Leukocyte-Suppressing Influences of Interleukin (IL)-10 in Cardiac Allografts

Insights from IL-10 Knockout Mice

Anne Räisänen-Sokolowski,*
Troels Glysing-Jensen,* and Mary E. Russell*†

From the Cardiovascular Biology Laboratory,* Harvard School of Public Health, and Brigham and Women's Hospital,† Harvard Medical School, Boston, Massachusetts

To investigate the role of interleukin (IL)-10 in late graft outcomes, we compared BALB/c donor hearts transplanted into immunosuppressed wild-type or IL-10 gene-deficient (–/–) C57BL recipients ($n = 49$) at 50 ± 5 days. There was prominent leukocyte infiltration and parenchymal destruction with more severe vascular occlusion in grafts from IL-10 –/– recipients. An occlusive CD45⁺ arteritis with medial necrosis occurred with IL-10 deficiency instead of the α -smooth muscle actin-rich arteriosclerosis seen in wild-type recipients. Increased interferon (IFN)- γ as well as Mac-1, inducible nitric oxide synthase, and allograft inflammatory factor-1 (but not CD3 and IL-4) transcript levels were seen in allografts from IL-10 –/– recipients as assessed by ³²P reverse transcription polymerase chain reaction. We then evaluated the contribution of IFN- γ -mediated responses by neutralizing IFN- γ . Anti-IFN- γ monoclonal antibody (MAb) treatment of IL-10 –/– recipients did not improve graft survival, parenchymal rejection, or occlusive arteritis, indicating that these processes are IFN- γ independent. However, medial smooth muscle cell loss in IL-10 –/– recipients was attenuated by anti-IFN- γ MAb. Hence, in this transplant model, IL-10 suppresses T cell and macrophage responses in the parenchyma and vasculature and confers a protective effect against late rejection. (*Am J Pathol* 1998, 153:1491–1500)

Interleukin (IL)-10 is a pleiotrophic cytokine that can be produced by T cells, B cells, macrophages, mast cells, and keratinocytes.¹ Reports of immunoregulatory properties of IL-10 include down-regulation of Th1-type cytokine production, suppression of macrophage and natural killer cell effector functions, and stimulation of B cell differentiation and immunoglobulin production.¹ The role of IL-10

in transplantation has been widely debated with contrasting results among the different experimental systems studied.² Elevated IL-10 expression in human and rodent allografts undergoing acute rejection suggested that IL-10 promotes alloimmune destruction.² However, up-regulated expression of IL-10 in allografts from tolerant, long-surviving recipients had led some to speculate that IL-10 may promote allograft survival.²

Studies where IL-10 levels have been manipulated in transplantation models have not resolved the confusion. Systemic administration of IL-10-Fc fusion protein accelerated graft failure in islet cell allografts,³ whereas pancreatic islet grafts overexpressing murine IL-10 had similar survival time to wild-type allografts.⁴ Retrovirus-mediated transfer of viral IL-10 gene into nonvascularized neonatal heart transplants prolonged graft survival.⁵ However, in that study, murine IL-10 had no survival benefit.⁵ The effect of systemic recombinant human IL-10 in a mouse heart transplant model appeared to depend on dosing and timing.⁶ Daily injection of a high dose (100 μ g/day) initiated on day 1 before the grafting shortened graft survival, whereas a lower dose (50 μ g/day) did not alter it. If IL-10 was given only perioperatively (days –1, 0, +1; 50 μ g/day), graft survival was improved. Hence, the efficacy of IL-10 manipulations (methods, doses, and timing) coupled with differences in transplant microenvironment may explain the inconsistent effects seen in graft survival to date.

By using mice with targeted gene deletion as recipients, we have recently shown that the presence of IL-10 is protective in a heterotopic cardiac mouse transplant model of late or attenuated rejection.⁷ After a 30-day course of T-cell-depleting immunosuppression, IL-10 –/– recipients rejected heterotopic mouse cardiac allografts twice as rapidly as wild-type controls.⁷ Grafts from IL-10 –/– recipients had prominent mononuclear cell infiltration, myocyte loss, and fibrosis. Hence, this earlier

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Address reprint requests to Dr. Mary E. Russell, Cardiovascular Biology Laboratory, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115. E-mail: russell@cviab.harvard.edu.

study demonstrated that when present, IL-10 had a suppressive influence on the alloimmune response that culminates in graft failure.

