Interferon- γ Acts Directly on Rejecting Renal Allografts to Prevent Graft Necrosis

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In transplant rejection interferon (IFN)- γ regulates the recipient immune response but also acts directly on IFN- γ receptors in the graft. We investigated these direct actions by comparing rejecting kidneys from donors lacking IFN- γ receptors (GRKO mice) or control donors (129Sv/J) in CBA recipients. Beginning day 5, 129Sv/J kidneys displayed high major histocompatibility complex (MHC) expression, progressive infiltration by inflammatory cells, but no thrombosis and little necrosis, even at day 21. GRKO kidneys showed increasing fibrin thrombi in small veins, peritubular capillary congestion, hyaline casts, and patchy parenchymal necrosis, progressing to near total necrosis at day 10. Terminal dUTP nick-end labeling assays were positive only in the interstitial infiltrate, confirming that massive cell death in GRKO transplants was not apoptotic. Paradoxically, GRKO kidneys showed little donor MHC induction and less inflammatory infiltration. Both GRKO and 129Sv/J allografts evoked vigorous host immune responses including alloantibody and mRNA for cytotoxic T cell genes (perforin, granzyme B, Fas ligand), and displayed similar expression of complement inhibitors (CD46, CD55, CD59). GRKO kidneys displayed less mRNA for inducible nitric oxide synthase and monokine inducible by IFN- γ but increased heme oxygenase-1 mRNA. Thus IFN- γ acting on IFN- γ receptors in allografts promotes infiltration and MHC induction but prevents early thrombosis, congestion, and necrosis. (*Am J Pathol* 2001, 158:215–226)

Interferon (IFN)- γ is a major player in host defense, graft rejection, and autoimmunity.^{1–6} IFN- γ acts through the IFN- γ receptor (IFN- γ R), composed of a ligand-binding chain R1 and an accessory chain R2,⁷ members of the same family as IFN- α/β R and IL-10R.^{8–12} IFN- γ generally promotes host defense and its absence creates a mild immunodeficiency. Humans with defects in IFN- γ R display increased susceptibility to mycobacterial infec-

tion.^{13,14} Mice with targeted disruption of the genes for IFN- γ (GKO mice),¹⁵ IFN- γ R1 (GRKO),¹⁶ or IFN- γ R2¹⁷ have multiple defects in immune function,^{18,19} including decreased nitric oxide (NO) and IgG2a production and increased susceptibility to mycobacteria,¹⁴ Leishmania,²⁰ and certain viruses.²¹ In addition to its role in immune regulation, IFN- γ differs from other cytokines in its extensive effect on nonmarrow derived cells, as evident in the unique role of IFN- γ in inducing major histocompatibility complex (MHC) expression in epithelial and endothelial cells. Mice lacking IFN- γ or IFN- γ R1 show decreased class I expression in the basal state, much less induction of MHC expression in response to stimuli, and decreased class I and II induction after local injury.^{22,23}

Thus, defects in IFN- γ or IFN- γ R genes were expected to impair allograft rejection. For example, IFN- γ should increase antigenicity because it increases the expression of MHC products, which are potent alloantigens and are massively increased in rejecting grafts^{24,25} and in the recipient tissues,^{26,27} in large part because of IFN- γ . IFN- γ promotes chronic immune injury to transplant vessels.^{28,29} In contrast, acute rejection is brisk in IFN- γ -deficient hosts,³⁰ associated with increased T cell proliferation and cytotoxic T lymphocyte generation.^{30–32} We have found that mice lacking IFN- γ reject with altered pathology in kidney or heart allografts, including thrombosis, congestion, and infarction in the rejecting organ despite having little MHC induction (P. F. Halloran, L. W. Miller, A. Battocchio, J. Urmson, L.-F. Zhu, N. M. Kneteman, and K. Solez: Unique roles of IFN- γ in graft rejection: induction of MHC expression and protection from early vascular injury. Submitted for publication.) The rejection is immune because it is absent in isografts and is blocked by immunosuppressive drugs. The ability of IFN- γ to restrain early tissue destruction could be relevant to the essential role of IFN- γ in some tolerance models.³² IFN- γ also plays a role in xenografts by preventing vascular injury.³³ These observations highlight the existence of an unexpected tendency of IFN- γ to

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protect allografts and concordant xenografts during the early posttransplant period. In later periods IFN- γ may promote graft injury.^{28,29}

While the best-studied effects of IFN- γ are on the immune response, IFN- γ can act directly on allografts independently of its effect on the immune system. To explore how such direct effects alter rejection, we examined the pathology of rejecting kidney allografts lacking IFN- γ R. We transplanted H-2^b kidneys from donors lacking or expressing IFN- γ R into allogeneic H-2^k recipients with normal IFN- γ production. Grafts with IFN- γ R showed typical interstitial infiltration and MHC induction at days 5, 7, and 10, but remained viable despite severe rejection at day 21. Grafts lacking IFN- γ R showed little MHC induction and reduced cellular infiltration but increased vascular injury as manifest by venous thrombosis, congestion of peritubular capillaries, and massive necrosis of parenchymal cells by days 7 to 10. Thus a major early effect of IFN- γ in acute rejection is a direct action on the IFN- γ R of graft that maintains graft viability during the intense inflammation of acute rejection by preventing thrombosis, congestion, and necrosis.

