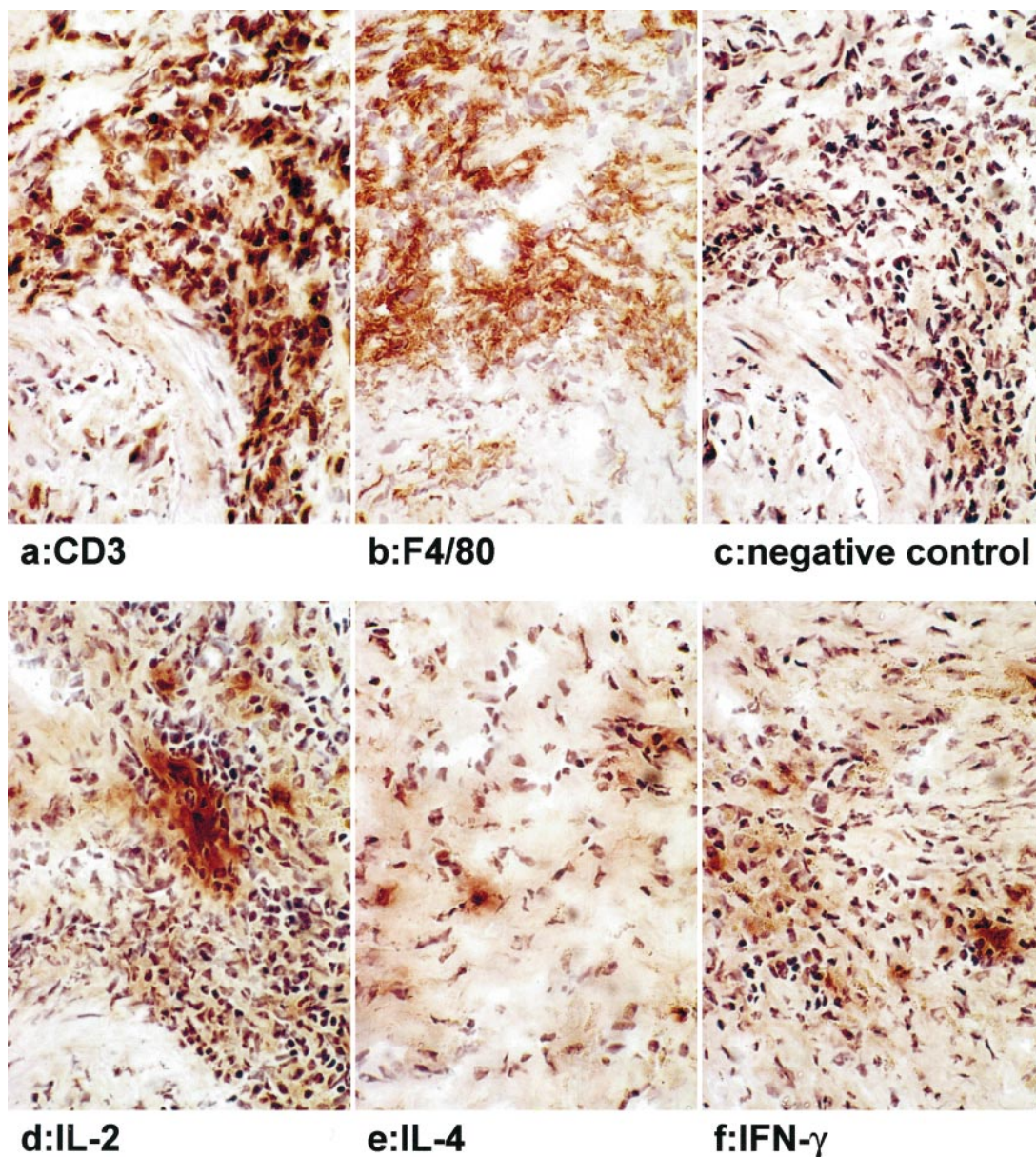


FIGURE 1. Representative immunostaining from frozen sections of an allograft placed in a wild-type recipient. Staining of allografts from other wild-type and IL-4^{-/-} recipients showed the same pattern with an exception of IL-4 staining, which was absent in the allografts from IL-4^{-/-} recipients. Panels *a* through *d* show serial sections of a perivascular region; *e* and *f* are adjacent areas of parenchymal tissue. *a* shows infiltrating CD3-positive cells around an occluded vessel, *b* shows extensive F4/80 positivity around the vessel, *c* is the negative control, *d* shows a cluster of IL-2-positive cells among the infiltrating cells, *e* shows a few IL-4-positive cells in a graft from a wild-type recipient, *f* shows some IFN- γ -positive cells in parenchymal tissue. Original magnification, $\times 400$.

($n = 68$) were affected by minor amounts of thickening ($p < 0.0001$ compared with both allograft groups).

Evaluation of the second hearts

We evaluated the histologic status of the second hearts used to assess donor Ag-specific tolerance. These were transplanted 80 days after the first transplant without further immunosuppression given to the recipients. These second donor-strain hearts harvested >30 days after challenge showed histologic features of parenchymal rejection similar to those in the primary hearts harvested at 110 days (data not shown). The extensive T cell and macrophage infiltrate seen in these well-beating grafts was equivalent to that seen at >110 days in primary cardiac allografts (Table II). Second donor-strain hearts showed similar RT-PCR patterns in both IL-4^{-/-} and wild-type recipients (Table III).