A number of mechanisms might be invoked to explain our findings. The original reports describing the phenotype of IL-10 $-/-$ mice indicated augmented cell-mediated immune responses consistent with loss of suppressing influences.⁸ Although the IL-10 knockout mice appeared normal at birth, a chronic inflammatory bowel disease developed with age (especially if maintained in conventional animal facilities).⁹ The chronic enterocolitis involved large numbers of infiltrating mononuclear cells, including macrophages and Th1-type T cells in the bowel. This indicated that IL-10-deficient mice had an aggravated leukocyte response to gut flora present in the conventional facility.⁸

Since then, IL-10 $-/-$ mice have been studied after other microbial challenges. Allergic bronchopulmonary aspergillosis, enterocolitis, and *Toxoplasma gondii* and *Trypanosoma cruzi* infections showed increased mortality and morbidity in IL-10 $-/-$ mice, whereas *Listeria monocytogenes*-infected mice were resistant to infection.¹⁰⁻¹⁵ The altered immune response was typically associated with increased production of pro-inflammatory cytokines, such as interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and IL-12. Findings in these infection models suggested that aggravated Th1-type responses develop when leukocyte-suppressive effects of IL-10 are lost. This could convert protective immunity to a pathological response that ultimately leads to tissue destruction.⁹

Less is known about the molecular mechanism(s) through which IL-10 alters the alloimmune response. Intragraft cytokine analysis of hearts placed in IL-10 $-/-$ recipients in our earlier study showed that early graft failure was associated with increased expression of IFN- γ .⁷ We have also recently shown that IFN- γ has a role in promoting graft arteriosclerosis.¹⁶ In cardiac allografts placed in IFN- γ $-/-$ recipients receiving T-cell-depleting immunosuppression, severity and frequency of vascular occlusion were significantly reduced as well as myointimal smooth muscle cell accumulation. Taken together, these findings suggest that augmented or accelerated Th1-type responses and interrelated macrophage activation might also promote graft loss in the setting of recipient IL-10 gene deletion.

To gain more insight about how IL-10 protects the graft from rejection, we compared graft outcomes in IL-10 $-/-$ recipients with wild-type recipients. Because graft failure was evident in IL-10 $-/-$ recipients at 55 days in our earlier study,⁷ we harvested grafts from wild-type recipients at this time point to have a time-matched control group and analyzed vascular as well as parenchymal features. Second, we addressed the contribution of IFN- γ -mediated Th1 responses to cardiac rejection in IL-10 $-/-$ recipients. Our approach was to determine whether neutralization of IFN- γ with anti-IFN- γ monoclonal antibody (MAb) treatment in IL-10 $-/-$ recipients would alter graft outcomes (survival, vascular and parenchymal histology, and inflammatory cell activation).

Materials and Methods

Murine Cardiac Transplantation

BALB/cByJ (H-2^d) donors were used for heterotopic cardiac transplantation¹⁷ into 6- to 8-week-old C57BL (H-2^b) recipients that were either wild type or had targeted gene disruption in IL-10 (C57BL/10J).⁸ IL-10 $-/-$ mice had been backcrossed seven times to C57BL background. All animals were maintained in a specific-pathogen-free (SPF) animal facility and appeared healthy at the time of the transplantation and harvest. The targeted gene disruption was confirmed using triple polymerase chain reaction (PCR) assays that amplify a portion of the neomycin cassette and a portion of the targeted exon as recommended by Jackson Laboratories (www.jax.org). All mice were purchased from Jackson Laboratories (Bar Harbor, ME).

To attenuate acute rejection, we treated recipients with MAb against CD4 (clone GK1.5, rat IgG2b; American Type Culture Collection (ATCC), Rockville, MD) and CD8 (clone 2.43, rat IgG2b; ATCC) for days 1 to 4, 7, 14, 21, and 28 after transplantation at the dose of 500 μ g/day/mouse of each MAb as previously described.⁷ We have previously shown that a gradual repopulation of T cells occurs after the cessation of the a 30-day course of anti-CD4 and anti-CD8 treatment. At day 55 after transplantation, flow cytometry of splenocytes demonstrated that CD4⁺ T cells were 48% of normal level and CD8⁺ T cells were 15% of normal level.¹⁶ Hence, the animals were immunosuppressed at the time of harvest.

Graft function was evaluated by regular palpation graded on a scale from 4 (functioning well) to 0 (no heart beat).¹⁷ Graft survival was defined as days after transplantation with palpation score of ≥ 1 . Wild-type controls were harvested electively at 55 days after transplantation to serve as time-matched controls for grafts placed in IL-10 $-/-$ recipients that began to fail by this time point. In our previous study,⁷ we compared wild-type and knockout allografts at a comparable functional endpoint, ie, when graft function decreased. Two of seventeen allografts placed in IL-10 $-/-$ recipients treated with anti-IFN- γ MAb rejected at 33 days after transplantation and were not included for further histological analysis. Hearts were collected and sections of grafts processed for the evaluation of histology and RNA extraction as previously described.¹⁸

Neutralization of IFN- γ Using MAb

To determine whether IFN- γ -mediated Th1 forces contribute to the accelerated rejection in IL-10 $-/-$ recipients, a subgroup of IL-10 $-/-$ recipients received anti-IFN- γ MAb (clone R4-6A2, rat IgG1; ATCC). All ascites was purified in a protein G column as previously described¹⁸ and administered days -1 and 1 and then bi-weekly until harvest (2 mg/mouse/week, intraperitoneally).