Materials and Methods

Mice

The original GRKO (129/Sv/Ev) mouse strain with disrupted IFN- γ R1-chain genes was generated by gene targeting in murine embryonic stem cells.¹⁶ The gene was disrupted by inserting the neomycin-resistance gene (*neo*^r) into exon V, which encodes an extracellular domain. Homozygous 129/Sv/Ev mice were provided to us through Dr. Michel Aguet (University of Zurich, Switzerland).

As controls with wild-type IFN- γ R1 genes, we obtained 129Sv/J mice from Jackson Laboratories (Bar Harbor, ME). The mice were maintained in the Health Sciences Laboratory Animal Services at the University of Alberta and were kept on acidified water. All experiments conformed to approved animal care protocols. As in many other transplant experiments with knockout mice,³⁴ the GRKO and 129Sv/J are H-2 identical but may differ at minor loci. Nevertheless the short time frame and the complete H-2 disparity in the present experiments make it highly unlikely that any minor differences influence this experiment.

Kidney Transplants

In anesthetized 129Sv/J and GRKO donor mice, the abdomen was opened by a midline incision and the left kidney was excised and preserved in cold lactate Ringers solution. The recipient CBA mice were similarly anesthetized and the right native kidney was excised. The donor kidney was then anastomosed heterotopically to the abdominal aorta and vena cava, without removing the recipient left kidney. The mice were allowed to recover and were killed at days 5, 7, 10, and 21 by cervical dislocation under anesthesia. Tissue samples were obtained for histological staining [hematoxylin and eosin

(H&E), periodic acid Schiff, and Martius scarlet blue], as well as indirect immunoperoxidase staining for class I and II antigen expression.

Banff Scoring System

Using a modified version of the Banff scoring system³⁵ two pathologists assigned scores for the lesions observed in whole kidney sections including cortex and outer medulla.³⁵ Additional findings not included in the Banff scoring system such as the extent of necrosis, peritubular capillary congestion, and cast formation were scored from 0 to 3 based on the percentage of parenchymal involvement (0, no changes; 1, <25% of the total parenchyma involved; 2, 25 to 75% of total parenchyma involved; 3, >75% of the total parenchyma involved). Venous thrombosis was first assessed by H&E stain and the presence of fibrin in the thrombus was confirmed by Martius scarlet blue stain for fibrin. For scores on venous thrombosis the number of veins showing venous thrombosis was counted in each specimen.

Antibodies

Monoclonal antibodies (mAb) were purified in our laboratory from supernatants of hybridoma cell lines, AF 6-120.1.2 (mouse IgG against mouse I-A^b), 20-8-4S (mouse IgG against mouse H-2K^bD^b), 11-4.1 (mouse IgG against mouse H-2K^k), 11-5.2.1.9 (mouse IgG against mouse I-A^k), M1/42.3.9.8 (rat IgG_{2a} against all mouse H-2 haplotypes), and M5/114.15.2 (rat IgG_{2b} against mouse I-A^{b,d,q} and I-E^{d,k}), obtained from American Type Culture Collection (Rockville, MD). Briefly, the hybridoma cell lines were maintained in tissue culture, and the supernatants containing AF 6-120.1.2 (anti-I-A^b) and 20-8-4S (anti-H-2K^bD^b), 11-4.1 (anti-H-2K^k), and 11-5.2.1.9 (anti-I-A^k) were purified by protein A chromatography. The supernatants containing M1/42.3.9.8 (anti-2 antigens all haplotypes) and M5/114.15.2 (anti-I-A^{b,d,q} and I-E^{d,k}) were ammonium-sulfate precipitated, and then the antibodies were obtained by purification with a DE52 anion exchanger column (Whatman, Hillsboro, OR) and by concentration with Amicon ultrafiltration. The protein concentration was adjusted to 1 mg/ml by a modified Lowry method and maintained at -70°C. Radioiodination was performed by the Iodogen method (Pierce Chemical Co., Rockford, IL).³⁶

Radiolabeled Antibody-Binding Assay

Anti-H-2K^bD^b mAb and anti-I-A^b mAb were radiolabeled with [¹²⁵I]iodide.³⁶ Tissues of individual mice were prepared as described previously.³⁷⁻³⁹ The tissue concentration was adjusted to 20 mg/ml and 5 mg of kidney tissue was aliquoted in triplicate and spun. The pellets were incubated on ice with ¹²⁵I-labeled mAb in 10% normal mouse serum (100,000 cpm per 100 μ l) with agitation for 1 hour. After washing, the pellets were counted in a γ counter and the nonspecific binding of a negative tissue was subtracted. Based on standard

