Neutrophils Mediate Parenchymal Tissue Necrosis and Accelerate the Rejection of Complete Major Histocompatibility Complex-Disparate Cardiac Allografts in the Absence of Interferon- γ

Masayoshi Miura,^{*,†} Tarek El-Sawy,^{†,‡} and Robert L. Fairchild^{*,†,‡}

From the Urological Institute* and Department of Immunology,[†] Cleveland Clinic Foundation, Cleveland; and the Department of Pathology,[‡] Case Western Reserve University School of Medicine, Cleveland, Ohio

A major feature of acute rejection of cardiac allografts is an intense mononuclear cell infiltration accompanied by interferon (IFN)- γ production. In the current study we tested the role of IFN- γ in acute rejection of allografts by comparing the histopathology of rejection in wild-type versus IFN- $\gamma^{-/-}$ recipients of major histocompatibility complex-mismatched cardiac grafts. Wild-type recipients rejected the allografts at days 8 to 9 after transplant but rejection was accelerated 2 to 3 days in IFN- γ -deficient recipients. During rejection in wild-type recipients, the allografts were heavily infiltrated with CD8⁺ T cells and other mononuclear cells. In contrast, allografts in IFN- γ -deficient recipients had few T cells but an intense neutrophil infiltration accompanied by extensive graft parenchymal necrosis. No difference in expression levels of neutrophil chemoattractants including Gro α /KC, MIP-2, GCP-2, and MIP-1 α , was observed in allografts retrieved from wild-type and IFN- $\gamma^{-/-}$ recipients. Depletion of neutrophils from IFN- γ -deficient recipients delayed rejection until days 8 to 10 after transplant and restored the histopathology of acute allograft rejection to that observed in allografts rejected by wild-type recipients. These results indicate the potent regulatory properties of IFN- γ during acute rejection directed at neutrophil infiltration into allografts and mediating graft tissue necrosis. (*Am J Pathol* 2003, 162:509–519)

Allogeneic organ transplantation is a commonly used therapy for end-stage diseases of organ failure. Although current immunosuppressive strategies have decreased early graft loss because of T-cell-mediated rejection, acute allograft rejection remains a serious problem.¹ In addition to causing early graft loss, acute rejection is a significant risk factor for the subsequent development of chronic rejection resulting in late graft loss.^{2,3} Acute al-

lograft rejection is an immune response mediated by the coordinated infiltration of alloantigen-primed T cells into the graft and their activation to express the effector functions mediating destruction of the graft tissue.^{4,5}

The ischemia/reperfusion injury and surgical trauma imposed on the graft induces a substantial tissue inflammatory response. Components of this inflammatory response stimulate both adhesion molecule expression and production of chemokines in the allograft.^{6–8} Chemokines that are produced early after transplantation include those that mediate recruitment of neutrophils (eg, Gro α and MIP-2) and macrophages (eg, MCP-1). The appearance of these chemokines is eventually followed by the production of chemoattractants directing the recruitment of antigen-primed T cells including the interferon (IFN)- γ -induced chemokines IP-10 and Mig. Recent studies have indicated a critical role for IP-10 and Mig in T cell infiltration and acute rejection of heart allografts in murine models.^{9,10} These observations suggest a role for IFN- γ in alloantigen-primed T cell infiltration into allografts during the acute rejection process. However, recent results have demonstrated the accelerated rejection of heart allografts by IFN- γ -deficient recipients.^{11,12} T cells from IFN- $\gamma^{-/-}$ allograft recipients display exaggerated alloreactive responses suggesting a regulatory role for IFN- γ in restricting the magnitude of the recipient T cell response to graft alloantigens.¹² Although these results predict an increased T cell infiltration into the allograft to mediate acute rejection, the mechanism underlying rejection of the heart allografts in these recipients remains unclear. The goal of the current study was to examine molecular and histopathological aspects of major histocompatibility complex (MHC)-mismatched cardiac allografts during rejection in IFN- $\gamma^{-/-}$ recipients to gain further insights into the potential roles for IFN- γ during the acute rejection process.

Supported by grants from the National Institutes of Health (AI40459) and the American Heart Association.

Accepted for publication October 29, 2002.

Address reprint requests to Robert L. Fairchild, Ph.D., NB3-79, Lerner Research Inst., Cleveland Clinic Fdn., 9500 Euclid Ave., Cleveland, OH 44195-0001. E-mail: fairchr@ccf.org.

Materials and Methods

Experimental Animals

A/J (H-2^a) and C57Bl/6 (H-2^b) were obtained through Dr. Clarence Reeder at the National Cancer Institute (Frederick, MD). C57Bl/6. $\text{IFN-}\gamma^{-/-}$ (GKO) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Adult males, 7 to 12 weeks old, were used throughout this study.