Serum was collected from each mouse at the time of the harvest. An indirect enzyme-linked immunosorbent assay (ELISA) was used to measure anti-IFN- γ MAb (rat IgG1) levels in anti-IFN- γ MAb-treated animals. Briefly, a

96-well plate was coated with recombinant mouse IFN- γ (1.0 μ g/ml; Genzyme, Cambridge, MA) in PBS (pH 9.0) overnight at room temperature. Additional binding sites were blocked with blocking buffer (0.017 mol/L $\text{Na}_2\text{B}_4\text{O}_7$, 0.12 mol/L NaCl, 0.05% TWEEN 20, 1 mmol/L EDTA, 0.25% bovine serum albumin, 0.05% NaN_3) for 30 minutes. Anti-IFN- γ MAb standards (range, 3 to 800 ng/ml) and mouse sera samples (dilution, 1:100 to 1:30,000) in triplicate were diluted in blocking buffer, applied to the wells, and incubated overnight at room temperature. Bound primary antibody was detected using a secondary antibody rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) followed by application of avidin-biotin complex (Vector Laboratories) according to the manufacturer's instructions. Horseradish peroxidase label was detected using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (ABTS substrate kit, Vector Laboratories). Negative controls included serum from naive IL-10 $-/-$ mice and from transplanted wild-type mice that had received only anti-CD4/CD8 therapy. Final concentrations of anti-IFN- γ MAb in serum were derived from a standard curve. Anti-IFN- γ MAb concentrations in anti-IFN- γ MAb-treated IL-10 $-/-$ recipients ranged from 59 to 425 μ g/ml (mean, 198 ± 131 μ g/ml; $n = 13$) in serum at the time of harvest.

Functional Assay for Anti-IFN- γ MAb Activity in the Sera

Activation of a murine macrophage cell line (RAW264.7, TIB 71; ATCC) by IFN- γ was used to test recipient sera at the time of harvest for anti-IFN- γ activity. RAW264.7 cells become activated after IFN- γ stimulation and produce nitric oxide products (nitrates and nitrites) that can be measured using the Griess reagent after treatment with nitrate reductase (colorimetric nitric oxide assay kit, Oxford Biomedical Research, Oxford, MI). All samples and control MABs were assayed in triplicate, and the mean was derived. Figure 1 depicts how serum (1:10 dilution) from anti-IFN- γ MAb-treated IL-10 $-/-$ recipients inhibited IFN- γ -mediated NO production in a dilution-dependent fashion to 15% of the control (recombinant IFN- γ -stimulated (100 U/ml) with no serum) level. Serum from nontransplanted IL-10 $-/-$ animals and irrelevant MAB (anti-ICAM-1, clone YN1/1.7.4, rat IgG2a; ATCC) did not reduce nitric oxide products. These findings confirm the neutralizing capacity of anti-IFN- γ MAB in the recipient at time of harvest.

Histological Analysis

The degree of acute rejection was evaluated and graded from paraffin sections using a modification of the International Society for Heart and Lung Transplantation (ISHLT) criteria.^{7,19} Coded samples were evaluated by two observers. The scale was from 0 to 4: 0, no rejection; 1, mild focal (A) or diffuse (B) perivascular and interstitial infiltration with no parenchymal necrosis; 2, moderate, unifocal infiltration with/without focal myocyte injury; 3, moderate multifocal (A) or diffuse (B) infiltration with myo-

Figure 1. Murine macrophage cells (RAW264.7) were stimulated with 100 U/ml recombinant IFN- γ (white column) to produce nitric oxide products measured by a modified Griess reaction. Nitrate/nitrite production was inhibited by serum from anti-IFN- γ MAb-treated animals (black columns) compared with high levels seen with control sera or irrelevant Ab (gray column). Data are shown as means of triplicates.

cyte injury; 4, severe rejection with aggressive, diffuse infiltration, edema, myocyte necrosis, hemorrhage, and vasculitis.

Immunostaining

CD45 MAB (clone 30F11.1, leukocyte common antigen (LCA), Ly-5; PharMingen, San Diego, CA) was used to identify leukocytes within the grafts as previously described.²⁰ Smooth muscle cell (SMC) accumulation, indicative of more advanced arteriosclerotic stages, was estimated by immunostaining for α -smooth muscle actin and desmin. Twenty-six grafts were stained using the previously described protocol with minor modifications.¹⁶ Briefly, Verhoeff's elastin-stained paraffin sections were blocked with 10% normal goat sera and then stained with α -smooth muscle actin antibody (clone 1A4, dilution 1:20,000; Sigma Chemical Co., St. Louis, MO) overnight at 4°C and detected using the avidin-biotin complex and 3-amino-9-ethylcarbazole substrate (Vectastain ABC kit, AEC-kit; Vector Laboratories) as described previously.¹⁶ Anti-desmin immunostaining was also performed on selected samples ($n = 3$) to confirm the presence or absence of neointimal and medial SMCs according to the manufacturer's instructions. Anti-human desmin MAB coupled with horseradish peroxidase (clone D33; Dako, Glostrup, Denmark) reacts with 53-kd intermediate filament protein in muscle cells. The reagent labels both

striated (skeletal and cardiac) cells and SMCs and shows a broad interspecies cross-reactivity.