There was prominent vascular thickening in second BALB/c (donor-strain) heart allografts from IL-4^{-/-} and wild-type recipients (Fig. 4). The severity of vascular thickening measured as the mean percent luminal occlusion was $63.3 \pm 17.2\%$ in second grafts ($n = 3$, 45 vessels) placed in IL-4^{-/-} recipients compared with $48.2 \pm 18.1\%$ in second grafts ($n = 3$, 55 vessels) placed in wild-type recipients ($p = ns$). The degree of vascular occlusion was similar to that in the primary heart grafts that had been in place for >110 days. This shows an accelerated alloimmune response to the second donor-specific challenge hearts despite continued graft function. Hence, in this study the recipient state of experimental tolerance did not completely protect the second donor-strain grafts from cardiac rejection given that there were features of acute and chronic rejection by histologic assessment.

Table II. Cell surface marker expression² in BALB/c allografts from wild-type or IL-4^{-/-} mice^a

Recipient	Heart/Day of Harvest	n	CD3	B220	F4/80	IL-2R
Wild type	Rejecting First heart <80 days	3	21.6 ± 6.6 ^b	1.6 ± 3	18.2 ± 15	1.4 ± 1.4
IL-4 ^{-/-}	Rejecting First heart <80 days	4	28.2 ± 9 ^{b,c}	2.2 ± 1.3	17 ± 5.5	1.5 ± 0.5
Wild type	Long term surviving First heart >100 days	5	16 ± 3.4	2.5 ± 2.8	10.6 ± 3.3	2.2 ± 1.9
IL-4 ^{-/-}	Long term surviving First heart >100 days	5	12.6 ± 0.5 ^b	3.3 ± 1.4	6 ± 2	1.7 ± 0.6
Wild type	Second heart, >30 days	3	14.2 ± 5.0	2.3 ± 2.0	11.3 ± 6	2.7 ± 2.0
IL-4 ^{-/-}	Second heart, >30 days	3	15.0 ± 1.6	3.9 ± 0.8	7.2 ± 3.6	2.6 ± 1.3
IL-4 ^{-/-}	isografts >100 days	6	2.3 ± 1.5	0	0.4 ± 0.1	0.7 ± 1.2

^a Data are expressed as mean number of cells ± SD/high powered field (×400) based on analysis of >10 fields/graft.

^b Significantly different to isografts, $p < 0.0024$ by multiple analysis of variance and Bonferroni correction.