Heterotopic Cardiac Transplant

Cardiac transplants were performed with microsurgical techniques using the method of Corry and co-workers.¹³ Briefly, donor and recipient mice were anesthetized with phenobarbital. Donor hearts were harvested after cool perfusion with heparinized lactated Ringer's solution and placed in chilled lactated Ringer's solution while the recipient mouse was prepared. The graft aorta was anastomosed to the recipient abdominal aorta and the graft pulmonary artery to the recipient inferior vena cava. On reperfusion, the transplanted hearts resumed spontaneous contraction. The strength of cardiac pulsation was graded each day by palpation. Rejection of cardiac grafts was considered complete by cessation of impulse and was confirmed visually for each graft by laparotomy. In C57Bl/6 recipients complete rejection of A/J cardiac grafts typically occurs between 8 and 10 days after transplantation and cardiac isografts function for more than 300 days.

Antibodies

Rat anti-mouse Ly-6G, RB6-8C5, was purified from hybridoma culture supernatants using Protein-G Sepharose. Mice were depleted of neutrophils by giving 100 μg aliquots of RB6.8C5 on two consecutive days. Control mice received rat IgG. This treatment resulted in <5% neutrophils in the peritoneal wash of mice 4 hours after thioglycollate injection as assessed by staining the peritoneal wash cells with Wright's stain. Depletion was also monitored by antibody staining and flow cytometry. Previous studies have shown that treatment with RB6.8C5 does not affect the viability of circulating or lymphoid T cell populations.^{7,14} For use in immunocytochemistry or flow cytometry GK1.5, rat anti-mouse CD4 monoclonal antibody (mAb) and PE-RB6-8C5 were obtained from Pharmingen (San Diego, CA); 53-6.7, rat anti-mouse CD8 mAb, and biotinylated rabbit anti-rat polyclonal antibody were purchased from DAKO (Carpinteria, CA); and, control rat IgG was purchased from R&D Systems (Minneapolis, MN).

RNA Extraction

Grafts were retrieved at several time points after transplant, immediately snap-frozen in liquid nitrogen and kept at -80°C until extraction. Grafts were pulverized into powder in liquid nitrogen and homogenized in 1 ml of

Figure 1. Rapid rejection of complete MHC-mismatched cardiac allografts by IFN- γ -deficient recipients. A/J hearts were heterotopically transplanted to groups of five WT or IFN- γ -deficient C57Bl/6 mice. Mean time of rejection in WT recipients was 8.47 ± 1.39 days after transplant (■). In contrast IFN- γ -deficient recipients rejected the allografts at 6.0 ± 0.7 days after transplant (●), significantly earlier than the WT recipients ($P = 0.001$ by unpaired t -test).

Trizol reagent (Life Technologies, Inc., Grand Island, NY). After phase separation and precipitation according to the manufacturer's protocol, RNA was resuspended in diethylpyrocabonate-treated H_2O and quantitated by spectrophotometry.

In Vitro Transcription

Multiprobe templates set mCK-5b consisting of lymphotactin; eotaxin; RANTES; macrophage inflammatory protein (MIP)-1 α , MIP-1 β , MIP-2; monocyte chemoattractant protein (MCP)-1; TCA-3; L32; and GAPDH was purchased from PharMingen (San Diego, CA). Template cDNAs for murine Mig (95 to 475) and murine IP-10 (86 to 548) were linearized for *in vitro* transcription.¹⁰ It should be noted that the IP-10 template used in these analyses was cloned from C57Bl/6 macrophages and is identical to the IP-10 sequence of A/J mice used as allograft donors in this study. Template cDNA for murine GAPDH was also purchased from Ambion Inc. (Austin, TX). ^{32}P -UTP radio-labeled anti-sense riboprobes for RNase protection assay (RPA) were synthesized and purified using the RiboQuant *In vitro* Transcription Kit (BD PharMingen, San Diego, CA), according to the manufacturer's protocol.

RNase Protection Assay

Intragraft expression of chemokine and chemokine receptor genes was quantified by RPA using RiboQuant RPA kits (PharMingen, San Diego, CA) according to the manufacturer's protocol. In brief, 10 μg of sample RNA was hybridized overnight at 56°C with the ^{32}P -labeled riboprobes. The samples were treated with RNase A/T1 cocktail and then with proteinase K. After extraction and precipitation the samples were run on a denaturing 5% polyacrylamide gel. The gel was transferred to filter paper, dried, and exposed to X-ray film and a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) for quantification. The intensity of each signal was measured with ImageQuant (Molecular Dynamics) and stan-

Figure 2. Different histopathology of rejection of complete MHC-mismatched cardiac allografts by WT *versus* IFN- γ -deficient recipients. A/J hearts were heterotopically transplanted to groups of WT or IFN- γ -deficient C57Bl/6 mice. Allografts were retrieved at day 7 after transplant from WT recipients and at day 5 from IFN- γ -deficient recipients, and formalin-fixed, paraffin-embedded graft sections were stained with H&E. The allografts from WT recipients had extensive infiltration with mononuclear cells typically observed during acute rejection. In contrast, the allografts from IFN- γ -deficient recipients had less mononuclear cell infiltration but diffuse thrombosis and hemorrhagic necrosis accompanied by polymorphonuclear cell infiltration. Original magnifications: $\times 50$ (a, b); $\times 200$ (c, d).

normalized to the intensity of the GAPDH signal for each sample. The mean chemokine/GAPDH signal intensity $\pm$ SD for each group of four samples was determined.