Quantitation and Characterization of Vascular Thickening

The severity of disease (percentage of luminal occlusion) was analyzed in Verhoeff's elastin-stained sections from each graft.^{18,21} Microscopic images of each elastin-stained vessel cross section ($n = 692$) were captured, and the percentage of luminal occlusion was tabulated by tracing the internal elastic lamina and the lumen with the ScionImage 1.60 software (National Institutes of Health, Bethesda, MD). The mean value for each individual graft was tabulated, and the mean $\pm$ SE for each group was reported.

Image analysis using ScionImage 1.60 (National Institutes of Health) was performed to measure percent area of α -smooth muscle actin positivity within the neointima and media.¹⁶ Only larger vessels (area delineated by internal elastic lamina $>350 \mu\text{m}^2$) with greater than 40% luminal occlusion were of sufficient resolution for measurement. The area staining for α -smooth muscle actin was determined by measuring the pixel area displaying the color intensity of immunopositive cells.

Reverse Transcriptase PCR

Reverse transcriptase (RT)-PCR provides a global measurement of inflammatory markers in a more quantitative manner than immunostaining of individual microscopic sections. To estimate the contribution of T cells and macrophages, we elected to measure corrected CD3 and Mac-1 transcript levels because the low resolution of immunostaining in frozen sections often precludes quantitation. Hence, to measure relative differences in transcript levels between cardiac transplants we used a semiquantitative ³²P-RT-PCR technique published in detail previously.^{18,21,22} Total RNA from 20 grafts was quantitated and reverse transcribed to cDNA simultaneously to generate a cDNA panel that allowed comparison among different groups. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a reference gene to normalize variations in cDNA or total RNA loading between samples. Transcript analysis was completed for CD3, Mac-1, IFN- γ , IL-4, TNF- α , inducible nitric oxide synthase (iNOS), and allograft inflammatory factor (AIF)-1. Primer sequences and PCR conditions were previously described^{7,18} except for the following: G3PDH, sense primer 5'-CAT CAA GAA GGT GGT GAA GCA GGC-3' and antisense primer 5'-TTG TGA GGG AGA TGC TCA GTG TTG G-3', with an annealing temperature of 58°C for 22 cycles; Mac-1, sense primer 5'-CAG AGG CTG TGA ATA TGT CCT TGG-3' and antisense primer 5'-GTC ATT GAA GGT GAA GTG AAT CCG-3', with an annealing temperature of 53°C for 28 cycles; AIF-1, sense primer 5'-GAC AGA CTG CCA GCC TAA GAC ACC-3' and antisense primer 5'-CCA AGT TTC TCC TGC AGC ATT CGC-3', with an annealing temperature of 60°C for 28 cycles; and macrophage lectin, sense primer 5'-

TCA AGA ACA ACG GCT CGG AAG TG-3' and antisense primer 5'-GAC ATC ATC ATT CCA GGG ACC ACC-3', with an annealing temperature of 57°C for 30 cycles. The mean was obtained from triplicate analyses of the same cDNA. Corrected values were derived by dividing the measured incorporated ³²P for the transcript of interest by the mean G3PDH value for the cDNA. Means $\pm$ SEM per group are reported.

Statistical Analysis

The product limit (Kaplan-Meier) estimate of the cumulative survival was assessed with the Breslow-Gehan-Wilcoxon test to evaluate significant differences in graft survival.²³ The histological parameters and RT-PCR data were subjected to multiple analysis of variance without replication (StatView 4.5, Abacus Concepts, Berkeley, CA). If 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.^{24,25}

Results

Accelerated Parenchymal and Vascular Rejection in IL-10 $-/-$ Recipients

We had shown previously that grafts in immunosuppressed IL-10 $-/-$ recipients had shorter survival times compared with wild-type recipients (55 days *versus* 99 days).⁷ To extend our earlier findings, we assessed the histological outcomes of graft rejection in transplanted hearts from IL-10-deficient recipients ($n = 12$) and hearts from the time-matched wild-type control group ($n = 20$) harvested at 55 days after transplantation. Cardiac allografts from IL-10 $-/-$ recipients developed severe cellular rejection with infiltrating CD45-immunopositive cells, interstitial widening, and patchy myocyte necrosis (Figure 2) indicative of late acute rejection. Sections from allografts in wild-type recipients at 55 days had focal patches of infiltrating mononuclear cells and large regions of preserved myocardial architecture with infrequent patches of myocyte fibrosis.

Quantitation of graft rejection using modified ISHLT grading¹⁹ ranging from 0 (normal) to 4 (severe) showed that IL-10 $-/-$ recipients had significantly higher mean rejection scores compared with time-matched wild-type controls (Figure 2). Heart transplants from IL-10 $-/-$ recipients had mean scores of 3.6 ± 0.1 ($n = 12$) compared with 2.0 ± 0.1 ($n = 20$) in wild-type controls ($P < 0.0001$). Thus, deficiency of IL-10 in the recipient environment accelerated parenchymal rejection compared with wild-type controls.

