Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Targeted Deletion of Fgl-2/Fibroleukin in the Donor Modulates Immunologic Response and Acute Vascular Rejection in Cardiac Xenografts

Michael Mendicino, MingFeng Liu, Anand Ghanekar, Wei He, Cheryl Koscik, Itay Shalev, Mojib Javadi, Julie Turnbull, Wenhao Chen, Laisum Fung, Seisuke Sakamoto, Phillip Marsden, Thomas K. Waddell, M. James Phillips, Reginald Gorczynski, Gary A. Levy and David Grant

Circulation 2005, 112:248-256: originally published online July 5, 2005
doi: 10.1161/CIRCULATIONAHA.105.534271
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Targeted Deletion of Fgl-2/Fibroleukin in the Donor Modulates Immunologic Response and Acute Vascular Rejection in Cardiac Xenografts

Michael Mendicino; MingFeng Liu; Anand Ghanekar, MD; Wei He; Cheryl Koscik; Itay Shalev; Mojib Javadi; Julie Turnbull; Wenhao Chen; Laisum Fung; Seisuke Sakamoto, MD; Phillip Marsden, MD; Thomas K. Waddell, MD, PhD; M. James Phillips, MD; Reginald Gorczynski, MD, PhD; Gary A. Levy, MD; David Grant, MD

Background—Xenografts ultimately fail as a result of acute vascular rejection (AVR), a process characterized by intravascular thrombosis, fibrin deposition, and endothelial cell activation.

Methods and Results—We studied whether targeted deletion of Fgl-2, an inducible endothelial cell procoagulant, (Fgl-$2^{-/-}$) in the donor prevents AVR in a mouse-to-rat cardiac xenotransplantation model. By 3 days after transplant, Fgl-$2^{+/+}$ grafts developed typical features of AVR associated with increased levels of donor Fgl-2 mRNA. Grafts from Fgl-$2^{-/-}$ mice had reduced fibrin deposition but developed cellular rejection. Treatment with a short course of cobra venom factor and maintenance cyclosporine resulted in long-term acceptance of both Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ grafts. On withdrawal of cyclosporine, Fgl-$2^{+/+}$ grafts developed features of AVR; in contrast, Fgl-$2^{-/-}$ grafts again developed acute cellular rejection. Rejecting Fgl-$2^{+/+}$ hearts stained positively for IgG, IgM, C3, and C5b-9, whereas rejecting Fgl-$2^{-/-}$ hearts had minimal Ig and complement deposition despite xenoantibodies in the serum. Furthermore, serum containing xenoantibodies failed to stain Fgl-$2^{-/-}$ long-term treated hearts but did stain wild-type heart tissues. Treatment of Fgl-$2^{-/-}$ xenografts with mycophenolate mofetil and tacrolimus, a clinically relevant immune suppression protocol, led to long-term graft acceptance.

Conclusions—Deletion of Fgl-2 ameliorates AVR by downregulation of xenoantigens and may facilitate successful clinical heart xenotransplantation. **(*Circulation*. 2005;112:248-256.)**

Key Words: apoptosis ■ endothelium ■ immunology ■ thrombosis ■ transplantation

Transplantation is the treatment of choice for patients with end-stage heart failure, but its application is limited by a severe shortage of available human organs. Xenotransplantation potentially offers an unlimited supply of organs.[1,2] Hyperacute rejection of porcine hearts transplanted into nonhuman primates, which occurs within minutes to hours of graft implantation, has been overcome through depletion of preformed xenoreactive antibodies, prevention of complement activation, and production of 1,3-galactosyltransferase–deficient pigs, but these strategies have failed to prevent acute vascular rejection (AVR).[3,4] AVR, which occurs days to weeks after transplantation in nonhuman primates, is characterized by endothelial cell (EC) activation, intravascular thrombosis, and fibrin deposition.[5–7]

Other investigators have shown that activation of graft EC during AVR leads to a loss of thromboregulatory molecules, including thrombomodulin, heparan sulfate, and CD39, and an increase in expression of tissue factor (TF).[8,9] We recently reported that porcine kidney xenografts undergoing AVR showed induction of a novel procoagulant Fgl-2 on graft vascular EC.[10] Fgl-2 belongs to a family of fibrinogen-like proteins (fibrinogen-related domain [FRED]) that includes fibrinogen, tenascin, ficolin, and angiopoietin.[11–13] FRED proteins participate in coagulation, cell adhesion, transendothelial migration, cell proliferation, and regulation of transcription factors.[14,15] Fgl-2 is expressed and differentially regulated in many cell types, including EC.[16] Membrane-associated Fgl-2 has prothrombinase activity, whereas secreted Fgl-2 has immunomodulatory activity.[17,18] Distinct from TF, Fgl-2 directly cleaves prothrombin to thrombin and plays a key role in the pathogenesis of some diseases

Received March 3, 2004; revision received January 5, 2005; accepted March 8, 2005.

From the Multi Organ Transplant Program, University Health Network (M.M., M.L., A.G., W.H., C.K., I.S., M.J., J.T., W.C., L.F., S.S., P.M., T.K.W., M.J.P., R.G., G.A.L., D.G.); Canadian Institutes of Health Research Group on Cellular and Molecular Mechanisms of Organ Injury (M.M., M.L., A.G., C.K., I.S., W.C., P.M., M.J.P., R.G., G.A.L.); Departments of Immunology (M.M., C.K., I.S., M.J., R.G., G.A.L.) and Surgery (A.G., T.K.W., R.G., D.G.), Faculty of Medicine, University of Toronto; Department of Pathology, Hospital For Sick Children (M.J.P.); and St Michael's Hospital and Department of Medicine, University of Toronto (P.M.), Toronto, Ontario, Canada. Dr Mendicino is a Canadian Institutes of Health Research Trainee in Regenerative Medicine.

Correspondence to Gary A. Levy, MD, Toronto General Hospital, 585 University Ave, 11C-1236, Toronto, Ontario, Canada M5G 2N2. E-mail glfgl2@att.global.net

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.105.534271**

characterized by intravascular thrombosis and fibrin deposition.

To study the role of Fgl-2 in AVR, we transplanted hearts from Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ donor mice[19] into Lewis rats. As confirmed in the studies reported below, these recipients exert a strong immunologic response to murine xenoantigens, resulting in a predictable and reproducible pattern of AVR similar to that seen in primates.[20]

Methods

Animals

The generation and characterization of mice with a targeted deletion of Fgl-2 have been described.[19] Male mice on a mixed C57/bl6×129/SvJ background were used for all experiments, and control Fgl-$2^{+/+}$ mice were age and litter matched. Inbred adult male Lewis rats were obtained from Charles River (Montreal, Quebec, Canada). All animals were housed and handled in accordance with guidelines set by the Canadian Council on Animal Care.

Mouse-to-Rat Cardiac Xenotransplantation and Immune Suppression

Animals were anesthetized with an intraperitoneal injection of Somnotol (MTC Pharmaceuticals) at a dose of 65 mg/kg for all procedures. Each donor mouse heart was removed as previously described and implanted heterotopically in the groin of a Lewis rat.[21] The ascending aorta and pulmonary artery of the donor heart were anastomosed to the recipient femoral artery and femoral vein, respectively. Grafts were observed for 30 minutes after reperfusion and subsequently were assessed daily by palpation. Cessation of graft function was inferred by an absence of palpable ventricular contractions and was confirmed by direct examination of the graft. For accommodation induction, reagents and protocol were as previously described.[22] Tacrolimus with mycophenolate mofetil was used to provide clinically relevant immune suppression.[23]

Serum Xenoantibody Levels

The levels of xenoreactive antibodies in the serum of rats before and after xenotransplantation with hearts from wild-type (Fgl-$2^{+/+}$) or knockout (Fgl-$2^{-/-}$) mice were quantified after binding to the surface of Fgl-$2^{+/+}$ heart tissue.[24] Briefly, heart tissue from Fgl-$2^{+/+}$ hearts was fixed with 10% formalin in PBS on glass slides. The slides were blocked for 1 hour with 2% goat serum in PBS followed by another 1 hour of blocking with 10% FBS in PBS. Serum from naive or transplanted rats was added for 1 hour at 37°C. After extensive washing, mouse anti-rat Ig–specific antibody (Becton Dickinson) was added for 1 hour at 37°C. After 3 washes, a horseradish peroxidase–conjugated goat anti-mouse antibody (Becton Dickinson) was added for 1 hour, then substrate was added and developed. Samples were transferred to 96-well plates and read in a Titerteck spectrophotometer. Units (μg/mL) were extrapolated from a standard curve derived with the use of known quantities of purified rat Ig antibodies.