Histology

For immunohistology, heart allografts were retrieved at various times after transplant, embedded in OCT compound (Sakura Finetek U.S.A., Torrance, CA), and snap-frozen in liquid nitrogen. Sections were cut at 8- μ m thickness and mounted onto slides. Slides were dried overnight, fixed in acetone for 10 minutes, and air-dried. Slides were immersed in phosphate-buffered saline (PBS) for 10 minutes and in 0.03% H_2O_2 for 10 minutes to eliminate endogenous peroxidase activity. As primary antibodies, GK1.5 and 53-6.7 were diluted at 5 μ g/ml and RB6-8C5 was diluted at 10 μ g/ml in 0.05% Tris-HCl buffer with 1% bovine serum albumin. Slides were stained for 1 hour at room temperature with the primary anti-leukocyte antibodies or with rat IgG as a control. After three washes

in PBS for 5 minutes each, all slides were incubated for 20 minutes with biotinylated rabbit anti-rat IgG, diluted 1:300 in the same buffer as the primary antibodies. After three washes in PBS, slides were incubated with streptavidin-horseradish peroxidase (DAKO) for 20 minutes. The substrate-chromogen solution was prepared by dissolving a 3,3'-diaminobenzidine, 10 mg tablet (Sigma Chemical Co., St. Louis, MO) in 15 ml of PBS and adding 12 μ l of 30% H_2O_2 just before use. After three washes in PBS for 5 minutes each, the 3,3'-diaminobenzidine solution was applied to the slides and incubated for 3 to 7 minutes. After a final wash in H_2O , slides were counterstained with hematoxylin, rinsed, and immersed in 37 nmol/L of NH_4OH for 10 seconds. For hematoxylin and eosin (H&E) staining, allografts were fixed with 10% buffered formalin and paraffin-embedded sections were stained with each dye for 3 minutes. Finally, the slides were dehydrated, coverslipped, and the slides were viewed with a light microscope and captured using Image Pro Plus (Media Cybernetics, Silver Spring, MD).

Figure 3. Infiltrating leukocyte populations in cardiac allografts retrieved from WT and IFN- γ -deficient recipients at the time of rejection. A/J hearts were heterotopically transplanted to groups of WT or IFN- γ -deficient (GKO) C57Bl/6 mice. Allografts were retrieved at day 5 from GKO and at day 7 from WT recipients. Frozen sections of the allografts were stained for immunohistology with anti-CD4 mAb (GK1.5), anti-CD8 α mAb (53-6.7), or anti-Ly-6G mAb (RB6-8C5). Strong CD8 $^{+}$ cell infiltration into allografts is readily apparent in graft retrieved from WT but not GKO recipients. Very few Ly-6G $^{+}$ cells (ie, neutrophils) are observed infiltrating allografts retrieved from WT recipients at the time of rejection. In contrast, intense neutrophil infiltration is observed in allografts retrieved from GKO recipients. Modest infiltration with CD4 $^{+}$ T cells was observed in allografts from both recipients. Original magnifications, $\times 200$.

Flow Cytometry

The presence of neutrophils in mice treated with control rat IgG or anti-Ly6G mAb RB6-8C5 was assessed by antibody staining and flow cytometry. Spleen cell suspensions were prepared and 106 cell aliquots were washed three times with staining buffer (Dulbecco's PBS with 2% fetal calf serum/0.2% NaN₃) and stained with PE-RB6-8C5 for 25 minutes on ice. After washing five times, the cells were analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). The neutrophils were gated in the forward scatter (FSC) $\times$ side scatter (SSC) plot and the gated cells analyzed in the FL2 channel for expression of Ly6G.

Results

Rejection of MHC-Mismatched Cardiac Grafts by IFN- $\gamma^{-/-}$ Recipients

The rejection of A/J (H-2^a) heart allografts in wild-type (WT) C57Bl/6 (H-2^b) versus C57Bl/6.IFN- $\gamma^{-/-}$ (GKO) recipients was compared. As shown in Figure 1, WT recipients rejected the complete MHC-mismatched A/J heart grafts between days 8 to 10 after transplant (mean time of rejection, 8.5 days). In contrast, rejection of the heart

allografts in GKO recipients occurred 2 to 3 days earlier than WT recipients (mean time of rejection, 6.0 days).

To examine potential differences in histopathological features of the rejecting allografts that might account for the accelerated rejection of the A/J grafts by GKO recipients, histological analyses were performed. A/J cardiac allografts were retrieved the day before completion of rejection, eg, at day 7 after transplant from WT recipients and at day 5 from GKO recipients, and formalin-fixed sections were stained with H&E. Sections of allografts from WT recipients indicated the characteristic extensive mononuclear cell infiltration into the allografts typically observed during acute rejection (Figure 2, a and c). In contrast, less cellular infiltration but intensive intragraft thrombosis and disseminated hemorrhagic necrosis were observed in sections of A/J heart allografts retrieved from GKO recipients (Figure 2, b and d).