^c Significantly different to grafts from IL-4^{-/-}-tolerant animals, $p < 0.0024$.

Second third-party (CBA) hearts placed in IL-4^{-/-} ($n = 2$) or wild-type ($n = 2$) recipients showed typical features of acute cardiac rejection, with multiple inflammatory cell foci, myocyte necrosis, vasculitis, and deposition of fibrin. Vessels ($n = 42$) in these grafts did not develop transplant arteriosclerosis characterized by myointimal thickening (data not shown). However, the onset of acute rejection appeared to be delayed in these grafts (11–18 days after transplantation) compared with those in untreated IL-4^{-/-} and wild-type mice (8–9 days). More detailed studies with harvest time- and T cell immunosuppression-matched controls are required to evaluate the impact of tolerance on second hearts.

Discussion

Our studies are the first to use genetically altered mice to manipulate levels of IL-4 for direct assessment of long term graft outcomes. The advantage of this approach is that targeted gene deletion produces a complete and sustained deficiency of recipient sources of IL-4 for the long period of time required for tolerance and chronic rejection studies. Three novel findings arose from

these studies, which focused on the subset of grafts that survived long term. First, there were no differences in the indices of tolerance between recipients with or without IL-4 deletion. Vascularized BALB/c cardiac allografts survived long term and accepted second BALB/c hearts, but rejected CBA hearts. Secondly, an inflammatory response, as determined by histologic criteria, develops in allografts even though recipient IL-4 is absent. Third, prominent vascular thickening develops in cardiac allografts from IL-4^{-/-} recipients. This shows that IL-4 is not required for the development of graft vascular disease, a hallmark of chronic rejection. Together, these findings argue against the hypothesis that IL-4-driven responses are primarily responsible for mediating either experimental tolerance or chronic rejection. Instead, alternative or compensatory cytokines are likely to contribute to these aspects of the response to allografts.

Our finding that IL-4 does not have an essential role in the development of vascular thickening in this model adds to a growing body of evidence from the studies examining the functional role of IL-4 in transplants. Others have demonstrated, and we have confirmed, that IL-4 is not required for acute cardiac, skin, or