Northern Blotting and Reverse Transcriptase–Polymerase Chain Reaction

Explanted grafts were snap-frozen in liquid nitrogen and preserved at −80°C. Total RNA was isolated with the use of Trizol reagent (Life Technologies) according to the manufacturer's instructions. mRNA was isolated with the use of the Oligotex mRNA purification kit (Qiagen). Northern blot was performed with standard procedures with the use of gene-specific primers for Fgl-2 (sense 5′-TGCCCACGCTGACCATCCA-3′ and antisense 5′-AGG GGTACCGATCGTTGTC-3′) and TF (sense 5′-CAGTTCATGGGGACGGAGA-3′ and antisense 5′-GGTTCTTTCTGCGCTTGCA-3′), and a 1-kb cDNA probe for GAPDH was obtained from Clontech. Membranes were hybridized with radiolabeled cDNA probes with the use of ExpressHyb hybridization solution (Clontech). ImageQuant 5.1 software (Molecular Dynamics) quantified results by using pixel number counts normalized to GAPDH counts with background correction. Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed with a 1-step RT-PCR kit (Qiagen) with gene-specific primers for mouse Fgl-2 (sense 5′-GCGGGAATGGAGGGAAT-3′ and antisense 5′-TATCGTTGCCCAACCAAAAT-3′), rat Fgl-2 (sense 5′-AGACCCTGGCGGGAAT-3′ and antisense 5′-CGGCTTTGTAGTCCTTCCAC-3′), and β-actin (sense 5′-ATGTTTGAGACCTTCAACAC-3′ and antisense 5′-CACGTCACACTTCATGATGGA-3′).

Histopathology and Immunopathology

For light microscopy, specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) or Martius scarlet blue stain by standard procedures. For transmission electron microscopy (TEM), specimens were treated as previously described.[25] For immunohistochemistry of recipient cell lineage, 6-μm cryostat sections were fixed in cold acetone and subsequently studied by a standard indirect avidin-biotin method (Histomouse-SP kit, Zymed). Anti-rat leukocyte common antigen (LCA or CD45, clone OX-1), anti-rat $\alpha\beta$ T-cell receptor (clone R73), anti-rat CD45RA (clone OX-33), anti-rat NKR-P1A (clone 10/78) mouse monoclonal antibodies, and anti-rat granulocyte (clone HIS48), a mouse polyclonal antibody, were all obtained from BD Pharmingen. Monoclonal mouse anti-rat ED1 (clone ED1) was obtained from Serotec. A multiply absorbed biotinylated polyclonal goat anti-mouse Ig (BD Pharmingen) was used as a secondary antibody in all cases. Rat immunoglobulin and complement were studied by immunofluorescence with FITC-labeled rabbit anti-rat IgG $F(ab')_2$ fragment and goat anti-rat IgM $F(ab')_2$ fragment (Jackson Immunoresearch Labs) or mouse anti-rat ED11 (rat reticulum) (Serotec) and mouse anti-human C5b-9 (Dako), respectively. FITC-labeled rat anti-mouse IgG $F(ab')_2$ fragment (Jackson Immunoresearch Labs) was used as the secondary antibody for rat complement. Isotype-matched nonimmunized mouse monoclonal antibodies were included as negative controls in each experiment. Terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) was performed with standard procedures on paraffin-embedded histological sections with the use of an automated in situ hybridization instrument (Discovery Ventana Medical Systems).

Stored serum samples from accommodated rats with functioning and rejecting grafts were incubated with frozen tissue sections at a 1:50 dilution for 2 hours and then studied with the use of immunofluorescence for rat immunoglobulin as described above.

Morphometric Analysis

Naive hearts and syngeneically transplanted hearts harvested on postoperative days 1, 2, and 3 were used as controls. Morphometric analyses of tissue sections were performed to quantify the histopathological changes in the ventricular myocardium, including thrombosis, total cellular infiltration, cardiomyocytolysis, and specific cell lineage infiltration in Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ cardiac xenografts on postoperative days 1, 2, and 3 (n=4 hearts per day per genotype).

Primary Mouse Aortic EC Culture, Immunoprecipitation, and Western Blot

Primary mouse aortic EC (MAEC) were harvested, cultured, and characterized as described.[26] MAEC were stimulated with 50% heat-inactivated Lewis rat serum or media alone for 24 hours for immunoprecipitation and analysis by SDS-PAGE gel electrophoresis, with the use of rabbit anti-mouse Fgl-2 polyclonal antibody (R1) for capture and with the same primary with horseradish peroxidase–conjugated donkey anti-rabbit IgG as a secondary antibody for detection.