Table 1. Sequence of Polymerase Chain Reaction Primers

Genes	5' Primer	3' Primer
Granzyme	5'-AGAGCAAGGACAACACTCTTGAC	5'-TTGAGGGGCTCACAGC
Perforin	5'-AACTCCCTAATGAGAGACGCC	5'-ATGCTCTGTGGAGCTGTAAAA
FasL	5'-GATTCCTCAAAATTGATCAGAGAGAG	5'-GATTCCTCAAAATTGATCAGAGAGAG
CD46	5'-CATACCTGATGATTGCTACCTG	5'-GGACATTGACCACTTTACACTC
CD55	5'-ATGATCCGTGGGCGGGCGCCT	5'-ATGCTCTGAGGTGGGCCGCAG
CD59	5'-CTAAGTCCTAGATGCCTCCTC	5'-TGAGCGTGTGAGAGTGGTAAA
HO-1	5'-TCGACAGCCCCACCAAGTTC	5'-GGAGCGGTGTCTGGGATGAG
NOS2	5'-TGGGAATGGAGACTGTCCAG	5'-GGGATCTGAATGTGATGTTTG
MIG	5'-GGACTCGGCAAATGTGAAGAAG	5'-AGCTATGAAGGAAAGGGACACT
IP10	5'-AAGAATGATGAGCAGAGATGTC	5'-CGTCGCACCTCCACATAG
HPRT	5'-GTTGGATACAGGCCAGACTTTGTTG	5'-GAGGGTAGGCTGGCCTATGGCT

curves, the change in specific cpm bound in this assay corresponds to approximately a sevenfold change in antigen output.³⁹

Indirect Immunoperoxidase Staining of Tissue Sections

Fresh frozen cryostat sections were fixed in acetone, then incubated with normal goat serum. The slides were incubated with rat mAb against class I (M1) and class II (M5) or with phosphate-buffered saline (PBS) as a control. The slides were then incubated with affinity purified peroxidase-conjugated goat anti-rat IgG F (ab')₂ fragment (ICN, Costa Mesa, CA). Immune complexes were visualized by the use of 3'3 diaminobenzidine tetrahydrochloride and hydrogen peroxide for the color reaction and then counterstained with hematoxylin. To count the number of infiltrating cells stained for various markers, 10 fields were counted and the results were expressed as the mean number per high-power field.

Assessment of Gene Expression

Total RNA was extracted and pooled from three kidneys that were harvested on day 7. RNA was transcribed into cDNA using Superscript reverse transcriptase (BRL, Burlington, Ontario) and amplified in a Perkin Elmer Cetus thermal cycler (Perkin Elmer Cetus, Emeryville, CA) using *Taq* DNA polymerase. The sequences of the polymerase chain reaction primers are shown in Table 1. The polymerase chain reaction products were Southern blotted and probed with radiolabeled oligonucleotide probes. Quantitation was performed by phosphorimaging of blots and analysis by ImageGuage Software (Fuji, CT).

The Terminal dUTP Nick-End Labeling (TUNEL) Assay

To stain apoptotic cells, we performed TUNEL of fragmented DNA on 3- μ m sections of paraffin-embedded tissue.^{40,41} Briefly, sections were deparaffinized in xylene for 5 minutes twice and then hydrated through a series of alcohols. To inactivate endogenous peroxidase, the sections were immersed in 1% H₂O₂ for 8 minutes at room temperature and then rinsed twice in distilled water. The sections were then treated with proteinase K (20 μ g/ml in

PBS) for 10 minutes at room temperature and then rinsed in PBS three times. The sections were air dried and then flooded with terminal transferase (TDT) buffer (30 mmol/L Tris-HCl, pH 7.2, 1 mmol/L CoCl₂, 140 mmol/L sodium cacodylate) for 30 minutes at room temperature. To begin labeling the TDT buffer was replaced with TDT buffer containing 0.25 nmol/ μ l biotin-16-dUTP and 0.25 U/ μ l TDT (Hoffmann-La Roche, Quebec). After a 1-hour incubation at 37°C in a humidified chamber the slides were rinsed twice in PBS for 5 minutes. Nonspecific staining was blocked by immersing the slides in 2% bovine serum albumin in PBS for 20 minutes at room temperature, followed by two rinses in PBS for 5 minutes. The slides were then incubated with the avidin-biotin complex (Vector Laboratories, Burlingame, CA) for 30 minutes and then rinsed in PBS two times for 5 minutes. The reaction was visualized using the diaminobenzidine substrate kit (Vector Laboratories). The slides were counterstained with methyl green, dehydrated through a series of alcohols and xylene, and mounted with Permount (Fisher, Nepean, ON). For negative controls TDT was omitted. Thymus from young mice or kidney sections treated with 10 to 1,000 ng/ml DNase I (Sigma Chemical Co., St. Louis, MO) were used as positive controls. Apoptotic cells were counted in 10 fields ($\times$ 200) in one kidney in each group.

Assessment of Cytotoxic Alloantibody

129Sv/J and GRKO spleen cells (10×10^6) were injected intraperitoneally into CBA mice, bled at day 7, boosted with 10×10^6 spleen cells and bled at day 14. Transplanted mice (CBA) with 129Sv/J or GRKO kidneys were bled at the time of harvest and their sera were assessed in a microcytotoxicity assay. Serum (2 μ l) and spleen cells (2 μ l) (2×10^6 /ml) were incubated in a 37°C CO₂ incubator for 30 minutes. Rabbit complement (2 μ l at 1:3 dilution) was added and incubated at room temperature for 90 minutes. Two μ l of 5% eosin and one drop of 10% buffered formalin were added to each well. The plates were flooded with formalin and examined under an inverted microscope to determine the percentage of dead cells.

Statistical Analysis

Values are given as mean $\pm$ SE in Table 2 and statistical analysis was performed using the Mann Whitney *U* test.