Prominent Luminal Occlusion Develops in IL-10 $-/-$ Recipients

To determine whether IL-10 also affected the graft vasculature, we analyzed and quantitated severity of vascu-

Figure 2. Cardiac allografts from IL-10 $-/-$ recipients had severe parenchymal rejection, including infiltrating leukocytes (immunopositive for CD45; arrowheads), interstitial widening, and myocyte destruction. The transplants from wild-type recipients had preserved myocardial structure with only focal inflammatory cell infiltrates (arrowheads). Immunostaining for CD45 with hematoxylin counterstaining; original magnification, $\times 125$. Rejection scoring with modified ISHLT index demonstrated significantly more severe parenchymal rejection in IL-10 $-/-$ group compared with wild-type group ($*P < 0.0001$). Number of animals: wild type ($n = 20$), IL-10 $-/-$ ($n = 12$). Data are given as means $\pm$ SEM.

lar thickening. As shown in Figure 3, allografts from IL-10 $-/-$ recipients had severe inflammatory cell infiltration around all graft vessels with prominent mononuclear cell accumulation within the expanded neointima. In contrast, in allografts from wild-type recipients, the lumen was occluded by dense fibrotic intimal thickening. Image analysis of all elastin-stained vessel cross sections ($n = 692$) was completed to derive mean percent luminal occlusion, an indicator of severity of vascular thickening. Transplants from IL-10 $-/-$ recipients had higher mean luminal occlusion, $69 \pm 4\%$ ($n = 12$ animals; $n = 165$ vessel cross sections) compared with $41 \pm 4\%$ in wild-type recipients ($n = 20$ and $n = 313$, respectively; $P = 0.0001$; Figure 3).

Anti-IFN- γ MAb Treatment Selectively Alters Vascular Outcomes without Changing Survival

To determine whether augmented IFN- γ -mediated Th1-type responses contributed to accelerated graft failure in the IL-10 $-/-$ recipients, we neutralized IFN- γ by administration of anti-IFN- γ MAb. In the IL-10 $-/-$ recipients

($n = 17$) receiving anti-IFN- γ MAb, mean heart transplant survival time was 51 ± 2 days, comparable to that in IL-10 $-/-$ recipients that did not receive anti-IFN- γ .⁷ Hence, inhibition of IFN- γ did not prolong graft survival. Second, microscopic analysis of grafts that survived 50 ± 5 days ($n = 15$) showed that neutralization of IFN- γ did not alter the histological outcome of transplants from IL-10 $-/-$ recipients. Transplanted hearts had severe late acute rejection depicted by mononuclear cell infiltration, interstitial widening, and tissue destruction even after anti-IFN- γ MAb treatment. Mean ISHLT scores were 3.3 ± 0.3 ($n = 15$) in MAb-treated IL-10 $-/-$ mice compared with 3.6 ± 0.1 ($n = 12$; $P = 0.2986$) in IL-10 $-/-$ mice without anti-IFN- γ MAb. Hence, cardiac transplants from IL-10 $-/-$ mice develop accelerated late acute rejection independent of IFN- γ . Furthermore, anti-IFN- γ MAb treatment in the IL-10 $-/-$ recipient subgroup did not change the degree of luminal occlusion ($64 \pm 6\%$; $n = 15$ animals and $n = 214$ vessel cross sections). Hence, the absence of IL-10 in the recipient environment was associated with pronounced luminal occlusion that was not reduced with anti-IFN- γ MAb treatment.

Figure 3. Photomicrograph of allograft from wild-type (WT) recipient (left panel) depicts fibrotic luminal occlusion demarcated by internal elastic lamina. There are few infiltrating inflammatory cells in perivascular area. In contrast, in allograft from IL-10 $-/-$ recipient (middle panel), luminal occlusion is composed of abundant mononuclear inflammatory cells, and perivascular area has numerous inflammatory cells. Verhoeff's elastin staining; original magnification, $\times 125$. Quantitative image analysis was used to evaluate percent luminal occlusion in graft vessels (right panel). Transplants from IL-10 $-/-$ had significantly more severe percent luminal occlusion (mean $\pm$ SEM) than wild-type controls at 55 days after transplantation.

Figure 4. Immunostaining was used to characterize cell composition in the allograft vasculature with prominent thickening. Leukocytes (CD45 staining, red-brownish color, upper row) were seen throughout the expanded neointima in all transplant groups, but they were the dominant cell type in IL-10 $-/-$ recipients (with or without anti-IFN- γ MAb). In contrast, the major cell type in vessels from wild-type recipients was α -smooth muscle actin positive (SMC; middle row), indicating arteriosclerotic intimal thickening in these allografts (asterisk). In contrast, grafts from IL-10 $-/-$ recipients had few SMCs in the neointima (asterisks). The leukocyte accumulation in these grafts represents vascular occlusion due to an arteritis. There was substantial α -smooth muscle actin loss in the media of graft vessels from IL-10 $-/-$ recipients (arrowhead). Anti-IFN- γ MAb treatment (arrowhead) preserved α -smooth muscle actin staining in the media. Desmin staining (bottom row) confirmed the staining pattern of α -smooth muscle actin. Negative control (bottom left corner) included omission of primary antibody. Hematoxylin counterstaining in CD45 and desmin, Verhoeff's elastin in α -smooth muscle actin; original magnification, $\times 125$.