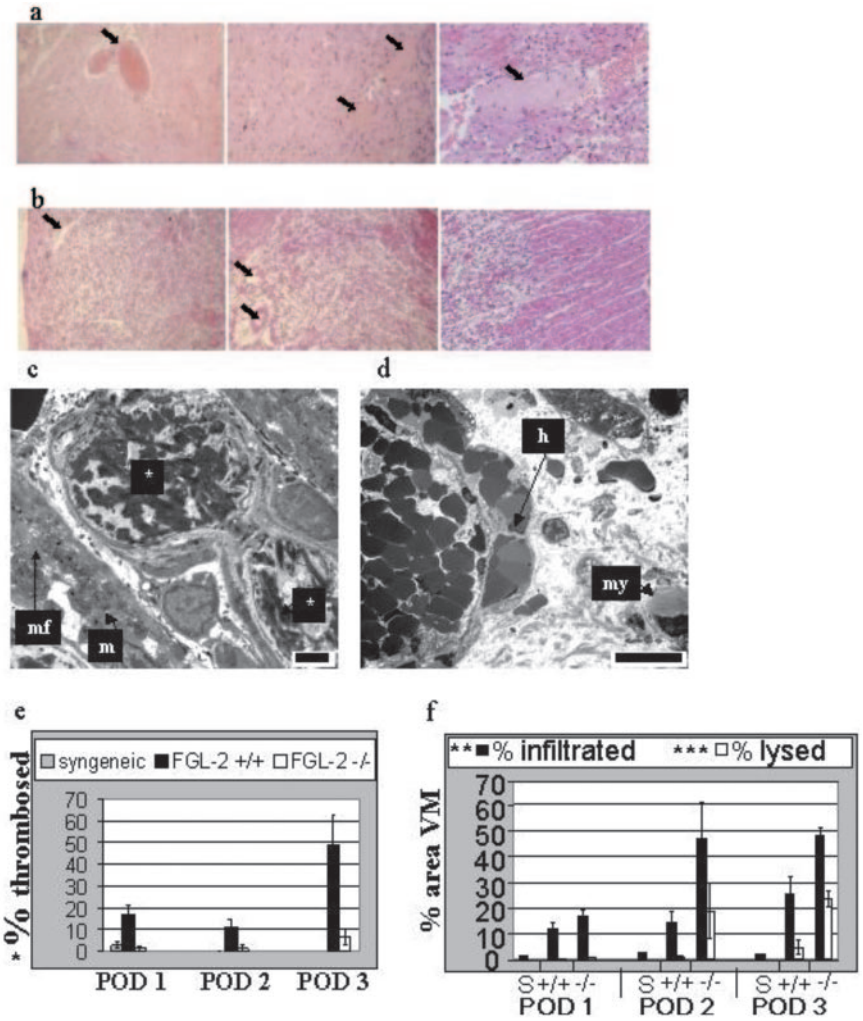

Figure 1. Effect of Fgl-2 deletion on cardiac xenografts. Three representative sections of myocardium from Fgl-$2^{+/+}$ (a) and Fgl-$2^{-/-}$ (b) myocardium are shown. a, In Fgl-$2^{+/+}$ grafts, vessels show thrombosis (arrows). b, In Fgl-$2^{-/-}$ grafts, vessels are patent (arrows), but there is myocytolysis with a dense infiltrate of inflammatory cells. a and b, Postoperative day 3. H&E stain; magnification ×100. c, Electron micrograph of Fgl-$2^{+/+}$ myocardium showing myocytes (mf indicates myofilaments; m, mitochondria) and occlusive vascular thrombosis (*fibrin thrombus). Bar=1 μm. d, Electron micrograph of Fgl-$2^{-/-}$ myocardium showing congestion and hemorrhage (h) and myocytolysis with myocyte remnant (my). Bar=10 μm. c and d, Postoperative day 2.5. Lead citrate stain. Bar=2 μm for c and 10 μm for d. e and f, Results of morphometric analysis of thrombosis (e) and myocytolysis and cell infiltration (f). Data are expressed as percent vessels thrombosed (mean±SEM) on each postoperative day (POD) ($P<0.0004$). Total cellular infiltration and cardiomyocytolysis are expressed as percent area of myocardium affected (area VM) (mean±SEM).

TNF-α Protein Assay

Hearts were homogenized on ice in T-PER reagent (Pierce) supplemented with protease inhibitor cocktail (BD Pharmingen), and samples were assessed for tumor necrosis factor-α (TNF-α) protein concentration by the rat TNF-α sandwich ELISA kit (Pierce) according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as mean±SEM for thrombosis, total cell infiltration, cardiomyocytolysis, and TNF-α protein expression or mean±SD for specific cell lineage infiltration. Survival of Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ cardiac xenografts was analyzed by Kaplan-Meier plots and compared with the Mantel-Cox log-rank test. A factorial ANOVA was used to compare genotype×postoperative day for histology findings. Linear regression analyses were used to examine the relationship between total cellular infiltration and cardiomyocytolysis.

Results

Fgl-$2^{-/-}$ Xenografts Were Protected From AVR but Developed an Accelerated Cellular Immune Response

Both Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ grafts recovered 3 days after transplant stained strongly for IgM and C3 but were negative for IgG and C5b-9, reflecting the innate humoral response that is induced in this xenograft model (data not shown). Survival of Fgl-$2^{-/-}$ grafts was not prolonged (3.2±0.4 versus 2.9±0.3 days) (n=10 per group; P=NS).

Cardiac xenografts from Fgl-$2^{+/+}$ littermate control mice developed typical features of AVR characterized by an extensive intravascular thrombosis seen on both H&E microscopy (Figure 1a) and TEM (Figure 1c). In contrast, the grafts from Fgl-$2^{-/-}$ mice showed minimal thrombosis and only focal hemorrhages (Figure 1b). Morphometric analysis showed a difference in thrombosed vessels ($P<0.0004$) (Figure 1e).

Although Fgl-$2^{-/-}$ grafts did not develop AVR, they showed dense infiltration by recipient mononuclear cells (Figure 1b). The cellular infiltration correlated spatially with areas of myocardial cell loss (cardiomyocytolysis) (Figure 1d). Morphometric analyses showed increased cellular infiltration and cardiomyocytolysis in the Fgl-$2^{-/-}$ grafts ($P<0.002$; $P<0.0005$) (Figure 1f). These assays were highly reproducible with <1% variability for all factors studied.

By immunohistochemistry, graft-infiltrating cells in Fgl-$2^{-/-}$ hearts were predominantly macrophages (Figure 2a) ($P<0.0006$) (Figure 2b). TUNEL analysis revealed that Fgl-$2^{-/-}$ grafts had increased apoptotic cardiomyocytes (Figure 3). TEM showed nuclear pyknosis, chromatin clumping, myofilament condensation, and residual cytoplasmic and

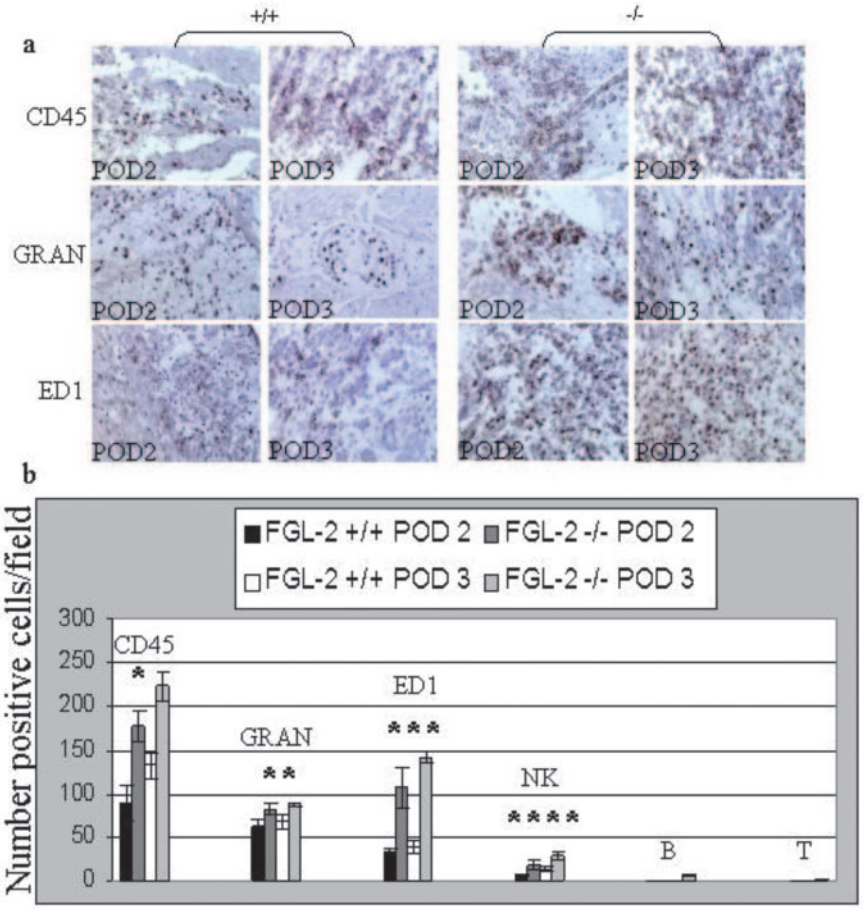

Figure 2. Comparison of recipient cell infiltration in Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ cardiac xenografts. a, Immunohistochemistry on postoperative days 2 and 3 for Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ cardiac xenografts for CD45 (all leukocytes), GRAN (neutrophils), and ED1 (macrophages). CD45RA (B cells), $\alpha\beta$ T-cell receptor (T cells), and NKR-P1A (natural killer [NK] cells) were also stained (pictures not shown). Magnification ×250. b, Morphometric analysis for specific cell lineage infiltration. Data are expressed on the *y* axis as number of positively stained cells per low-powered field (mean±SD). The *x* axis is the marker used. *$P<0.0001$; **P 0.0001; ***$P<0.0006$; ****$P<0.00016$. POD indicates postoperative day.

nuclear fragments (Figure 1d). Graft-infiltrating macrophages were in close contact with apoptotic cardiomyocytes (Figure 3b). In contrast, grafts from Fgl-$2^{+/+}$ donors revealed intact cardiomyocytes containing swollen mitochondria with dense matrical granules and disrupted myofilaments, consistent with ischemic necrosis (Figure 1a and 1c). In grafts from Fgl-$2^{-/-}$ mice, venulocapillary EC apoptosis was seen only in areas of heavy infiltration of macrophages, which was associated with high TNF-α protein expression (Figure 3c). In contrast, minimal macrophage infiltration and TNF-α protein expression were seen in grafts from Fgl-$2^{+/+}$ mice ($P<0.038$) (Figure 3d), which instead demonstrated vascular EC apoptosis adjacent to areas with intravascular thrombosis (Figure 3c).