Table 2. Comparison of the Gradings of the Histologic Lesions in GRKO *versus* 129 Kidneys

	Day	Score	GRKO mean of score ± SE (<i>P</i> value)*	Score	129 mean of score ± SE	<i>P</i> value†
Kidney weight	5		306 ± 37		279 ± 29	0.2
	7		484 ± 87		317 ± 67	0.001
Peritubular capillary congestion	5	2,2,0,1,1	.2 ± 0.3	0,1,0,0,0	0.2 ± 0.2	0.05
	7	2,3,3,3,3,2,2	2.63 ± 0.1 (<i>P</i> =0.008)	1,0,1,0,0,1,1,1,1	0.67 ± 0.1 (<i>P</i> =0.1)	<0.001
Venous thrombosis	5	0,3,0,5,0	1.6 ± 2.3	0,0,0,0,0	0	0.13
	7	3,5,2,14,9,12,5,4	6.75 ± 4.4 (<i>P</i> =0.03)	0,0,0,0,0,0,0,0,0	0 (<i>P</i> =1.0)	<0.001
Necrosis	5	0,0,0,0,0	0	2,0,0,0,0	0.4 ± 0.4	0.3
	7	0,2,1,2,2,2,2,1	1.5 ± 0.2 (<i>P</i> =0.005)	0,3,0,0,0,0,0,0,1	0.44 ± 0.3 (<i>P</i> =0.9)	0.02
Casts	5	0,0,0,0,0	0	0,0,0,0,0	0	1
	7	2,3,2,3,3,3,3,1	2.5 ± 0.2 (<i>P</i> =0.002)	0,1,0,0,0,0,1,1,0	0.33 ± 0.1 (<i>P</i> =0.1)	<0.001
Interstitial infiltrate	5	1,1,2,1,1	1.2 ± 1.2	2,2,2,1,2	1.8 ± 0.2	0.07
	7	2,3,2,2,2,2,1,2	2 ± 0.1 (<i>P</i> =0.02)	2,3,2,1,1,2,3,3,3	2.2 ± 0.2 (<i>P</i> =0.2)	0.4
Glomerulitis	5	1,1,0,0,0	0.4 ± 0.2	0,0,0,0,0	0	0.13
	7	0,1,0,0,3,3,1,1	1.13 ± 0.4 (<i>P</i> =0.3)	1,1,1,0,0,0,1,1,1	0.67 ± 0.1 (<i>P</i> =0.02)	0.6

*Significant difference between day 5 and day 7.

†Significant difference between 129 and GRKO analysis determined by Mann-Whitney U test. No arteritic lesion was found in GRKO and 129 animals at day 5 and 7. Number of animals: day 5, 129 = 5; GRKO = 5; day 7, 129 = 9; GRKO = 8.

Analysis of variance was performed in Table 3. A *P* value of 0.05 was considered to show a significant difference between the two groups.

Results

We transplanted the donor kidneys into the right side after removing the right recipient kidney, preserving the left recipient kidney to maintain renal function and as a control. This model permitted us to study the evolution of the changes in the graft without the complicating influence of host uremia, because the residual native kidney maintained normal renal function. The right transplant and the left native kidney were then studied 5, 7, 10, or 21 days after transplantation.

Pathology

Syngeneic control kidney transplants were performed with 129/J into 129/J, GRKO into GRKO, and GRKO into 129Sv/J mice. None of these transplants showed rejection or thrombosis at day 7. The left native kidneys were normal throughout and served as controls.

All 129Sv/J and GRKO kidney allografts showed rejection by day 5, progressing at the later times. The rejecting 129Sv/J and GRKO allografts were enlarged compared to the recipient left kidneys but GRKO transplants were more enlarged than 129Sv/J, particularly at day 7 (Table 2). The 129Sv/J transplants showed more mononuclear interstitial infiltrate but little necrosis, peritubular capillary congestion, or cast formation (Figure 1A). The infiltrate was present at day 5 and increased at day 7. There was mild tubulitis and no arteritis at days 5 and 7, although venulitis was common. Despite the mononuclear infiltrate, the 129Sv/J's at days 5, 7, and 10 displayed little parenchymal cell death (Table 2 and Figure 1A). By day 21 all 129Sv/J kidneys showed severe tubulitis and heavy infiltrate (Figure 1B). No 129Sv/J transplants showed venous thrombosis at days 5, 7, 10, or 21.

In GRKO kidneys the inflammatory infiltrate was less than in 129Sv/J kidneys (Table 2). However, beginning at day 5 and increasing at day 7 GRKO kidneys showed mural fibrin thrombi in small veins, peritubular capillary congestion, and cast formation in tubules (Figure 1, C and D). Inflammation in small veins (venulitis) was common in GRKO and 129Sv/J kidneys (Figure 1E). Venous

Table 3. Composition of the Interstitial Infiltrate in Rejecting Kidneys at Days 5 and 7*