To analyze these histological differences in greater detail, immunocytochemical staining was performed to test cellular infiltration into the grafts. Allografts were retrieved at day 4 after transplant from both groups, at day 5 from GKO recipients and at day 7 from WT C57Bl/6 recipients. Frozen sections were prepared and stained with mAbs to detect graft-infiltrating neutrophils (Ly-6G $^{+}$), CD4 $^{+}$, and CD8 $^{+}$ T cells. Representative sections are shown in Figure 3. On the day before completion of

Figure 4. T cell and neutrophil infiltration into cardiac allografts in WT versus IFN- γ -deficient recipients. The number of positively staining T cells and neutrophils was counted in 10 random fields at an original magnification of $\times 200$, from two different sections from four different allografts retrieved from WT and IFN- γ -deficient recipients. For WT recipients, allografts were retrieved at days 4, 5, 6, and 7 after transplant (■) and for IFN- γ -deficient recipients at days 4 and 5 (●). The numbers of positively stained cells were counted and the data expressed as mean number positively staining cells per field $\pm$ SD. **a:** There is a significantly larger number of CD8⁺ cells in the allografts from WT recipients on day 5 after transplant than in allografts from IFN- γ -deficient recipients at day 5 after transplant ($P < 0.01$, unpaired *t*-test). **b:** There is no difference in the number of infiltrating CD4⁺ T cells between the two groups. **c:** There is a significantly larger number of neutrophils infiltrating allografts from IFN- γ -deficient recipients at day 5 after transplant when compared to the allografts from WT recipients at the time of rejection ($P < 0.01$, unpaired *t*-test).

rejection by WT recipients, intense CD8⁺ cell infiltration into the A/J cardiac allografts was apparent. CD4⁺ T cell infiltration was also evident but at much lower levels than CD8⁺ cells. In contrast, CD8⁺ cell infiltration into A/J allografts retrieved from GKO recipients was strikingly reduced. CD4⁺ cell infiltration into allografts retrieved from GKO recipients was equivalent to that of allografts from WT recipients. Surprisingly, an intense neutrophil infiltration into allografts retrieved from GKO recipients the day before rejection was observed that was absent in allografts retrieved from WT recipients at either day 4 after transplant (not shown) or at day 7 after transplant, the day before completion of rejection in WT recipients.

To support these histological findings, the number of positively stained cells was counted in 10 random fields from two different sections from four different allografts. At day 5 after transplant there was a fourfold decrease in the number of CD8⁺ T-cell-infiltrating allografts retrieved from GKO recipients when compared to allografts retrieved from WT recipients (Figure 4a). Infiltration by CD4⁺ T cells was observed at low levels in allografts from both WT and GKO recipients (Figure 4b). At days 4 and 5 after transplant neutrophil infiltration into allografts re-

trieved from WT recipients was low (eg, 0 to 4 neutrophils per microscopic field on each day) and this infiltration increased modestly by day 7 after transplant. In comparison to neutrophil infiltration at the time of rejection in WT recipients, neutrophil infiltration into allografts retrieved from GKO recipients was increased fourfold to fivefold when examined the day before rejection was complete in these recipients (Figure 4c).

Expression of Neutrophil and T Cell Chemoattractants in IFN- γ -Deficient Recipients of Cardiac Allografts

The striking infiltration of neutrophils into allografts from GKO recipients suggested a potential difference in the levels of neutrophil chemoattractant production in allografts from WT versus GKO recipients. This was examined by testing the temporal mRNA expression of several different chemokines in allografts from each of the recipients. Expression of the neutrophil chemoattractants Gro α (KC), MIP-2, GCP-2, and MIP-1 α were at equivalent levels or lower in allografts from GKO recipients when

Figure 5. Temporal expression of neutrophil attractant chemokines in cardiac iso- and allografts. RNA was isolated from groups of iso- and allografts (●) from WT recipients and from A/J grafts from IFN- γ -deficient recipients (▲) on the indicated day after transplant. Expression of chemokine genes was tested by ribonuclease protection assay. The intensity of chemokine signal in the grafts was measured and standardized to the GAPDH signal for each sample. Data are presented as the mean intensity of four different samples SD and are representative of three individual RPA analyses. Expression of neutrophil chemoattractants, KC (Gro α), MIP-2, GCP-2, and MIP-1 α was at equivalent or lower levels in allografts from GKO recipients when compared to levels in WT recipients.

compared to WT recipients (Figure 5). Because there was decreased T cell infiltration into the allografts from GKO recipients when compared to WT recipients, the expression of T cell chemoattractants was also tested (Figure 6). As expected for IFN- γ induced chemokines, expression of Mig was undetectable. Increased expression of IP-10 was detected at equivalent levels in allografts from WT and GKO recipients at 6 hours after transplant. After that time point this expression decreased and was maintained at background levels in the GKO recipients whereas IP-10 expression reappeared in allografts in WT recipients at high levels beginning at day 3 after transplant. Among the T cell chemoattractants, lymphotactin was the only T cell chemoattractant that was expressed at equivalent levels in allografts from WT and GKO recipients throughout the study period. Chemoattractants for macrophages such as MIP-1 β and MCP-1 were also decreased in cardiac allografts from GKO recipients when compared to WT recipients (not shown).