AVR Was Associated With Increased Fgl-2 mRNA and Protein Levels in Wild-Type Donor Grafts and Primary EC, Respectively

Fgl-2 mRNA progressively increased beginning on postoperative day 1, whereas TF mRNA levels decreased compared with baseline levels (Figure 4a). By densitometry, a 3.3-fold increase in Fgl-2 mRNA levels and a 1.6-fold decrease in TF mRNA levels were detected by postoperative day 1 (Figure 4b). Semiquantitative RT-PCR, performed with the use of primer sets specific for either mouse or rat Fgl-2, showed minimal levels of rat Fgl-2 mRNA within the graft (Figure 4c), whereas mouse Fgl-2 levels increased, showing that the induced Fgl-2 mRNA was of donor origin. MAEC propagated from Fgl-$2^{+/+}$ mice showed induced Fgl-2 protein expression in cell lysates after incubation with heat-inactivated Lewis rat serum, whereas Fgl-$2^{-/-}$ MAEC showed no Fgl-2 protein expression (Figure 4d).

Effect of Treatment With Cobra Venom Factor and Cyclosporine on Graft Survival

Animals receiving cardiac xenografts were treated with cyclosporine, cobra venom factor, or a short course of cobra venom factor combined with maintenance cyclosporine (Table and Figure 5). Treatment with cobra venom factor and cyclosporine alone had minimal effects on survival or histology compared with untreated controls (Figures 1 and 5). Induction with cobra venom factor and maintenance cyclosporine resulted in long-term acceptance of cardiac xenografts from both Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ donors. At time of the animals' death on postoperative day 60, these grafts showed nearly normal histology (Figure 5).

Withdrawal of cyclosporine at postoperative day 60 led to rejection within 11 days in both groups (Table) (P=NS). Rejected Fgl-$2^{+/+}$ grafts had intravascular thrombosis and a mixed cellular infiltrate (Figure 5), whereas grafts from Fgl-$2^{-/-}$ mice had a histology similar to acute allograft rejection characterized by vasculitis/endotheliitis and a dense cellular infiltrate (Figure 5), comprising primarily $\alpha\beta$ T-cell receptor plus T cells (data not shown).

The Fgl-$2^{+/+}$ grafts were strongly positive for both rat IgM and IgG, as well as C3 and C5b-9. Martius scarlet blue staining confirmed extensive fibrin deposition (Figure 6). In

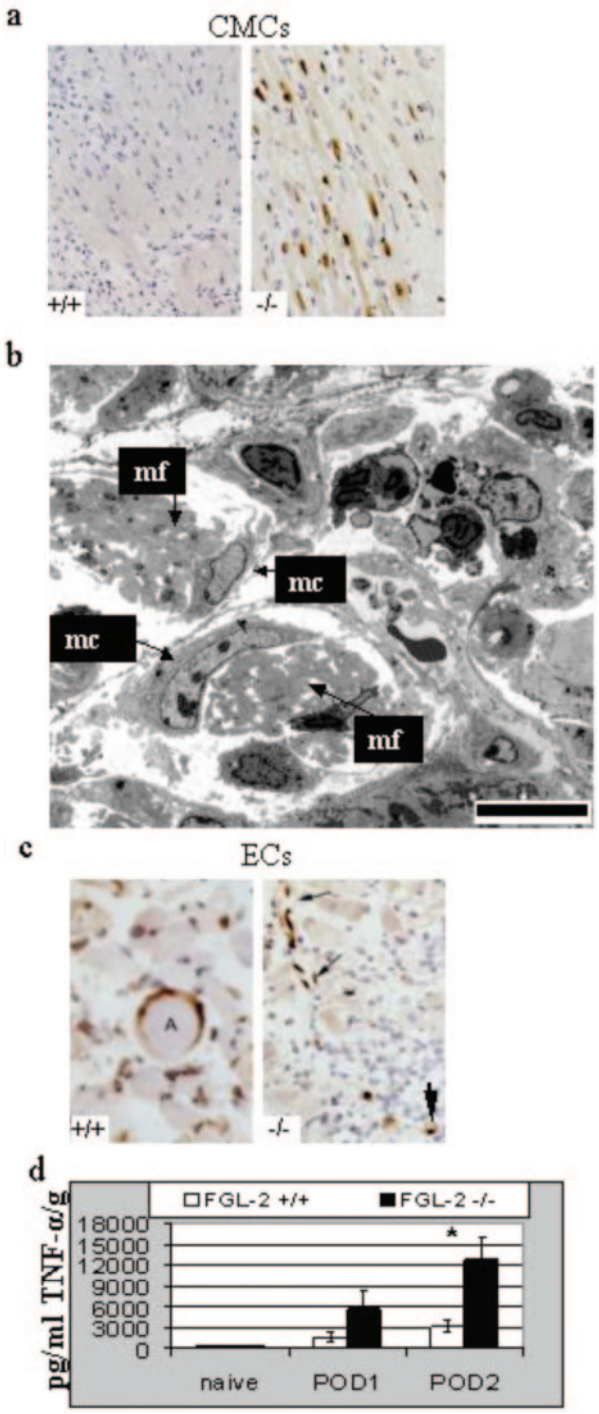

Figure 3. Comparison of apoptosis and TNF-α protein levels in Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ cardiac xenografts. a through c, Representative TUNEL stain on postoperative day 3 for Fgl-$2^{+/+}$ cardiac xenografts and TUNEL and TEM on postoperative day 3 for Fgl-$2^{-/-}$ cardiac xenografts, showing cardiomyocytes (CMCs) (a and b) and vascular EC (c) apoptosis. b, Electron micrograph (Fgl-$2^{-/-}$) showing cell-cell contact between macrophage (mc) and myocardial cell fragment (mf) in late stage of apoptosis (bar=10 μm). a and c are all $\times$250 except lower left micrograph at $\times$400. c, A thrombosed arteriole from an Fgl-$2^{+/+}$ graft is shown. Black arrows indicate venulocapillary EC apoptosis in Fgl-$2^{-/-}$ grafts. d, TNF-α protein expression in Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ cardiac xenografts. Data are expressed on the *y* axis as pg/mL TNF-α protein per gram wet tissue (mean$\pm$SEM). The *x* axis is postoperative day. *$P<0.038$.

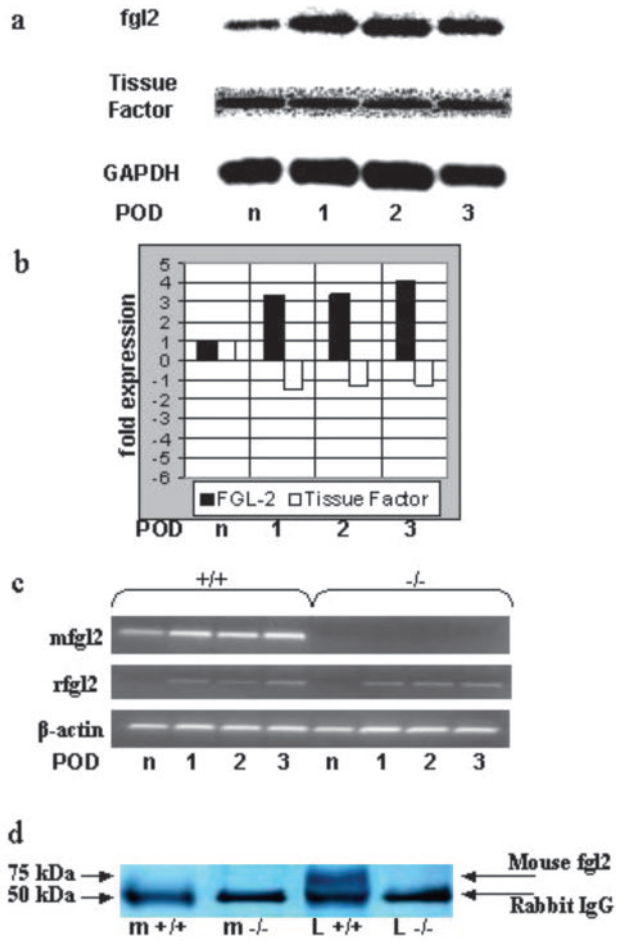

Figure 4. Fgl-2 expression in vascular endothelium of mouse-to-rat cardiac xenografts in vivo and in vitro. a, mRNA Northern blot demonstrating Fgl-2 and TF transcript levels in Fgl-$2^{+/+}$ cardiac xenografts, with GAPDH as a loading control. n indicates a naive heart; POD, postoperative day. b, Densitometric quantification of blot in panel a, based on fold expression of induced divided by constitutive transcript levels. c, Semiquantitative RT-PCR demonstrates donor and recipient contributions of mFgl-2 transcript levels from Fgl-$2^{+/+}$ cardiac xenografts, with β-actin as a loading control. d, Western blot of an $\approx$70-kDa Fgl-2 protein immunoprecipitated from Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ MAEC, stimulated with 50% Lewis rat serum (heat-inactivated) (L) or media control (m) for 24 hours.