No. of cells stained for	Day	Interstitial cells/HPF (mean ± SD)		% change GRKO <i>versus</i> 129	<i>P</i> value†
		GRKO	129		
CD45	5	196 ± 46.4	262 ± 36.7	(−25%)	0.03
	7	118 ± 34.5	336 ± 74.6	(−63%)	0.0001
CD3	5	40 ± 12.9	52 ± 20.0	(−40%)	0.02
	7	39 ± 11.0	197 ± 75.9	(−80%)	0.0001
CD8	5	14 ± 15.2	27 ± 25.7	(−48%)	0.38
	7	24 ± 10.7	101 ± 35.2	(−78%)	0.003
CD4	5	4 ± 3	6 ± 4.2	(−33%)	0.29
	7	0.1 ± 0.4	8 ± 6.8	(−98%)	0.0001
Class II	5	59 ± 33.2	42 ± 27.6	(+40%)	0.64
	7	79 ± 63.8	64 ± 32.4	(+23%)	0.01

*Day 5, GRKO *n* = 5; 129 *n* = 4; day 7, GRKO *n* = 8; 129 *n* = 8; day 10, GRKO *n* = 1; 129 *n* = 1 (both necrotic).

†Significant difference between GRKO and 129 by analysis of variance.

Figure 1. Pathological findings in 129Sv/J and GRKO transplanted right kidneys at days 5, 7, and 10. **A:** 129Sv/J transplant at day 7 showing moderate interstitial infiltrate. The parenchyma is well preserved. A vein with venulitis and without thrombosis is shown (**asterisk**) (H & E; original magnification, $\times 250$). **B:** 129Sv/J transplant at day 21 showing arteritis (**arrow**) and a mild interstitial infiltrate, and viable renal parenchyma (H & E; original magnification, $\times 250$). **C:** GRKO transplant at day 7 showing peritubular capillary congestion and tubular casts in nonnecrotic areas (H & E; original magnification, $\times 250$). **D:** GRKO transplant at day 7 showing parenchymal necrosis in a congested background (H & E; original magnification, $\times 250$). **E:** GRKO transplant at day 5 showing mural thrombus in a vein (**arrow**) and venulitis (H & E; original magnification, $\times 250$). **F:** GRKO transplant at day 7 showing fibrin within a venous thrombus (**asterisk**) stained orange-red and located in a vein (**arrows**). Peritubular capillaries are engorged with red blood cells (yellow) (Martius scarlet blue; original magnification, $\times 250$). **G:** GRKO transplant at day 5 showing apoptotic cells (**arrowheads**) only in the peritubular capillaries (TUNEL; original magnification, $\times 160$). **H:** GRKO transplant at day 7 showing apoptotic interstitial cells (**arrowheads**) in an area of parenchymal necrosis (TUNEL; original magnification, $\times 160$).

Figure 2. Comparison of 129Sv/J and GRKO transplanted right kidneys stained for class I (M1) and II (M5) rat monoclonals. **A:** 129Sv/J kidneys stained with M1, showing intense staining of the tubular epithelium (arrow) and the interstitial infiltrate. **B:** 129Sv/J kidneys stained with M5, showing staining of the tubular epithelium (arrow) and of the interstitial infiltrate. **C:** GRKO kidneys stained with M1, showing prominence of interstitial staining (arrows) but little or no tubular positivity (asterisks). **D:** GRKO kidneys stained with M5, showing mild interstitial staining with little tubular staining (original magnification, $\times 160$).

thrombosis was found in two of five GRKO transplants at day 5 (Figure 1E) and in all eight GRKO transplants at day 7 (Table 2) (Figure 1F). By day 7 GRKO transplants showed extensive parenchymal cell necrosis (Figure 1D), which was massive at days 10 and 21 (not shown). There was little tubulitis and no arteritis in GRKO kidneys at days 5 and 7 (Table 2).

Electron microscopy of 129Sv/J and GRKO transplants at days 5 and day 7 confirmed that the peritubular capillaries were congested in the GRKO transplants, but neither 129Sv/J nor GRKO kidneys displayed interstitial hemorrhage. In both 129Sv/J and GRKO transplants interstitial inflammatory cells were shown to be outside of peritubular capillaries. The electron microscopic assessment of renal tubular epithelium showed changes compatible with ischemic necrosis (data not shown).

TUNEL Assay

We used the TUNEL assay to clarify the character of cell death in 129Sv/J and GRKO transplants. Apoptosis was frequent among the inflammatory cells located in the interstitium and the peritubular capillaries at day 5 (Figure 1G) and at day 7 (Figure 1H) but was not seen in the parenchymal cells, even in areas of extensive cell death.

Thus by TUNEL assay the loss of parenchymal viability was not because of apoptosis.

Immunostaining of the Infiltrate

We counted the infiltrating mononuclear cells by indirect immunoperoxidase staining at days 5 and 7 (Table 3). The rejecting GRKO kidneys contained fewer cells staining for CD3, CD8, CD4, and CD45 than did the rejecting 129Sv/J kidneys, particularly at day 7. In contrast class II-positive interstitial cells tended to be increased in GRKO kidneys.