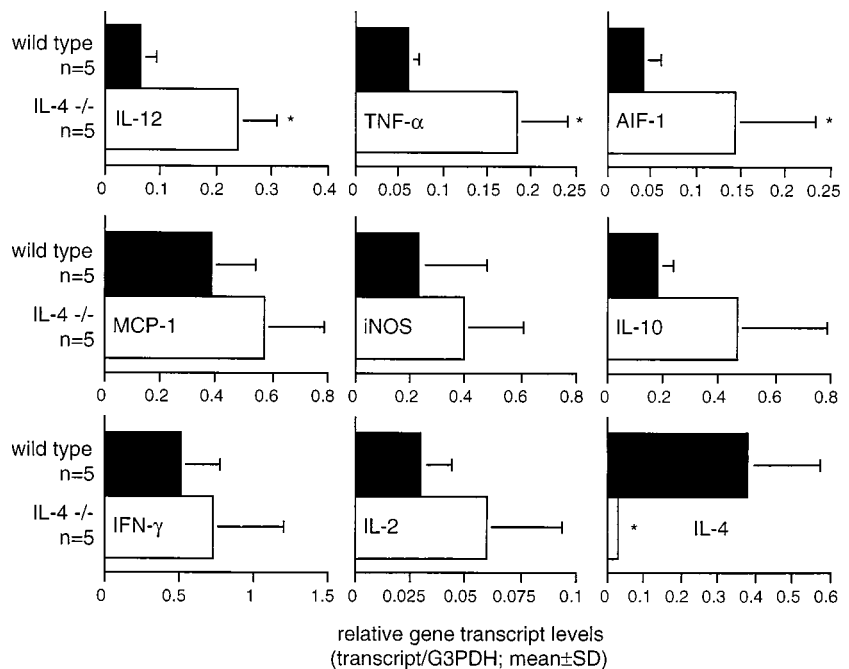
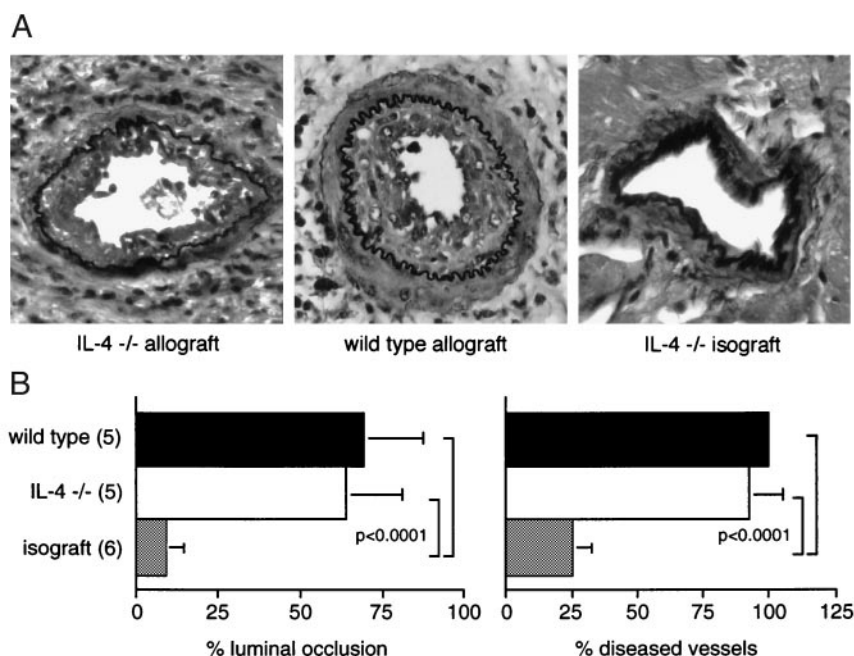


FIGURE 2. Corrected transcript levels for macrophage activation markers and T cell makers. Significant (*) increases in mean transcript levels for IL-12, TNF- α , and AIF-1 were found in the group of allografts from IL-4^{-/-} mice compared with wild-type recipients. T cell activation markers, IL-2, IFN- γ , and IL-10, were not significantly different. Each bar represents the mean ± SD of all grafts in each subgroup.

FIGURE 3. A, Vessel morphology in allografts from IL-4^{-/-} and wild-type recipients and in IL-4^{-/-} isografts. Representative vessels showing prominent vascular thickening in allografts from IL-4^{-/-} (left) and wild-type (middle) recipients with no intimal thickening in isografts (right). Verhoeff's elastin staining; original magnification, ×400. B, Quantitative analysis of the severity and frequency of vascular thickening in primary BALB/c hearts. No significant differences in the percent luminal occlusion was seen in allografts from IL-4^{-/-} and wild-type recipients, whereas isografts had significantly lower levels than allografts. Each bar represents the mean ± SD of all grafts in each group. The number of animals per group is given in parentheses.



pancreatic islet rejection in nonimmunosuppressed recipients (22, 23, 37). Studies by Lakkis et al. demonstrated that CTLA4 Ig prolonged cardiac graft survival to a similar extent in wild-type and IL-4^{-/-} recipients. Tolerance tests employing challenge with second graft donor tissue were not performed, but the findings did indicate that IL-4 was not obligatory for long term graft acceptance (23, 37). This is similar to the results of our previous study, using a 30-day course of immunosuppression, showing that allografts placed in IL-4^{-/-} recipients had graft survival equal to that in wild-type animals (24). In the same study we demonstrated that gene deletion of IFN- γ or IL-10 in the recipient environment was associated with decreased allograft survival. Hence, in all three processes (long term graft survival, acute rejection, and chronic rejection), removal of functional IL-4 did not inhibit the cellular processes leading to these disorders, suggesting that IL-4 is neither necessary nor sufficient to sustain these responses.