contrast, grafts from Fgl-$2^{-/-}$ mice had minimal immunoglobulin and complement deposition and scant macrovascular fibrin. Nevertheless, the latter grafts had cardiomyocyte apoptosis by TUNEL staining (Figure 6). In a pilot study, treatment of rats with mycophenolate mofetil and tacrolimus daily led to long-term acceptance of all Fgl-$2^{-/-}$ heart grafts (>21 days), whereas 33% of Fgl-$2^{+/+}$ grafts underwent vascular rejection (Table).

Serum xenoantibodies from rats transplanted with Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ hearts were measured by ELISA (Figure 7). As expected, at time 0, levels of preformed xenoantibodies were high. Subsequent levels were lower, reflecting possible adsorption by the graft and/or reduced production. Reduced levels of xenoantibodies were detected in the Fgl-$2^{-/-}$ recipients, but antibody was present (Figure 7I). Fgl-$2^{-/-}$ grafts recovered from rats that had received long-term immunosup-

Effect of Recipient Immunosuppression on Survival and Pathology

Donor Genotype	Recipient Treatment	Survival (POD)	Pathology Appearance
Fgl-2$^{+/+}$	CsA (n=4)	3, 3, 3, 3	AVR
Fgl-2$^{-/-}$	CsA (n=3)	3, 3, 3	Cellular rejection
Fgl-2$^{+/+}$	CVF (n=4)	4, 5, 5, 6	AVR
Fgl-2$^{-/-}$	CVF (n=4)	4, 4, 5, 6	Cellular rejection
Fgl-2$^{+/+}$	CsA+CVF (n=3)	60,* 60,* 60*	Normal
Fgl-2$^{-/-}$	CsA+CVF (n=3)	60,* 60,* 60*	Normal
Fgl-2$^{+/+}$	CsA+CVF; CsA withdrawal POD 60 (n=4)	70, 67, 69, 71	AVR
Fgl-2$^{-/-}$	CsA+CVF; CsA withdrawal POD 60 (n=3)	66, 69, 69	Allo-like rejection
Fgl-2$^{+/+}$	MMF+TAC (n=3)	6, 15, >21	AVR
Fgl-2$^{-/-}$	MMF+TAC (n=3)	>21, >21, >21	

Rats receiving Fgl-2$^{+/+}$ or Fgl-2$^{-/-}$ grafts were treated with cyclosporine A (CsA) alone, cobra venom factor (CVF) alone, CsA and CVF in combination, or mycophenolate mofetil (MMF) and tacrolimus (TAC) in combination per protocol. Survival and pathology of Fgl-2$^{+/+}$ and Fgl-2$^{-/-}$ cardiac xenografts in immunosuppressed Lewis rat recipients in all treated groups are shown.

*Postoperative day (POD) 60 was chosen as an arbitrary reference to long-term survival.

pression failed to stain with rat serum containing xenoreactive antibodies (Figure 7F and 7H). Serum from rats that had received Fgl-2$^{+/+}$ hearts or Fgl-2$^{-/-}$ hearts produced similar staining of tissue sections from naive Fgl-2$^{+/+}$ hearts (Figure 7A to 7E and 7G).

Discussion

The present data demonstrate an important role for Fgl-2 in the pathophysiology of xenograft AVR and provide a rationale for targeting Fgl-2 to facilitate successful human xenotransplantation. Fgl-2 mRNA expression was upregulated in association with the progression of AVR in donor grafts from Fgl-2$^{+/+}$ mice in a mouse-to-rat cardiac xenograft model. With no immune suppression, both Fgl-2$^{+/+}$ and Fgl-2$^{-/-}$ grafts were rejected by day 3. The Fgl-2$^{+/+}$ grafts had vascular thrombosis and fibrin deposition and other classic features of AVR, whereas the Fgl-2$^{-/-}$ heart grafts had patent blood vessels with minimal fibrin deposition but notable monomorphic cellular infiltrates, cardiomyocytolysis, and EC apoptosis, features consistent with an accelerated form of cellular rejection. Two injections of cobra venom factor and maintenance cyclosporine led to normal myocardial histology and long-term acceptance of both the Fgl-2$^{+/+}$ and Fgl-2$^{-/-}$ hearts. Withdrawal of cyclosporine at day 60 led to rejection of the Fgl-2$^{+/+}$ and Fgl-2$^{-/-}$ grafts within 11 days. The Fgl-2$^{+/+}$ rejected grafts showed pathological features of AVR, including thrombosis and immunoglobulin deposition, whereas the Fgl-2$^{-/-}$ grafts showed features of acute cellular rejection with minimal immunoglobulin deposition. Pilot studies using a clinically relevant immunosuppressive protocol consisting of tacrolimus with mycophenolate mofetil in rats transplanted with Fgl-2$^{-/-}$ grafts resulted in long-term acceptance, whereas some of the Fgl-2$^{+/+}$ grafts underwent AVR.