Assessment of MHC Expression by Immunostaining and Radiolabeled Antibody-Binding Assay

The pattern of MHC expression was determined by indirect immunoperoxidase staining. We used rat monoclonal anti-class I (M1) and anti-class II (M5), plus peroxidase-labeled goat anti-rat IgG (Figure 2). Class I and II was strongly expressed in the tubules, glomeruli, and arterial endothelium of rejecting 129Sv/J kidneys. The tubule staining was localized to the basolateral aspect

Figure 3. MHC product expression by radiolabeled antibody-binding assay in transplants and in recipient tissues. **A:** Donor MHC expression was assessed in the CBA recipient native (Nat) kidney and in the donor transplant (Tx) kidney using antibodies for class I (H-2K^bD^b) and class II (I-A^b), specific for 129Sv/J and GRKO donors. **B:** Recipient MHC expression was assessed in the native and transplanted kidneys using antibodies for class I (H-2K) and class II (I-A^k) specific for recipient kidneys. The fold increase (in brackets) for recipient class I and II was the increase over the normal CBA controls. The analysis of variance showed a significant difference between 129Sv/J and GRKO donor class I ($P < 0.0001$) and class II ($P = 0.0001$). There was a significant difference between 129Sv/J and GRKO recipient class I ($P = 0.02$) but not for class II ($P = 0.09$). Significant differences in 129Sv/J and GRKO transplants were determined by analysis of variance.

(Figure 2, A and B). The intensity and extent of MHC expression in nonnecrotic tubules was much less in GRKO kidneys (Figure 2, C and D), and the expression was mainly confined to the cellular infiltrate. M5 stained some tubules of the GRKO kidneys, predominantly those that were dilated or damaged. The MHC expression patterns were also confirmed with mouse monoclonals against donor and recipient class I and class II, plus goat anti-mouse IgG (data not shown).

We assessed MHC induction in rejecting transplants in a radiolabeled antibody-binding assay, using allospecific mouse monoclonals against class I or class II of H-2^b (donor type, Figure 3A) or H-2^k (recipient type, Figure 3B). The extent of MHC induction was calculated by a standard curve, and is shown as a number above the bar indicating fold induction over normal kidney controls. Donor (H-2^b) class I and II expression was strongly induced in 129Sv/J transplants but was much less increased in GRKO transplants (Figure 3A). The MHC induction in 129Sv/J transplants was 10 times greater for class I and 4.5 times greater for class

II than in GRKO transplants. Donor class I or class II was not induced in the native kidney of the recipient, as expected.

Recipient type H-2^k class I and class II in rejecting GRKO and 129Sv/J transplants was increased (Figure 3B), because of the infiltrating recipient cells. Recipient MHC was also strongly induced in the native kidney of CBA recipients rejecting either 129Sv/J or GRKO transplants (Figure 3B). We have previously established that systemic MHC induction in allogeneic responses is highly dependent on IFN- γ .^{22,26} Thus the MHC induction in the native kidney confirms that both 129Sv/J and GRKO allografts evoked IFN- γ production.

Alloantibody Responses of CBA Mice Against 129Sv/J or GRKO

Because vascular injury and necrosis in renal transplants suggests alloantibody-mediated damage,⁴² we investigated the alloantibody response, either to spleen cell

Figure 4. Alloantibody responses of 129Sv/J and GRKO mice to CBA alloantigens. **A:** 129Sv/J and GRKO mice were immunized intraperitoneally with 10^7 CBA spleen cells at day 0 and boosted at day 7, and were bled at day 7 and day 14. **B:** CBA recipients of 129Sv/J ($n = 4$) or GRKO ($n = 5$) kidneys were assessed for alloantibody responses at day 7, and tested against 129Sv/J spleen cell targets. No sera had activity against CBA targets (control not shown). Mean cytotoxicity was higher for all GRKO sera than 129Sv/J at dilutions 1:18 and 1:54. $P < 0.05$ (analyzed by analysis of variance).

immunizations or to kidney allografts. CBA mice were immunized intraperitoneally with 10^7 129Sv/J or GRKO spleen cells, and boosted with a second injection at day 7 (Figure 4A). The first injection of GRKO or 129Sv/J spleen cells evoked equivalent cytotoxic alloantibody production in CBA mice. The second injection increased the cytotoxic activity more in 129Sv/J than in GRKO. Thus the absence of IFN- γ R in the immunizing cells did not prevent the initial alloantibody response but may reduce the secondary response.

We compared the donor-specific cytotoxic alloantibody responses in CBA recipients bearing 129Sv/J versus GRKO transplants (Figure 4B). CBA recipients had 129Sv/J- or GRKO-specific alloantibody demonstrable at day 7. Antibody titers were lower in mice rejecting transplants than in mice immunized with spleen cells. The cytotoxic antibody titer was higher in hosts rejecting GRKO transplants compared to 129Sv/J transplants.

Gene Expression in Rejecting GRKO versus 129Sv/J Allografts

We studied the mRNA levels for cytotoxic T-cell effector molecules, complement inhibitory proteins, and the endothelial protective gene heme oxygenase-1 (HO-1) in the transplant.^{43,44} Rejecting 129Sv/J and GRKO kidneys both showed increased expression of FasL, perforin, and granzyme B mRNA compared to control kidneys, with no obvious differences between GRKO kidneys and 129Sv/J kidneys (Figure 5A). All kidneys had abundant mRNA for complement inhibitory proteins CD46, CD55, and CD59, with no differences between rejecting and control kidneys or between GRKO and 129Sv/J transplants (Figure