Cellular Characterization of Neointima

Immunostaining of consecutive paraffin sections was used to characterize the composition of the vascular thickening that developed in cardiac allografts. Figure 4 contains selected vessels with prominent vascular occlusion to illustrate the altered cellular composition among different groups. In wild-type recipients, there were a few inflammatory cells within the neointima

identified by CD45 (LCA) staining. In contrast, vascular thickening in transplants from IL-10 $-/-$ recipients with or without anti-IFN- γ MAb treatment was dominated by CD45-immunopositive cells. Vessel neointimal α -smooth muscle actin staining was sparse in IL-10 $-/-$ recipients. In contrast, in wild-type recipients there was abundant intense staining for α -smooth muscle actin interspersed with CD45 throughout the neointima (Figure 4).

Figure 5. Quantitative image analysis of α -smooth muscle actin staining showed that transplants from IL-10 $-/-$ recipients had significantly less α -smooth muscle actin positivity in the neointima than wild-type animals ($*P < 0.0001$). Loss of α -smooth muscle actin in the media was evident in IL-10 $-/-$ recipients, but anti-IFN- γ MAb treatment prevented medial SMC loss. The number of vessel cross sections analyzed per group is shown in the columns.

Detailed quantitative image analysis of 153 vessel cross sections confirmed that heart transplants placed in wild-type recipients developed significantly more SMCs in the neointima. This was not the case in grafts from IL-10 $-/-$ recipients. IL-10 $-/-$ recipients with anti-IFN- γ MAb treatment displayed only $11 \pm 2\%$ ($n = 9$ animals and $n = 67$ vessel cross sections) neointimal α -smooth muscle actin positively. Those without anti-IFN- γ MAb treatment showed a reduction to $8 \pm 1\%$ ($n = 6$ and $n = 65$) α -smooth muscle actin positivity compared with $34 \pm 5\%$ ($n = 5$ and $n = 21$) in wild-type recipients ($P < 0.0001$ for both; Figure 5).

Anti-desmin staining confirmed the neointimal staining pattern seen with α -smooth muscle actin (Figure 4). Hence, vascular thickening developing in allografts from IL-10 $-/-$ recipients was due to a monocytic arteritis rather than the classic arteriosclerosis seen in wild-type recipients characterized by accumulation of myointimal cells.

Characterization of Vascular Media

α -Smooth muscle actin staining revealed striking differences in the vascular patterns seen in vessel medial layers among groups. Concentric medial staining delineating the vessels was clear in the grafts from wild-type and IL-10 $-/-$ recipients treated with anti-IFN- γ MAb but diminished in the IL-10 $-/-$ group. To examine this, we quantitated α -smooth muscle actin positivity in medial areas. In the IL-10 $-/-$ group, 28% (18/65) of the external elastic lamina (EEL) was destroyed and did not permit quantitative analysis. In the anti-IFN- γ MAb treatment group, 16% (11/67) of the transplants had disruptions of the EEL, whereas in the wild-type group, none of the vessels had EEL destruction (0%, 0/21). Image analysis (Figure 5) showed significantly lower SMC positivity in the vascular media in the IL-10 $-/-$ group, $33 \pm 2\%$ ($n = 47$

vessel cross sections), compared with the wild-type group ($69 \pm 3\%$; $n = 21$; $P < 0.0001$). Anti-IFN- γ MAb administration to IL-10 $-/-$ recipients resulted in higher SMC positivity in the media ($57 \pm 3\%$; $n = 56$; $P < 0.0136$ compared with untreated IL-10 $-/-$ recipients). Hence, IL-10 deficiency was associated with CD45-immunopositive vascular thickening and medial cell loss, most likely due to medial necrosis. Anti-IFN- γ treatment in IL-10 $-/-$ recipients partially inhibited development of medial SMC loss in the vascular wall but not CD45 $^{+}$ cell accumulation.

To reaffirm the role of IFN- γ in medial necrosis associated with accelerated rejection, we performed image analysis of α -smooth muscle actin positivity in vessels from rejecting IFN- γ $-/-$ recipients originally transplanted for another study.⁷ Quantitative image analysis of α -smooth muscle actin immunopositivity in this study also showed that graft vessels from IFN- γ $-/-$ recipients had significantly more SMCs in the vascular medial layer than vessels from wild-type recipients ($31 \pm 3\%$ ($n = 27$) versus $19 \pm 3\%$ ($n = 25$); $P = 0.0057$). These two studies illustrate that IFN- γ deficiency protected cardiac graft vessels from medial necrosis.