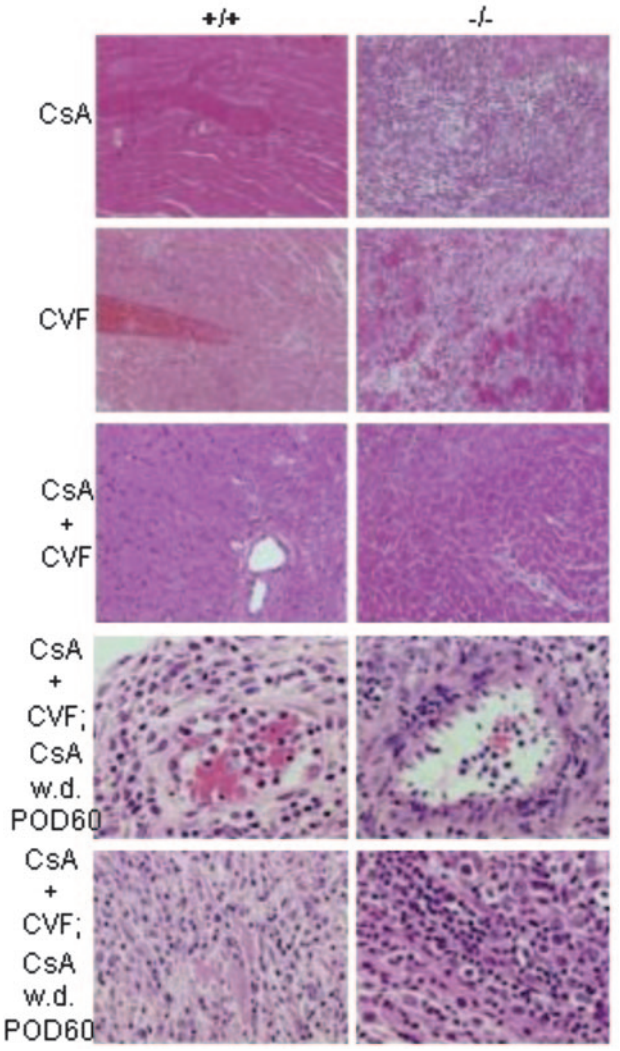

Figure 5. Comparison of Fgl-2$^{+/+}$ (left) and Fgl-2$^{-/-}$ (right) grafts treated with various immunosuppressive protocols. Panels 1 and 2: Fgl-2$^{+/+}$ and Fgl-2$^{-/-}$ grafts treated with either cyclosporine (CsA) alone or cobra venom factor (CVF) alone rejected with typical features of untreated controls (Figure 1). Fgl-2$^{+/+}$ grafts developed AVR including vascular thrombosis, whereas Fgl-2$^{-/-}$ grafts showed myocytolysis, hemorrhage, and heavy cellular infiltrates. Panel 3, A short course of cobra venom factor and maintenance cyclosporine (combined treatment) resulted in normal histology of both Fgl-2$^{+/+}$ and Fgl-2$^{-/-}$ grafts at animals' death on postoperative day 60. Panels 4 and 5, Rats transplanted with Fgl-2$^{+/+}$ or Fgl-2$^{-/-}$ grafts and first treated with a short course of cobra venom factor in conjunction with cyclosporine, as in panel 3. Withdrawal of cyclosporine on postoperative day 60 led to rejection within 11 days. Rejecting Fgl/2$^{+/+}$ grafts showed a pattern consistent with AVR, with vascular thrombosis and cellular infiltrate, mostly macrophages. In contrast, rejecting Fgl-2$^{-/-}$ grafts showed vasculitis (endotheliitis) and cellular infiltrate, predominantly T cells with eosinophils compatible with allograft rejection. Magnification, rows 1 to 3, ×100; row 4, ×400; row 5, ×250.

The present data show that deletion of Fgl-2 from the donor endothelium ameliorates thrombosis and fibrin deposition in cardiac xenografts. Serum containing xenoreactive antibodies failed to bind to Fgl-2$^{-/-}$ grafts recovered from rats that had been treated with long-term immunosuppression, suggesting that deletion of Fgl-2 and long-term immunosup-

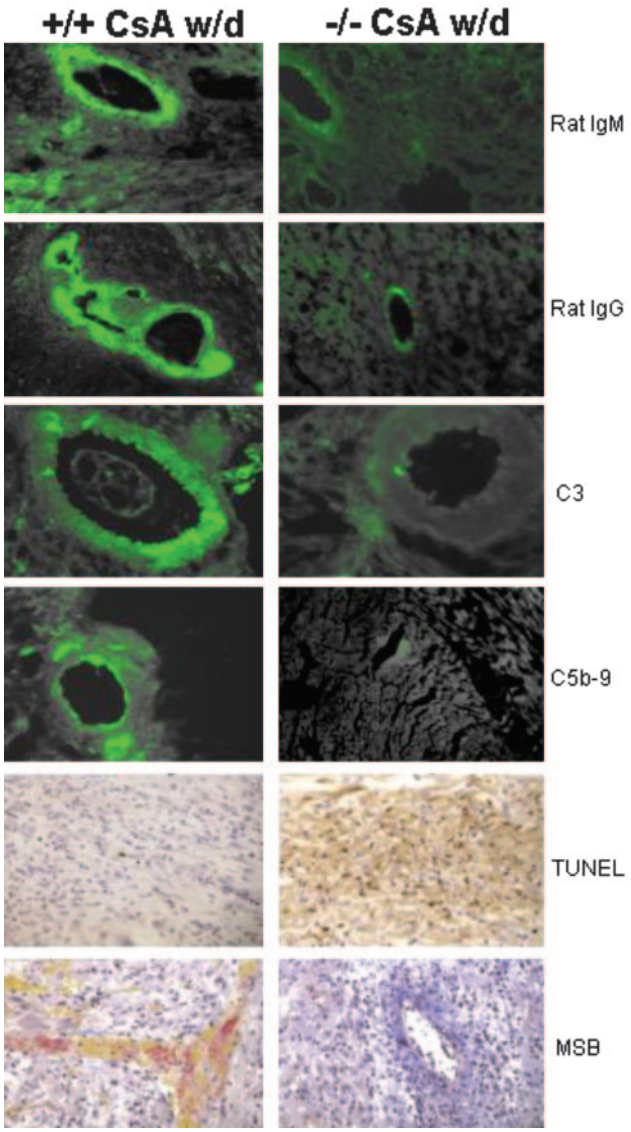

Figure 6. Effects of a short course of cobra venom factor and maintenance cyclosporine, which was withdrawn at day 60 on the immunopathology of Fgl/2$^{+/+}$ (+/+ CsA w/d) and Fgl-2$^{-/-}$ (−/−CsA w/d) rejecting grafts. Tissues were stained for rat IgM, IgG, C3, C5b-9, apoptosis by TUNEL, or fibrin. The Fgl-2$^{+/+}$ graft vessels were strongly positive by immunofluorescence for IgM, IgG, C3, and C5b-9. TUNEL staining was markedly positive in Fgl-2$^{-/-}$ grafts, whereas fibrin deposition (pink) and thrombosis was prominent in Fgl-2$^{+/+}$ grafts. Magnification in all panels ×200. MSB indicates Martius scarlet blue.

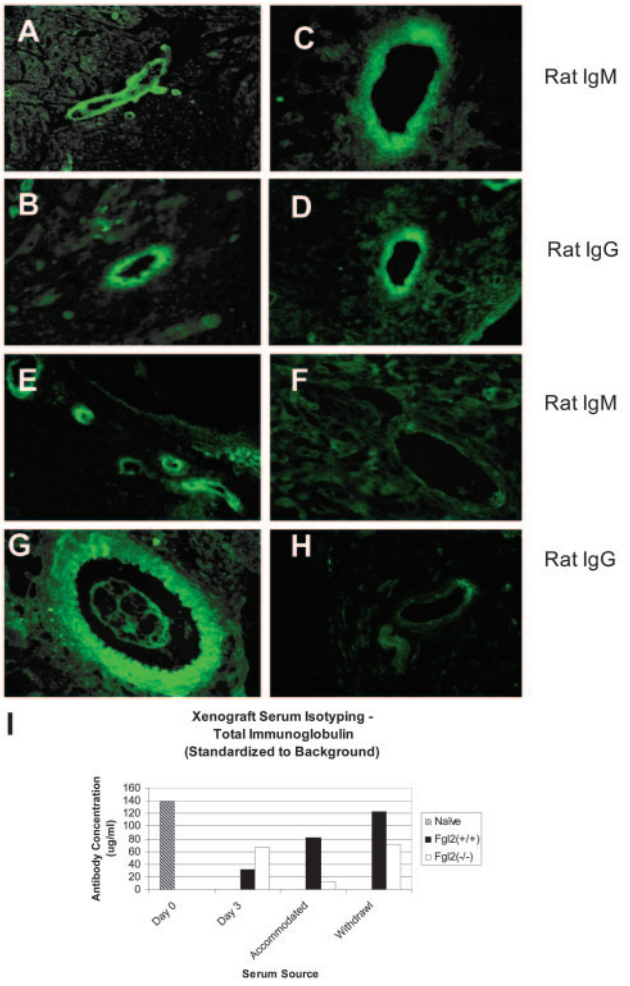

Figure 7. Immunopathology of transplanted and accommodated heart xenografts. Serum at animals' death was collected from rats that received Fgl-2$^{+/+}$ and Fgl-2$^{-/-}$ mouse hearts and were treated for 60 days with cyclosporine A, which was then stopped. IgM (A and C) and IgG (B and D) xenoantibodies were present in recipients of both Fgl-2$^{+/+}$ and Fgl-2$^{-/-}$ hearts, as evidenced by strong staining of vascular endothelium of tissue sections from naive Fgl-2$^{+/+}$ hearts. Accommodated Fgl-2$^{+/+}$ hearts stained positively for both IgM (E) and IgG (G), whereas accommodated Fgl-2$^{-/-}$ hearts failed to stain for either IgM (F) or IgG (H) (magnification ×200). Levels of total immunoglobulins in serum from naive rats and rats receiving Fgl-2$^{+/+}$ and Fgl-2$^{-/-}$ hearts while on cyclosporine A (accommodated) and after withdrawal of immunosuppression at time of rejection were detected in an ELISA assay (I).