Figure 5. The evaluation of mRNA for perforin, granzyme B, FasL, CD46, CD55, CD59, HO-1, NOS2, MIG, and IP10 in normal and rejecting kidneys. GRKO or 129Sv/J kidney was transplanted in CBA mice and harvested at day 7. reverse transcriptase-polymerase chain reaction was used to amplify mRNA using sequence specific primers (**A:** perforin, granzyme B, and FasL; **B:** CD46, CD55, CD59, HO-1, NOS2, MIG, and IP10). Polymerase chain reaction product was Southern blotted and probed with internal oligomers. HPRT was used as a loading control. Phosphorimaging was used to analyze the relative intensity of hybridization between the 129Sv/J transplants and GRKO transplants. The ratio of the phosphorimaging in 129Sv/J kidney to GRKO kidney was approximately twofold higher for NOS2, 2.6-fold for IP10, and threefold for MIG. For HO-1, the GRKO kidney gave a 2.7-fold greater hybridization than 129Sv/J kidney.

5B). HO-1 mRNA was increased in rejecting 129Sv/J and GRKO transplants, but the increase was greater in the GRKO than in the 129Sv/J.

We also examined the mRNA for the IFN- γ -inducible chemokines—monokine inducible by IFN- γ (MIG) and IFN- γ -inducible protein (IP-10)—and for nitric oxide synthetase (NOS2). MIG and IP-10 mRNA levels were strongly induced in rejecting transplants, but the levels were lower in GRKO transplants than in 129Sv/J transplants (Figure 5B). Rejecting GRKO kidneys also displayed lower levels of mRNA for NOS2.

Phosphoimaging analysis was used to estimate the relative intensity of hybridization between the 129Sv/J and GRKO transplants at day 7. The expression of NOS2, IP-10, and MIG was twofold, 2.6-fold, and 3.3-fold lower, respectively, and HO-1 expression was 2.7-fold higher in rejecting GRKO kidney allografts compared to 129Sv/J allografts.

Discussion

The present study establishes that IFN- γ strikingly affects the evolution of tissue injury in a rejecting allograft by a direct action on the graft that prevents thrombosis, congestion, and necrosis during acute rejection. From day 5 allografts lacking IFN- γ Rs in hosts with intact IFN- γ -IFN- γ R systems showed increased venous thrombosis, peritubular congestion, casts, and massive cell death by necrosis. There was decreased T-cell interstitial infiltrate; reduced MHC class I and II induction in the transplant; decreased expression of MIG, IP-10, and NOS2; and increased expression of HO-1. The host MHC expression in the host tissues provided evidence for abundant IFN- γ production and effect in the hosts, making it unlikely that a lack of host IFN- γ production could contribute to these effects. Cytotoxic T-cell gene expression and alloantibody responses were strong in both GRKO and 129Sv/J transplants. The findings indicate that IFN- γ acts on IFN- γ Rs in the grafted tissue to maintain the circulation of the allograft during acute rejection. The IFN- γ may act by modulating graft susceptibility to immune injury or by altering the host immune response.

Although it is possible that grafts lacking IFN- γ Rs evoke an altered host immune response, there was no obvious difference in the immune response evoked by allografts with and without IFN- γ Rs to explain the differences in tissue injury. These CBA hosts produce IFN- γ and have normal IFN- γ Rs, and produce abundant IFN- γ as witnessed by the induction of host MHC expression in the normal native kidney, an effect that is highly dependent on IFN- γ .^{22,26} The expression of the CTL effector genes perforin, granzyme B, and FasL was increased both in 129Sv/J and GRKO grafts at day 7. Alloantibody responses were readily induced by immunization with either both GRKO or 129Sv/J spleen cells, and during rejection of both GRKO or 129Sv/J grafts with and without IFN- γ Rs. Hosts rejecting GRKO kidneys showed higher alloantibody levels than hosts rejecting 129Sv/J transplants, but this could reflect increased absorption to the 129Sv/J kidneys because of their high MHC expression,

rather than increased production. In studies currently in progress we have shown that IgG is deposited on the basolateral membrane of the tubules of wild-type rejecting kidneys but not GRKO kidneys, supporting the possibility of absorption of anti-MHC antibody to host MHC induced by IFN- γ . However, we have not yet proven by elution that this IgG is indeed anti-MHC. Moreover, the modest difference in titer of antibody between hosts rejecting wild-type *versus* GRKO kidneys is probably insufficient to explain the extreme differences in pathology, although we cannot exclude the possibility that different rates and sites of alloantibody deposition contribute to these differences.

A working hypothesis for the excessive necrosis in GRKO allografts is that during acute rejection the lack of IFN- γ Rs renders the grafts susceptible to early thrombosis, congestion, and ischemic necrosis. The TUNEL assays showed little apoptosis of epithelial cells, but do not exclude apoptosis of endothelial cells in small vessels as a mechanism of the venous thrombosis, because TUNEL assays may miss such changes. One mechanism by which IFN- γ could modulate the pathology of rejection is induction of NOS2 and nitric oxide production. The grafts lacking IFN- γ Rs displayed lower levels of mRNA for NOS2. Host inflammatory cells presumably contribute to NOS2 expression and NO production, but the donor vascular cells are unable to respond to IFN- γ and thus should have less NOS2 expression and impaired NO production. Nitric oxide has potential for protective or aggressive effects in graft rejection. NO limits inflammation at endothelial surfaces,⁴⁵ and inhibits apoptosis of some cell types,⁴⁶ including endothelial cells,⁴⁷ despite promoting apoptosis in other cell types.⁴⁸ Inhibitors of NOS tend to make rejection worse, suggesting a protective role.⁴⁹ On the other hand, acute graft rejection is actually attenuated rather than accelerated in hosts lacking NOS2, and thrombosis is not increased,⁵⁰ indicating that NOS2 alone is probably not the critical mediator of the protective effects of IFN- γ , although it may protect against transplant arteriosclerosis.⁵¹ The complex and contradictory effects of NOS2 and NO in graft rejection models invites us to leave the issue of the role of NOS2 unresolved at present.⁵² Moreover, some effects of NOS2 and NO may be dependent on the microenvironment of the organ transplanted.^{46,47}