Lack of Macrophage Suppression in IL-10 $-/-$ Recipients

To identify potential molecular pathways contributing to changes in the parenchyma and vasculature in IL-10 $-/-$ recipients, we used semiquantitative RT-PCR to assess intragraft transcript levels for selected inflammatory factors. Transcript levels for CD3 and Mac-1 were used to estimate overall inflammatory infiltration. IFN- γ and IL-4 (signature Th1 and Th2 cytokines) and macrophage activation products iNOS, AIF-1, and TNF- α were evaluated to characterize cytokine profiles of inflammatory cells. Figure 6 demonstrates that intragraft transcript levels for Mac-1, but not CD3, were significantly higher in IL-10 $-/-$ recipients with or without anti-IFN- γ MAb than in wild-type controls. These findings suggest that of the numerous infiltrating inflammatory cells in IL-10 $-/-$ recipients, many were macrophages. The observation of increased IFN- γ , but comparable IL-4 levels, confirmed our previous finding⁷ showing accelerated Th1-like responses without significant alteration in Th2-like responses in allografts from IL-10 $-/-$ recipients. Immunostaining confirmed the presence of the IFN- γ protein in mononuclear cells in the parenchymal tissue.⁷ These cells were small and lacked granules, suggesting that they were lymphocytes and not natural killer cells. Evaluation of selective macrophage activation markers showed that anti-IFN- γ MAb treatment significantly decreased transcript levels for IFN- γ -responsive factors, ie, iNOS and AIF-1, but did not alter transcripts for TNF- α or macrophage lectin. These findings suggest that, when present, IL-10 suppresses macrophage activation and perhaps cytotoxic activities mediating medial necrosis in cardiac transplants from IL-10 $-/-$ recipients.

Thus, cardiac allografts from IL-10 $-/-$ recipients develop a vascular occlusion from an arteritis characterized by increased infiltration of leukocytes in the neointima

Figure 6. Intragraft transcript analysis by semiquantitative RT-PCR. Transcript levels normalized against G3PDH for Mac-1- and IFN- γ -responsive macrophage markers were higher in allografts from IL-10 $-/-$ recipients than in allografts from wild-type recipients. The number of infiltrating CD3 cells was similar in the groups, but Th1-type cytokine (IFN- γ) was higher in IL-10 $-/-$ groups. Th2-type cytokine (IL-4) was similar in all groups. * $P < 0.017$ compared with wild-type controls; * $P < 0.017$ compared with IL-10 $-/-$. Results are given as means $\pm$ SEM for all grafts (n in parentheses) after triplicate analysis.

and loss of SMCs in the media. Inhibition of IFN- γ either by MAb administration or gene deletion was able to diminish medial SMC destruction but not neointimal leukocyte expansion.

Discussion

Here, we characterize leukocyte-antagonizing properties of IL-10 after alloimmune injury produced by transplantation. By comparing time-matched cardiac allografts from IL-10 knockout and wild-type recipients, we show that cardiac allograft rejection is accelerated. Despite T-cell-depleting immunosuppression, there was increased mononuclear cell infiltration and activation with graft destruction. This accelerated form of late rejection produces more luminal occlusion in grafts from the IL-10 $-/-$ recipients. A prominent mononuclear cell arteritis was seen in the IL-10 $-/-$ group compared with the SMC-rich arteriosclerosis seen in graft vessels from wild-type recipients. This increased mononuclear cell response in the IL-10 $-/-$ recipients is not simply the result of an augmented IFN- γ -mediated Th1 response but is associated with macrophage activation. Neutralization of IFN- γ did not prevent parenchymal rejection or vascular occlusion but did inhibit loss of medial SMCs from the

vessel wall. Taken together, our findings indicate that the presence of IL-10 protects allografts from rejection by inhibiting leukocyte accumulation and infiltration, potentially by altering macrophage activation.

IL-10 Suppresses Inflammatory Responses after Transplantation

In this study, we extend our earlier findings using IL-10 knockout mice to show that IL-10 had a protective influence on cardiac transplantation. There was prominent inflammatory cell infiltration and accelerated rejection in IL-10 $-/-$ recipients. To determine whether IFN- γ -mediated Th1-type responses may have contributed to graft failure, we neutralized IFN- γ with MAb in the group of IL-10 $-/-$ recipients. Treatment with MAb did not improve graft survival or reduce immune cell infiltration and parenchymal rejection but prevented medial damage. This finding is consistent with earlier studies showing that the role of IFN- γ may be different in parenchymal (or acute) rejection compared with vascular forms of rejection.^{7,16,26,27} Hence, gene deletion of IL-10 in concert with IFN- γ blockade was not sufficient to suppress leukocyte activation and infiltration associated with an alloimmune response. These findings indicate that the protective effect of IL-10 is not simply from the suppression of IFN- γ -associated Th1 responses and that other immune forces are likely to contribute.

A number of other mechanisms might confer these protective effects of IL-10 in our model. Areas to be considered include inhibition of other Th1 responses, leukocyte adhesion and migration,²⁸ T cell and/or macrophage cytotoxicity,¹ macrophage deactivation,²⁹ suppression of antigen-specific responses,³⁰ regulation of humoral responses,^{1,31} or even regulation of IL-10 receptor levels. As a first step in examining T cell and interrelated macrophage pathways, we performed intragraft analysis. In grafts from IL-10 $-/-$ recipients, there was an increase in IFN- γ -associated Th1-type cytokine levels in concert with higher levels for a number of macrophage factors, including Mac-1, AIF-1, and iNOS, but not macrophage lectin or TNF- α . These findings would argue that IL-10 has leukocyte/macrophage suppressive properties that protect heart transplants from rejection. Although anti-IFN- γ MAb treatment did not decrease the number of macrophages infiltrating (reflected by Mac-1 transcript levels) into allografts from IL-10 recipients, it decreased their activation level (as reflected by AIF-1 and iNOS transcript levels). In addition, further descriptive and function studies with the IL-10 knockouts can be used to investigate other mechanisms (monokines, chemokines, and growth factors) through which IL-10 may regulate macrophage function, leukocyte adhesion, and humoral components of the alloimmune response.