pression leads to a functionally important (in terms of avoidance of antibody-mediated vascular rejection) down-modulation of xenoantigen expression. Interestingly, this change in xenoantigen expression occurred after sensitization (implantation of the graft), which is also a feature of other models of chronic antigenemia, including tumors and chronic parasitemias.[27,28] Unpublished studies in our laboratory have shown that xeno AVR is not prevented with the use of Fgl-2$^{-/-}$ mice as recipients, which tests the role of Fgl-2 production by host immune cells in AVR. A similar experiment has been reported by others with negative results in an allograft model.[29] Hancock et al,[29] using a different construct to block Fgl-2 expression, found that targeted deletion of Fgl-2 in the recipient in an allogeneic model failed to prevent fibrin deposition, supporting our hypothesis that Fgl-2 production by donor EC is more important than Fgl-2 production by host immune cells in the pathogenesis of AVR.

In contrast to previous reports, the present data suggest that Fgl-2 may be a more important procoagulant than TF in xenograft AVR.[30] We found that TF mRNA levels declined over time as AVR progressed, whereas Fgl-2 mRNA levels increased postoperatively. These findings may explain why inhibition of TF activity by the nematode anticoagulant peptide NAPc2[31,32] failed to control AVR in pilot mouse-to-rat cardiac xenotransplantation studies in our laboratory (data

not shown). The diverging pattern of total graft mRNA TF and Fgl-2 expression during xenograft rejection suggests that these genes respond to different regulatory stimuli, consistent with in vitro studies that have shown that induction of TF expression on porcine EC by human serum is predominately mediated by antibody and complement,[33,34] whereas Fgl-2 expression is not induced by media containing complement or isolectin B_4 (a substitute for anti–α-Gal–specific preformed antibody).[10]

Targeted deletion of Fgl-2 modified AVR through effects on thrombosis, apoptosis, and cellular recruitment. Although untreated Fgl-$2^{-/-}$ xenografts showed minimal evidence for thrombosis, they were ultimately rejected by 3 to 4 days after transplantation by a form of cellular rejections. Rejected untreated Fgl-$2^{-/-}$ xenografts had increased apoptosis of the cardiomyocytes and vascular EC, which spatially correlated with the intensity of the macrophage infiltration. In contrast, in the Fgl-$2^{+/+}$ grafts, cardiomyocytes and vascular EC appeared to be damaged by a combination of apoptosis and necrosis, which was predominantly localized to the myocardium, where it was spatially associated with local vascular occlusion by thrombi, suggesting ischemia as the likely effector mechanism.

Our data suggest that the increased cardiomyocyte apoptosis observed in the Fgl-$2^{-/-}$ grafts may be due to the cytotoxic effects of the graft-infiltrating macrophages. Cardiomyocyte damage appeared to involve cell-cell contact–dependent ligand-receptor interactions, resulting in phagocytosis based on the TEM studies. TNF-α may be the effector molecule in this process because protein expression was significantly higher in rejected Fgl-$2^{-/-}$ grafts compared with Fgl-$2^{+/+}$ grafts and TNF-TNF R1 interactions are known to be proapoptotic in many disease states.[35–37] Alterations in the expression of vascular adhesion molecules in the Fgl-$2^{-/-}$ grafts may contribute to the recruitment of macrophages to the Fgl-$2^{-/-}$ grafts. Another possibility is that the Fgl-2 protein itself contributes to the regulation of apoptotic cell death, as reported with other fibrinogen-like proteins.[38–40] Alternatively, deletion of Fgl-2 from cardiomyocytes, which constitutively express Fgl-2 normally, may make these cells more susceptible to apoptosis.

Long-term survival of both Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ cardiac xenografts with normal histology was achieved with the use of an immunosuppressive protocol previously described to induce accommodation of mouse-to-rat xenografts.[41–43] When cyclosporine was withdrawn at 60 days, both Fgl-$2^{+/+}$ and Fgl-$2^{-/-}$ grafts failed. The Fgl-$2^{+/+}$ grafts once again developed AVR characterized by heavy deposits of IgM, IgG, C3, and C5b-9, leading to widespread vascular thrombosis similar to that observed in untreated Fgl-$2^{+/+}$ grafts. In contrast, the Fgl-$2^{-/-}$ grafts developed features of acute cellular allograft rejection[44] with an extensive vasculitis/endotheliitis that was characterized by a predominately lymphocytic infiltrate with minimal microvascular thrombosis and minimal deposition of immunoglobulin.

Serum containing xenoreactive antibodies recovered from rats that had transplanted Fgl-$2^{+/+}$ hearts failed to stain tissue sections from Fgl-$2^{-/-}$ long-term treated hearts but stained wild-type heart tissues. It is interesting that the grafts were still lost to a cellular immune response despite apparent downregulation of xenoantigens on the vascular endothelium of the graft. We speculate that internalization of these antibody-reactive xenoepitopes from the cell surface may subject them to intracellular processing pathways, resulting in re-presentation as processed peptides suitable for determinant recognition by T-cell receptors. Downregulation of xenoantibody levels resulting from the loss of xenoantigens is another mechanism that may have contributed to the change in phenotype of rejection. Ongoing fine-specificity mapping of the epitopes recognized by xenoantibodies and the antigen-recognizing T cells will elaborate on this possibility.

In conclusion, the most important finding of the present study is that targeted deletion of Fgl-2 changes the pattern of rejection from an acute vascular process (AVR) that is very difficult to prevent to a form of cellular rejection that is more amenable to treatment with clinically relevant immune suppression. If these findings can be reproduced in higher-order primates, then targeting Fgl-2 may, in combination with other current strategies, permit successful clinical heart xenotransplantation.

Acknowledgments

This work was supported by the Heart and Stroke Foundation of Ontario, Canada, and the Canadian Institutes of Health Research. We thank Lily Morikawa and Patricia Feugas for specimen processing, Michael Ho for paraffin section immunohistochemistry, and Mingda Shi for technical assistance.

References

1. Grant D, Mendicino M, Levy G. Xenotransplantation: just around the corner? *Surgery*. 2001;129:243–247.
2. Appel JZ III, Buhler L, Cooper DK. The pig as a source of cardiac xenografts. *J Card Surg*. 2001;16:345–356.
3. Rosengard AM, Cary NR, Langford GA, Tucker AW, Wallwork J, White DJ. Tissue expression of human complement inhibitor, decay-accelerating factor, in transgenic pigs: a potential approach for preventing xenograft rejection. *Transplantation*. 1995;59:1325–1333.
4. Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y, Ayares DL. Production of alpha 1,3-galactosyltransferase–deficient pigs. *Science*. 2003;299:411–414.
5. Robson SC, Cooper DK, d'Apice AJ. Disordered regulation of coagulation and platelet activation in xenotransplantation. *Xenotransplantation*. 2000;7:166–176.
6. Goddard MJ, Dunning J, Horsley J, Atkinson C, Pino-Chavez G, Wallwork J. Histopathology of cardiac xenograft rejection in the pig-to-baboon model. *J Heart Lung Transplant*. 2002;21:474–484.
7. Platt JL. The immunological hurdles to cardiac xenotransplantation. *J Card Surg*. 2001;16:439–447.
8. Pearson JD. Endothelial cell function and thrombosis. *Baillieres Best Pract Res Clin Haematol*. 1999;12:329–341.
9. Bach FH, Robson SC, Ferran C, Winkler H, Millan MT, Stuhlmeier KM, Vanhove B, Blakely ML, van der Werf WJ, Hofer E. Endothelial cell activation and thromboregulation during xenograft rejection. *Immunol Rev*. 1994;141:5–30.
10. Ghanekar A, Mendicino M, Liu H, He W, Liu M, Zhong R, Phillips MJ, Levy GA, Grant DR. Endothelial induction of fgl2 contributes to thrombosis during acute vascular xenograft rejection. *J Immunol*. 2004;172:5693–5701.
11. Ruegg CR, Chiquet-Ehrismann R, Alkan SS. Tenascin, an extracellular matrix protein, exerts immunomodulatory activities. *Proc Natl Acad Sci U S A*. 1989;86:7437–7441.
12. Smiley ST, King JA, Hancock WW. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol*. 2001;167:2887–2894.

13. Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science*. 1999;286:2511–2514.
14. Liu X, Piela-Smith TH. Fibrin(ogen)-induced expression of ICAM-1 and chemokines in human synovial fibroblasts. *J Immunol*. 2000;165:5255–5261.
15. Sitrin RG, Pan PM, Srikanth S, Todd RF III. Fibrinogen activates NF-kappa B transcription factors in mononuclear phagocytes. *J Immunol*. 1998;161:1462–1470.
16. Liu M, Leibowitz JL, Clark DA, Mendicino M, Ning Q, Ding JW, D'Abreo C, Fung L, Marsden PA, Levy GA. Gene transcription of fgl2 in endothelial cells is controlled by Ets-1 and Oct-1 and requires the presence of both Sp1 and Sp3. *Eur J Biochem*. 2003;270:2274–2286.
17. Chan CW, Chan MW, Liu M, Fung L, Cole EH, Leibowitz JL, Marsden PA, Clark DA, Levy GA. Kinetic analysis of a unique direct prothrombinase, fgl2, and identification of a serine residue critical for the prothrombinase activity. *J Immunol*. 2002;168:5170–5177.
18. Chan CW, Kay LS, Khadaroo RG, Chan MW, Lakatoo S, Young KJ, Zhang L, Gorczynski RM, Cattral M, Rotstein O, Levy GA. Soluble fibrinogen-like protein 2/fibroleukin exhibits immunosuppressive properties: suppressing T cell proliferation and inhibiting maturation of bone marrow–derived dendritic cells. *J Immunol*. 2003;170:4036–4044.
19. Marsden PA, Ning Q, Fung LS, Luo X, Chen Y, Mendicino M, Ghanekar A, Scott JA, Miller T, Chan CW, Chan MW, He W, Gorczynski RM, Grant DR, Clark DA, Phillips MJ, Levy GA. The Fgl2/fibroleukin prothrombinase contributes to immunologically mediated thrombosis in experimental and human viral hepatitis. *J Clin Invest*. 2003;112:58–66.
20. Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, Grey ST, Colvin RB, Choi AM, Poss KD, Bach FH. Expression of heme oxygenase-1 can determine cardiac xenograft survival. *Nat Med*. 1998;4:1073–1077.
21. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice: the role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation*. 1973;16:343–350.
22. Miyatake T, Koyamada N, Hancock WW, Soares MP, Bach FH. Survival of accommodated cardiac xenografts upon retransplantation into cyclosporine-treated recipients. *Transplantation*. 1998;65:1563–1569.
23. Ginesta MM, Ribas Y, Mollevi DG, Vidal A, Manez R, Figueras J, Jaurrieta E. Acute xenograft rejection, late xenograft rejection and long term survival xenografts in the hamster-to-rat heart transplantation model: histological characterisation under low-dose of FK506. *APMIS*. 2002;110:737–745.
24. Parker W, Bruno D, Holzknecht ZE, Platt JL. Characterization and affinity isolation of xenoreactive human natural antibodies. *J Immunol*. 1994;153:3791–3803.
25. Levy GA, Liu M, Ding J, Yuwaraj S, Leibowitz J, Marsden PA, Ning Q, Kovalinka A, Phillips MJ. Molecular and functional analysis of the human prothrombinase gene (HFGL2) and its role in viral hepatitis. *Am J Pathol*. 2000;156:1217–1225.
26. Kreisel D, Krupnick AS, Szeto WY, Popma SH, Sankaran D, Krasinskas AM, Amin KM, Rosengard BR. A simple method for culturing mouse vascular endothelium. *J Immunol Methods*. 2001;254:31–45.
27. Sadick MD, Heinzel FP, Shigekane VM, Fisher WL, Locksley RM. Cellular and humoral immunity to Leishmania major in genetically susceptible mice after in vivo depletion of L3T4+ T cells. *J Immunol*. 1987;139:1303–1309.
28. Slansky JE, Rattis FM, Boyd LF, Fahmy T, Jaffee EM, Schneck JP, Margulies DH, Pardoll DM. Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. *Immunity*. 2000;13:529–538.
29. Hancock WW, Szaba FM, Berggren KN, Parent MA, Mullarky IK, Pearl J, Cooper AM, Ely KH, Woodland DL, Kim IJ, Blackman MA, Johnson LL, Smiley ST. Intact type 1 immunity and immune-associated coagulative responses in mice lacking IFN gamma–inducible fibrinogen-like protein 2. *Proc Natl Acad Sci U S A*. 2004;101:3005–3010.
30. Nagayasu T, Saadi S, Holzknecht RA, Plummer TB, Platt JL. Expression of tissue factor mRNA in cardiac xenografts: clues to the pathogenesis of acute vascular rejection. *Transplantation*. 2000;69:475–482.
31. Ansell JE, Weitz JI, Comerota AJ. Advances in therapy and the management of antithrombotic drugs for venous thromboembolism. *Hematology (Am Soc Hematol Educ Program)*. 2000;266–284.
32. Girard TJ, Nicholson NS. The role of tissue factor/factor VIIa in the pathophysiology of acute thrombotic formation. *Curr Opin Pharmacol*. 2001;1:159–163.
33. Saadi S, Holzknecht RA, Patte CP, Stern DM, Platt JL. Complement-mediated regulation of tissue factor activity in endothelium. *J Exp Med*. 1995;182:1807–1814.
34. Fecke W, Long J, Richards A, Harrison R. Protection of hDAF-transgenic porcine endothelial cells against activation by human complement: role of the membrane attack complex. *Xenotransplantation*. 2002;9:97–105.
35. Yeh WC, Hakem R, Woo M, Mak TW. Gene targeting in the analysis of mammalian apoptosis and TNF receptor superfamily signaling. *Immunol Rev*. 1999;169:283–302.
36. Ferrari R. The role of TNF in cardiovascular disease. *Pharmacol Res*. 1999;40:97–105.
37. Meldrum DR. Tumor necrosis factor in the heart. *Am J Physiol*. 1998;274(pt 2):R577–R595.
38. Kim I, Moon SO, Han CY, Pak YK, Moon SK, Kim JJ, Koh GY. The angiopoietin-tie2 system in coronary artery endothelium prevents oxidized low-density lipoprotein-induced apoptosis. *Cardiovasc Res*. 2001;49:872–881.
39. Liu W, Ahmad SA, Reinmuth N, Shaheen RM, Jung YD, Fan F, Ellis LM. Endothelial cell survival and apoptosis in the tumor vasculature. *Apoptosis*. 2000;5:323–328.
40. Rubel C, Fernandez GC, Dran G, Bompadre MB, Isturiz MA, Palermo MS. Fibrinogen promotes neutrophil activation and delays apoptosis. *J Immunol*. 2001;166:2002–2010.
41. Soares MP, Lin Y, Sato K, Stuhlmeier KM, Bach FH. Accommodation. *Immunol Today*. 1999;20:434–437.
42. Bach FH, Ferran C, Hechenleitner P, Mark W, Koyamada N, Miyatake T, Winkler H, Badrichani A, Candinas D, Hancock WW. Accommodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. *Nat Med*. 1997;3:196–204.
43. Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH, Soares MP. Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. *J Immunol*. 2001;166:4185–4194.
44. Pietra BA, Gill RG. Immunobiology of cardiac allograft and xenograft transplantation. *Semin Thorac Cardiovasc Surg Pediatr Card Surg Annu*. 2001;4:123–157.