Our findings support the idea that IL-4^{-/-} mice have a selective defect in Th2 responses as proposed by Kopf and coworkers in their analysis of IL-4 knockout mice (20). They found that Th2 responses in vitro to anti-CD3-mediated activation were impaired, but delayed-type hypersensitivity responses were intact. Interest-

ingly, IL-4^{-/-} mice are resistant to killing or abscess formation from *Salmonella typhimurium*, showing that IL-4 may actually promote instead of protecting against the pathologic process to this insult (38).

Factors other than IL-4 are likely to play a role in allograft acceptance and development of vascular thickening. We have examined only IL-4-mediated responses, but other inflammatory mediators alone or in combination may contribute to allograft acceptance or chronic rejection. One major activity associated with the Th2-type response is deactivation or suppression of macrophage responses. To screen for possible compensatory factors, we used RT-PCR assays to compare relative transcript levels in a series of graft cDNAs from each tolerant recipient group. Our evaluation of selected macrophage factors showed differences in allografts from IL-4^{-/-} and wild-type recipients, indicating that macrophage suppression was altered by IL-4 deficiency. We found an increase in monokines (IL-12, AIF-1, and TNF- α) in IL-4^{-/-} recipients compared with wild-type animals. However, transcript levels for monocyte chemoattractant protein-1 and inducible nitric oxide synthase were similar in IL-4^{-/-} and wild-type recipients. These findings suggest that the lack of IL-4 interrupted only some of the macrophage suppression pathways. It is possible that these or other macrophage factors may have contributed to vascular thickening in the absence of IL-4.

Interestingly, we did not see an increase in other T cell cytokines, i.e., IL-2, IFN- γ , and IL-10, in primary heart allografts from IL-4^{-/-} recipients compared with that in wild-type animals. This was concordant with the study by Lakkis et al. (23) in which long surviving hearts from IL-4^{-/-} and wild-type mice treated with CTLA4-Ig did not have significant differences in transcripts for IL-2, IL-10, IL-13, or IFN- γ . These findings are inconsistent with the hypothesis that IL-4 inhibits Th1-like responses. However, it is possible that other factors have taken the suppressive role of IL-4 in the knockout animals. Taken together, the search for compensatory factors will ultimately depend upon establishing causal linkages demonstrating that putative factors control the processes of tolerance and/or transplant arteriosclerosis. This will require more functional studies demonstrating that the absence of a factor interrupts the process and that its presence promotes it.

Table III. Gene transcript levels in second allografts from wild-type and IL-4^{-/-} recipients^a

Gene Transcript	Wild Type (n = 3)	IL-4 ^{-/-} (n = 3)	p Value
IL-12	0.261 ± 0.052	0.354 ± 0.064	0.1252
IFN- γ	0.676 ± 0.023	0.882 ± 0.274	0.2645
IL-2	0.097 ± 0.050	0.066 ± 0.016	0.3521
IL-4	0.244 ± 0.038	0.043 ± 0.017	0.0011 ^b
IL-10	0.473 ± 0.202	0.404 ± 0.251	0.7308
TNF- α	0.176 ± 0.064	0.206 ± 0.057	0.5743
AIF-1	0.098 ± 0.005	0.143 ± 0.072	0.3399
iNOS	0.514 ± 0.223	0.424 ± 0.179	0.6122
MCP-1	0.512 ± 0.207	0.624 ± 0.311	0.6289

^a Data are expressed as mean relative transcript level ± SD (gene transcript level/G3PDH transcript level) based on analysis of triplicate cDNA/graft. Three grafts were analyzed in both groups.

^b Difference statistically significant by multiple analysis of variance and Bonferroni correction.