Vascular Changes in Heart Transplants from IL-10 $-/-$ Recipients

The graft vasculature is the first site of interaction between donor and recipient immune cells. To determine

whether the suppressive effects of IL-10 altered vascular outcomes, we quantitated luminal occlusion and studied the cell composition within the vessels. We showed that graft vessels from IL-10 $-/-$ recipients had an increase in the severity of vascular occlusion compared with wild-type recipients. However, the luminal narrowing was due to a profound neointimal accumulation of mononuclear cells. Graft vessels from IL-10 $-/-$ recipients were characteristic of an arteritis with a paucity of SMCs and a dominance of leukocytes in the neointima coupled with a striking loss of staining in the medial layer. This pattern recapitulates features of acute vascular rejection outlined in the Banff schema used for kidney allografts. Intimal thickening with transmural leukocyte infiltration and medial SMC necrosis are hallmarks of the severe intimal arteritis in that schema.³² Hence, in the setting of IL-10 deficiency, there was accelerated vascular rejection. On the other hand, we found that graft vessels from wild-type recipients develop the characteristic SMC-rich neointima seen with arteriosclerotic syndromes. These cardiac graft vessels have features of chronic vascular rejection described in kidney transplants characterized by luminal occlusion primarily due to concentric myofibrotic thickening.³² Hence, one could speculate that the presence of IL-10 may be required to allow development of the fibrous intimal thickening characteristic of chronic vascular disease.

IFN- γ Promotes Medial Destruction in the Graft Vascular Wall

In graft vessels from wild-type recipients, the medial SMC layer is protected from destruction as indicated by immunostaining for SMC markers. Yet, there is an alloimmune response sufficient to promote development of a SMC-rich neointima. In comparison, graft vessels from IL-10 $-/-$ recipients have severe medial SMC loss in concert with parenchymal heart rejection. IFN- γ contributes to SMC loss in the media, in that the blockade of functional IFN- γ either by anti-IFN- γ MAb treatment or gene targeting significantly diminished medial destruction in our model. Medial necrosis has been commonly seen in isolated vessel transplant models where immunosuppression is withheld (aortic³³ and carotid²⁰) and active acute rejection is not controlled. Taken together, these findings suggest that inflammatory forces that participate in active acute rejection contribute to medial SMC loss. Studies examining graft vascular changes should distinguish between vessel occlusion secondary to active rejection (arteritis and medial necrosis) and chronic fibrotic thickening. The mechanisms underlying these two processes are likely to be distinct even though they may overlap depending on the immunosuppressive state.

A potential mechanism through which monoclonal anti-IFN- γ therapy may protect medial SMCs from destruction includes inhibition of production of reactive nitrogen intermediates. This hypothesis is supported by the finding that neutralization of functionally active IFN- γ decreases intragraft iNOS transcript levels in MAb-treated animals (regardless of high IFN- γ transcript levels). Recently, Ste-

fano et al³⁴ demonstrated that IL-10-induced nitric oxide release inhibited adherence of monocytes and granulocytes in explanted human saphenous veins. Other monocyte-mediated activities, which may contribute to cytotoxicity and tissue destruction, include secretion of neutral proteases, arginase, thymidine, complement components, and reactive oxygen intermediates.³⁵

IFN- γ -mediated SMC loss in the media may also arise, in part, through programmed cell death, apoptosis. Cytotoxic T cells may have directly contributed to apoptosis through perforin-granzyme, Fas/Fas-ligand, or TNF receptor pathways to induce apoptosis.³⁶ *In vitro* studies have demonstrated that reactive oxygen species³⁷ or cytokines, particularly IFN- γ , can stimulate apoptosis in SMCs.³⁸ Apoptosis may be a potential regulator in the vessel wall, balancing cell proliferation and thus helping to maintain normal vessel architecture.³⁹

Our studies using IL-10 $-/-$ mice provide new insight on the role of IL-10 in organ transplantation. In IL-10 $-/-$ recipients there is a sustained and complete reduction of immune cell sources of IL-10 after transplantation that is not dependent on delivery route, dosing, or timing of the agent. We have clearly demonstrated that IL-10 has protective roles in heart transplantation through leukocyte suppression. These findings are consistent with studies showing that IL-10 $-/-$ mice have aggravated immune responses in infectious disease models where increased mortality and morbidity were associated with aberrant Th1-type immune responses.^{11,12,14,15} We have shown after alloimmune injury that IL-10 diminishes leukocyte infiltration into the parenchyma and graft vasculature and promotes medial SMC integrity through IFN- γ inhibition. One could speculate that IL-10 may promote development of transplant arteriosclerosis by attenuating cytokine-mediated inflammatory destruction in the vasculature. This might allow the vascular wall to develop a fibrotic, healing response to injury and, hence, promote myointimal thickening.

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