Depletion of Neutrophils Restores the Survival of Cardiac Allografts in GKO Recipients

To directly test if the rapid rejection of cardiac allografts in GKO recipients was mediated by the intense neutrophil infiltration, antibody-mediated depletion of recipient neutrophils was performed before transplant surgery. Recipients were treated with anti-Ly6G mAb RB6-8C5 or control rat IgG on days -2, -1, 0, 1, 2, 4, and 6 after transplant. Spleen cells from sentinel mice treated with control rat IgG or RB6-8C5 were stained with RB6-8C5 and analyzed by flow cytometry to monitor the presence of neutrophils in the treated groups (Figure 7). Treatment with RB6-8C5 resulted in complete depletion of Ly6G^{high}-expressing cells from the spleen. However, a population expressing low levels of Ly6G was detected in the RB6-8C5-treated group but not in the control group.

Depletion of recipient neutrophils restored the survival of the allografts in the GKO recipients to the time that rejection was observed in the WT recipients, days 7 to 10

Figure 6. Temporal expression of T-cell attractant chemokines in cardiac iso- and allografts. RNA isolated from groups of iso- and allografts shown in Figure 5 was tested by ribonuclease protection assay for the expression of T-cell attractant chemokines. The intensity of chemokine signal in the grafts was measured and standardized to the GAPDH signal for each sample. Data are presented as the mean intensity of four different samples SD and are representative of three individual RPA analyses. With the exception of IP-10 and RANTES expression at 6 hours after transplant, expression of IP-10, Mig, and RANTES was at lower levels in allografts from IFN- γ -deficient recipients when compared to levels in WT recipients.

after transplant (Figure 8). At day 9 after transplant, heart allografts were retrieved from the neutrophil-depleted GKO recipients and paraffin-embedded sections were prepared and stained with H&E. Depletion of GKO recipient neutrophils abrogated the graft parenchymal necrosis and restored the mononuclear cell infiltration observed during acute rejection of the allografts in WT recipients (Figure 9A). Frozen sections prepared from these grafts at the time of rejection were stained with antibodies to examine infiltration of the allografts by neutrophils and T cells. As previously observed, intense neutrophil infiltration was observed in A/J allografts from GKO recipients at the time of rejection (day 6) when compared to rejecting allografts from WT recipients at day 8 after transplant (Figure 9B). The presence of neutrophils in A/J heart allografts from IFN- γ -deficient recipients treated with RB6-8C5 was almost completely absent with few Ly6G⁺ cells detectable in the rejecting allografts at day 9 after transplant. GKO recipient treatment with RB6-8C5 also restored the intense CD8⁺ T cell infiltration into the A/J heart allografts that was observed in allografts from the WT recipients (Figure 9C).

Discussion

IFN- γ production by alloantigen-primed T cells is often observed in allografts during acute rejection.^{15,16} In this context, studies demonstrating the accelerated rejection of complete MHC-mismatched heart allografts in IFN- γ ^{-/-} (ie, GKO) recipients were somewhat surprising.^{11,12} Associated with this accelerated rejection was the unregulated expansion of alloantigen-reactive T cells suggesting that the unregulated expansion of T cells might mediate the accelerated rejection observed in IFN- γ ^{-/-} recipients. Based on these results, experiments in the current study revealed that CD8⁺ and CD4⁺ T-cell infiltration into cardiac allografts in GKO recipients is actually decreased at the time of rejection when compared to T cell infiltration into allografts in WT recipients. These results indicate that despite the unregulated expansion of T cells in the IFN- γ -deficient recipients few of the T cells are directed to infiltrate the cardiac allograft at the time the grafts are rejected.

In contrast to the few T cells observed in the cardiac allografts retrieved from IFN- γ -deficient recipients at the

Figure 7. Depletion of neutrophils by treatment with anti-Ly6G mAb. Groups of IFN- γ -deficient mice were treated with 150 μ g of RB6-8C5 intraperitoneally on days -2, -1, 0, 1, 2, 4, and 6 to deplete neutrophils or with rat IgG as a control. On day 7, spleen cell aliquots were stained with PE-RB6-8C5 and analyzed by flow cytometry. The FSC $\times$ SSC plot was used to gate the neutrophil population and then the intensity of anti-Ly6G staining was assessed in a FL2 histogram of unstained cells and stained cells from control and RB6-8C5-treated mice.

time rejection is complete, an intense neutrophil infiltration was observed that was not observed in the WT recipients. Associated with this neutrophil infiltration was histological evidence of severe hemorrhagic necrosis of the cardiac allograft parenchyma. Studies using allografts from IFN- γ $R^{-/-}$ donors have also reported this histopathology during acute rejection of murine renal allografts.¹⁷ Results in the current report provide strong evidence that this intense neutrophil infiltration mediates the histopathology and accelerated rejection of the A/J heart allografts in the IFN- γ -deficient recipients. Depletion of recipient neutrophils restored the time of allograft rejection to that observed in WT recipients and the level of T cell infiltration was similar in the allografts from WT and IFN- γ -deficient recipients at the time of rejection. This depletion also abrogated the allograft parenchymal necrosis observed during rejection in the GKO recipients and the histology observed at the time of rejection was similar to that observed in rejecting allografts in WT recipients. In addition to the almost complete absence of neutrophils in rejecting allografts, it is worth noting that recipient treatment with the anti-Ly6G mAb resulted in the absence of peripheral cells expressing high levels of the determinant but that cells expressing low levels of Ly6G were observed in these treated animals. The expression of Ly6G has been shown to increase with neutrophil maturation,¹⁸ suggesting that the Ly6G^{low}-expressing