No direct anticoagulant effect of IFN- γ on platelets or on coagulation is established, and the hosts in this model should regulate host platelet and coagulation functions normally. Moreover, there is no excess of thrombosis in mice lacking IFN- γ or IFN- γ Rs or in our syngeneic control transplants lacking IFN- γ Rs.

The reduction in expression of IFN- γ -inducible chemokines such as MIG and IP-10 may have either protective or injurious effects. Reduced induction of IP-10 and MIG in grafts lacking IFN- γ Rs could alter the rejection process, eg, by reducing the mononuclear infiltrate of cells with CXCR3 receptors. At least one model of rejection shows that IFN- γ participates in graft destruction via the induction of MIG.⁵³ Thus the lack of MIG and IP-10 induction in GRKO should have protected rather than injured the graft. We also considered the possibility that the

damage and thrombosis in the vasculature reflect dysregulated diapedesis because of a lack of appropriate chemokine milieu, but the pathology showed that the leukocytes in the allograft had crossed into the interstitium and did not show excessive endothelialitis.

Given the potency of antibody to damage endothelium in both allografts and xenografts, we considered the possibility that the thrombosis in GRKO mice reflected antibody injury because of disturbed expression of complement regulatory proteins or HO-1. Wang et al³³ concluded that IFN- γ protects concordant rat xenotransplants against vascular injury by antibody, supporting the concept that IFN- γ may limit antibody injury to vessels. In our experiments we found no changes in expression of complement regulatory proteins in GRKO kidneys, confirming previous conclusions that these proteins are not regulated by IFN- γ .⁵⁴⁻⁵⁷ Deficiency in the endothelial protective effect of HO-1 could not be incriminated in the thrombosis of GRKO kidneys because the GRKO actually had higher levels of HO-1, perhaps as a response to the injury to the tissue.⁵⁸ Thus antibody injury remains a potential cause of the excessive thrombosis, but by an undefined mechanism.

Of the genes in the graft that may mediate the effect of IFN- γ -IFN- γ R on the pattern of graft rejection, one candidate is the MHC genes themselves. Induction of intense MHC expression during graft rejection is often assumed to favor rejection because MHC products are powerful alloantigens and key antigen presenting structures. However, allogeneic MHC can also lead to tolerance or anergy, and MHC negatively regulates natural killer receptors, at least *in vitro*. High MHC expression in less critical tissue components such as tubule epithelium could temporarily divert immune effectors such as alloantibody or CTL from engaging the critical endothelial cells of the vasculature. Such effects could be mediated either by membrane-bound MHC or by soluble MHC released from the graft. This effect would be temporary, because high MHC expression could facilitate later invasive lesions such as tubulitis and endothelialitis. The fact that alloantibody levels are lower in hosts rejecting 129Sv/J grafts than in hosts rejecting GRKO grafts (which have much less MHC expression) could be used to support this explanation. Thus low MHC expression in rejecting GRKO transplants might favor vascular injury either by a reduced immune-modulating role on antibody and CTL mechanisms, or by reduced negative signaling to natural killer cell inhibitory receptors.

There are some parallels between the present results and those described by Pober et al,⁵⁹ in that both reflect direct effects of IFN- γ on graft vessels. The latter system demonstrated throughout a longer time frame that IFN- γ could directly lead to arteriosclerosis. Other observations also suggest a similar adverse long-term effect of IFN- γ on arteries in grafts.^{28,29} Thus, the early benefits of IFN- γ actions during acute rejection may be followed by direct deleterious effects throughout a longer time frame.

The ability of IFN- γ to act directly on the target tissue to prevent vascular thrombosis and maintain tissue viability invites the question of whether this mechanism operates in other inflammatory diseases. Because a similar effect operates in concordant xenografts,³³ it is possible that

one reason for severe vascular injury in discordant xenografts is the species specificity of IFN- γ Rs, making discordant xenografts comparable to GRKO grafts. Moreover, in models of autoimmune disease, hosts lacking IFN- γ or IFN- γ Rs sometimes display excessive tissue injury that is usually attributed to lack of immunoregulatory effects of IFN- γ on lymphocytes, eg, in experimental arthritis,^{60,61} encephalitis,⁶² and uveitis.⁶³ Thus the principle is that IFN- γ can directly affect the target tissue in a disease process independent of its effects on the host immune response, reminding us to avoid attributing IFN- γ effects in disease states exclusively to its direct effects on inflammatory cells.

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