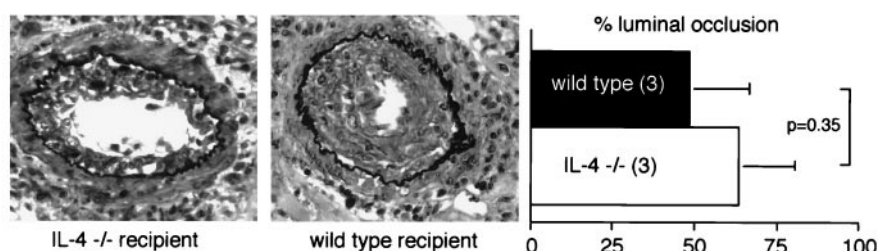


FIGURE 4. Vascular analysis of secondary heart allografts from challenged recipients. Donor-strain allografts developed prominent vascular thickening in both IL-4^{-/-} (left panel) and wild-type (middle panel) recipients within 30 days after transplantation. Quantitative morphometric analysis (right panel) showed no significant difference in the percent luminal occlusion in vessels from allografts in IL-4^{-/-} and wild-type recipients. Each bar represents the mean \pm SD from all grafts in each recipient group.

Descriptive studies first implicated IL-4 as a key mediator for tolerance based on the presence of IL-4 gene transcripts, protein product, or both in long surviving allografts from tolerant rats and mice (4, 6, 10). IL-4 expression was also increased in cardiac allografts undergoing chronic rejection, and the levels were reduced in concert with arteriosclerosis by various immunosuppressive strategies (13, 19). Our studies using IL-4^{-/-} recipients have brought new important functional data to this area in the transplant field.

In this study, experimental induction of peripheral tolerance, defined as long term function of a primary allograft with acceptance of a secondary donor allograft and rejection of a third party graft (34, 35) could be achieved in >50% of IL-4^{-/-} and wild-type recipients after a 30-day course of immunosuppression. Despite continued graft function, both primary and second hearts showed evidence of ongoing rejection: mononuclear infiltration, inflammatory activation, and vascular thickening. Others have also shown that rat kidney grafts can develop primary graft destruction and chronic rejection and yet fulfill the conventional definition of peripheral tolerance by accepting donor second grafts and rejecting third party grafts (39).

These findings raise an interesting question: have these recipients achieved a clinically applicable level of tolerance? We are of the opinion that the presence of active rejection is incompatible with the tolerant state. In addition to acceptance of a second donor graft and rejection of a third party graft, the parenchymal and vascular tissue should be substantially free of histologic signs of acute and chronic rejection. Hence, the definition of tolerance in experimental model systems should be redefined. As we and others using peripheral tolerance models have not typically achieved this degree of peripheral tolerance, the question of whether true peripheral tolerance can prevent the development of chronic rejection is still unanswered.

Gene manipulation of IL-4 (deletion in our study or addition in others (40)) has failed to modify rejection responses. Along these lines, Davies et al. reported that anti-IL-4 mAb treatment could not break established peripheral tolerance in vivo. In studies of the transfer of infectious tolerance, only partial inhibition of transferable cell activity was seen. They concluded that IL-4 played a role in tolerance, but the tolerant state was not maintained by the continuous systemic presence of IL-4 (41). Thus, it appears that IL-4 is not the sole mediator of peripheral tolerance.

In conclusion, long term graft survival can be induced in MHC class I- and II-mismatched cardiac allograft recipients regardless of recipient IL-4 deficiency. However, histologic analysis showed that true peripheral tolerance was not achieved despite preserved graft function. Although similar patterns were seen in histology and immunohistochemistry among allografts from IL-4^{-/-} and wild-type recipients, the increased sensitivity of the RT-PCR anal-

ysis of heart transplants revealed impaired macrophage suppression in IL-4^{-/-} recipients compared with that in wild-type recipients. These or other potential pathways may have contributed to the ongoing alloimmune response observed in these long term surviving heart transplants. In addition, even though the recipient environment was devoid of IL-4, cardiac allografts developed prominent vascular thickening associated with chronic rejection. Further functional studies need to be completed to define the cytokines alone or in combination that mediate tolerance and chronic rejection.

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