Figure 8. Depletion of neutrophils restores the survival of cardiac allografts in IFN- γ -deficient recipients to the time of rejection observed in WT recipients. Groups of five IFN- γ -deficient recipients were treated with 150 μ g of RB6-8C5 intraperitoneally on days -2, -1, 0, 1, 2, 4, and 6 to deplete neutrophils (○). Another group of IFN- γ -deficient recipients received 150 μ g of rat IgG as a control (●). Neutrophil depletion restored the survival of the allografts in the IFN- γ -deficient recipients to equivalent times as allograft survival in the WT recipients (■).

cells observed in RB6-8C5-treated animals may be an immature neutrophil population developing in response to the depletion of mature neutrophils. Similar populations of immature neutrophils may represent the banded, neutrophil-like cells observed in rejecting A/J allografts from GKO recipients that did not stain positively with RB6-8C5 in the immunohistochemical analyses shown in Figure 3. Allografts engineered to ectopically express FasL are quickly rejected by a mechanism that includes intense neutrophilic infiltration and tissue necrosis that is reminiscent of the histopathology observed in cardiac allografts in GKO recipients as reported in the current study.^{19,20} Overall, these results indicate a unique and severe acute rejection pathology mediated by unregulated neutrophil infiltration and activity in heart allografts.

Neutrophils are among the earliest leukocytes to traffic to inflammatory sites.²¹ Neutrophils are potent amplifiers of early inflammation in both isografts and allografts but their presence in grafts is relatively short-lived. Similarly, reperfusion of ischemic tissues quickly induces production of neutrophil chemoattractants and neutrophil infiltration into the parenchyma of ischemic tissues.²²⁻²⁴ Depletion of neutrophils or antagonism of neutrophil chemoattractant chemokines in animal models of ischemia/reperfusion attenuates this tissue injury indicating the ability of neutrophils to mediate tissue pathology in transplanted tissues.^{22,25-27} Although the intensity of neutrophil infiltration into cardiac grafts in WT recipients subsides with the resolution of this early inflammation, the intensity of this infiltration continues to increase in the allografts in GKO recipients.

Figure 9. Depletion of neutrophils restores the histopathology of acute rejection of allografts in GKO recipients. Groups of IFN- γ -deficient recipients were treated with 150 μ g of RB6-8C5 intraperitoneally on days -2, -1, 0, 1, 2, 4, and 6 to deplete neutrophils or with rat IgG as a control. Allografts were retrieved from control-treated GKO recipients at day 6, from RB6-8C5-treated recipients at day 9, and from WT recipients at day 8 after transplant. **A:** Paraffin-embedded, formalin-fixed sections were stained with H&E. Depletion of neutrophils restores the mononuclear cell graft infiltration and attenuates the diffuse thrombosis and hemorrhagic necrosis observed in allografts from control-treated GKO recipients. **B:** Frozen sections were stained for neutrophils using anti-Ly6G mAb. Treatment with RB6-8C5 resulted in an almost complete absence of neutrophil infiltration into the rejecting allografts from the treated GKO recipients. **C:** Frozen sections were stained for CD8 α (53-6.7) or CD4 (GK1.5). As shown in Figure 3, allografts rejected by GKO recipients show diminished infiltration with T cells. However, depletion of neutrophils in these recipients restored CD8⁺ T cell infiltration into the allografts to levels observed in allografts from WT recipients at the time of rejection. Original magnifications: $\times 200$ (A, B); $\times 400$ (C).

Wildtype

GKO

GKO α -Neutrophil

Wildtype

CD8

GKO

GKO α -Neutrophil

CD4

The factors directing the intense neutrophil infiltration into A/J heart allografts in GKO recipients remains unknown. Several studies have indicated the ability of IFN- γ to inhibit the transcription of chemokines including the neutrophil chemoattractant KC.^{28,29} This led us to examine the possibility that expression of KC and MIP-2 were unregulated in the allografts in GKO recipients. Surprisingly, there was no observable increase or maintenance of neutrophil chemoattractant mRNA levels in the allografts indicating that dysregulated production of neutrophil chemoattractants such as KC and MIP-2 do not mediate the sustained neutrophil infiltration into the heart allografts in GKO recipients. IFN- γ also inhibits the expression of both E- and P-selectins on activated endothelial cell surfaces.³⁰ The absence of recipient-derived IFN- γ may result in unregulated selectin expression on allograft endothelial cells and uninhibited infiltration of neutrophils into the allograft. Although only low numbers of CD8⁺ T cells infiltrated the grafts, the intense neutrophil infiltration into these allografts was dependent on T cells because recipient depletion of T cells abrogated this infiltration and resulted in long-term survival (eg, >60 days after transplant) of the allografts (data not shown). In addition to chemokines, neutrophils trafficking into parenchymal tissues can be directed by many other mediators, such as C5a,³¹ and the unregulated production of such a mediator in cardiac allografts in GKO recipients may account for the intense neutrophil graft infiltration observed in GKO recipients.

Recent studies from Bishop and co-workers³² have indicated increased neutrophil and eosinophil infiltration into BALB/c hearts transplanted to B6.IFN- $\gamma^{-/-}$ recipients. In contrast to the current studies, WT and IFN- γ -deficient recipients rejected the BALB/c cardiac grafts at similar times and the impact of neutrophil graft infiltration was not tested. Histological evaluation of tissue sections from the A/J allografts retrieved from the GKO recipients in the current study revealed very few if any eosinophils (data not shown). Consistent with this result, the expression of the eosinophil chemoattractant eotaxin in the allografts was very low, at the same levels as observed in allografts from WT recipients (data not shown). The source of the allograft in these studies as a C5-deficient donor may account at least in part for the absence of eosinophils observed in the A/J allografts during rejection by the GKO recipients.^{33,34}

At the time of rejection in GKO recipients, cardiac allografts had low levels of infiltration with CD4⁺ and CD8⁺ T cells. The factors directing the T cell infiltration into the allografts in the absence of IFN- γ -inducible factors is unclear. Recent studies from this laboratory have indicated a dominant role of Mig in the recruitment of alloantigen-primed T cells into cardiac allografts¹⁰ and the low numbers of T cells at days 5 to 6 after transplant in the GKO recipients may reflect the absence of Mig in the allografts. In contrast, IP-10 is induced by tumor necrosis factor- α as well as by IFN- γ ³⁵ and was expressed at equivalent levels in cardiac allografts in GKO and WT recipients at early times after transplant. In addition, lymphotactin was expressed at equivalent levels in allografts from the different sets of recipients. Finally, it is

possible that the unregulated expansion of CD8⁺ T cells in GKO recipients overcomes the requirement for Mig and/or other T cell chemoattractants.

The ability of components of the innate immune system to control the activation and effector function of adaptive immune system components has recently attracted considerable interest. The results of the current study indicate that regulatory interactions between the two systems is also maintained in the reverse direction. Transplantation of MHC-disparate hearts into IFN- γ -deficient recipients resulted in dysregulated neutrophil infiltration into the graft and these leukocytes mediated the striking parenchymal tissue necrosis observed in the grafts. These results indicate a critical role for IFN- γ in regulating neutrophil infiltration into allografts. Recent studies from this laboratory have demonstrated early effects of IFN- γ on neutrophils infiltrating into cardiac allografts.¹⁰ Neutrophils are a major source of Mig in cardiac allografts in WT recipients at day 3 after transplant. On the basis of these results we have proposed that T cell production of IFN- γ at the graft endothelial surface binds to proteoglycans on the surface of the endothelial cells and is bound by neutrophils undergoing arrest on the allograft vascular endothelium. The results of the current report indicate that in addition to stimulating the production of Mig, binding of IFN- γ by neutrophils has effects that regulate the intensity of infiltration into the allograft. Definition of this regulatory mechanism should be important for the design of strategies to regulate neutrophil-mediated histopathology in many inflammatory processes.

References

1. Hosenpud JD, Bennett LE, Keck BE, Fiore B, Boucek MM, Novick RJ: The registry of the international society for heart and lung transplantation. Fifteenth official report—1998. *J Heart Lung Transplant* 1998, 17:656–668
2. Matas AJ, Gillingham KJ, Humar A, Dunn DL, Sutherland DER, Najarian JS: Immunologic and nonimmunologic factors. Different risks for cadaver and living donor transplantation. *Transplantation* 2000, 69: 54–58
3. Matas AJ, Gillingham KJ, Payne WD, Najarian JS: The impact of an acute rejection episode on long-term renal allograft survival (t1/2). *Transplantation* 1994, 57:857–859
4. Hall B: Cells mediating allograft rejection. *Transplantation* 1991, 51: 1141–1151
5. Rosenberg AS, Singer A: Cellular basis of skin allograft rejection: an in vivo model of immune-mediated tissue destruction. *Annu Rev Immunol* 1993, 10:333–358
6. Fuggle SV, Sanderson JB, Gray DWR, Richardson A, Morris PJ: Variation in expression of endothelial adhesion molecules in pretransplant and transplanted kidneys: correlation with intragraft events. *Transplantation* 1993, 55:117–123
7. Morita K, Miura M, Paolone DR, Engeman TM, Kapoor A, Remick DG, Fairchild RL: Early chemokine cascades in murine cardiac grafts regulate T cell recruitment and progression of acute allograft rejection. *J Immunol* 2001, 167:2979–2984
8. Pelletier RP, Morgan CJ, Sedmak DD, Miyake K, Kincade PW, Ferguson RM, Orosz CG: Analysis of inflammatory endothelial changes, including VCAM-1 expression, in murine cardiac grafts. *Transplantation* 1993, 55:315–320
9. Hancock WW, Gao W, Csizmadia V, Faia KL, Shemmeri N, Luster AD: Donor-derived IP-10 initiates development of acute allograft rejection. *J Exp Med* 2001, 193:975–980
10. Miura M, Morita K, Kobayashi H, Hamilton TA, Burdick MA, Strieter RM, Fairchild RL: Monokine induced by IFN- γ is a dominant factor

- directing T cells into murine cardiac allografts during acute rejection. *J Immunol* 2001, 167:3494–3504
11. Halloran PF, Miller LW, Urmson J, Ramassar V, Zhu L-F, Kneteman NM, Solez K, Afrouzian M: IFN- γ alters the pathology of graft rejection: protection from early necrosis. *J Immunol* 2001, 166:7072–7081
12. Konieczny BT, Dai Z, Elwood ET, Saleem S, Linsley PS, Baddoura FK, Larsen CP, Pearson TC, Lakkis FG: IFN- γ is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. *J Immunol* 1998, 160:2059–2064
13. Corry RJ, Winn HJ, Russell PS: Primarily vascularized allografts of hearts in mice. *Transplantation* 1973, 16:343–350
14. Dilulio NA, Engeman TM, Armstrong D, Tannenbaum C, Hamilton TA, Fairchild RL: Gro α -mediated recruitment of neutrophils is required for elicitation of contact hypersensitivity. *Eur J Immunol* 1999, 29:3485–3495
15. Dallman MJ, Larsen CP, Morris PJ: Cytokine gene transcription in vascularized organ grafts: analysis using semiquantitative polymerase chain reaction. *J Exp Med* 1991, 174:493–496
16. Morgan CJ, Pelletier RP, Hernandez CJ, Teske DL, Huang E, Ohye R, Orosz CG, Ferguson RM: Alloantigen-dependent endothelial phenotype and lymphokine mRNA expression in rejecting murine cardiac allografts. *Transplantation* 1993, 55:919–923
17. Halloran PF, Afrouzian M, Ramassar V, Urmson J, Zhu L-F, Helms LMH, Solez K, Kneteman NM: Interferon- γ acts directly on rejecting renal allografts to prevent graft necrosis. *Am J Pathol* 2001, 158:215–226
18. Hestdal K, Ruscetti FW, Ihle JN, Jacobsen SEW, Dubois CM, Kopp WC, Longo DL, Keller JR: Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *J Immunol* 1991, 147:22–28
19. Takeuchi T, Ueki T, Nishimatsu H, Kajiura T, Ishida T, Jishage K, Ueda O, Suzuki H, Li B, Miyama N, Kitamura T: Accelerated rejection of Fas ligand-expressing heart grafts. *J Immunol* 1999, 162:518–522
20. Turvey DE, Gonazlez-Nicolini V, Kingsley CI, Larregina A, Morris PJ, Castro MG, Lowenstein PR, Wood KJ: Fas ligand-transfected myoblasts and islet cell transplantation. *Transplantation* 2000, 69:1972–1976
21. Schleimer RP, Freeland HS, Peters SP, Brown KE, Derse CP: An assessment of the effects of glucocorticoids on degranulation, chemotaxis, binding to vascular endothelium and formation of leukotriene B₄ by purified human neutrophils. *J Pharmacol Exp Ther* 1989, 250:598–605
22. Miura M, Fu X, Zhang Q-W, Remick DG, Fairchild RL: Neutralization of Gro α and macrophage inflammatory protein-2 attenuates renal ischemia/reperfusion injury. *Am J Pathol* 2001, 159:2137–2145
23. Rabb H, O'Meara YM, Maderna P, Coleman P, Brady HR: Leukocytes, cell adhesion molecules and ischemic acute renal failure. *Kidney Int* 1997, 51:1463–1468
24. Takada M, Nadeau KC, Shaw GD, Marquette KA, Tilney NL: The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. *J Clin Invest* 1997, 99:2682–2690
25. Colletti LM, Kunkel SL, Walz A, Burdick MD, Kunkel G, Wilke CA, Strieter RM: Chemokine expression during hepatic ischemia/reperfusion-induced lung injury in the rat: the role of epithelial neutrophil activating protein. *J Clin Invest* 1995, 95:134–141
26. Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN: Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am J Physiol* 1987, 253:H699–H703
27. Seekamp A, Mulligan MS, Till GO, Ward PA: Requirements for neutrophil products and L-arginine following ischemia-reperfusion of rat hind limbs. *Am J Pathol* 1993, 142:1217–1226
28. Horton MR, Burdick MD, Strieter RM, Bao C, Noble PW: Regulation of hyaluronan-induced chemokine gene expression by IL-10 and IFN- γ in mouse macrophages. *J Immunol* 1998, 160:3023–3030
29. Ohmori H, Hamilton TA: IFN- γ selectively inhibits lipopolysaccharide-inducible JE/monocyte chemoattractant protein-1 and KC/Gro/melanoma growth-stimulating activity gene expression in mouse peritoneal macrophages. *J Immunol* 1994, 153:2204–2212
30. Melrose J, Tsurushita N, Liu G, Berg EL: IFN- γ inhibits activation-induced expression of E- and P-selectin on endothelial cells. *J Immunol* 1998, 161:2457–2464
31. Baldwin WM, Larsen CP, Fairchild RL: Innate immune responses to transplants: a significant variable with cadaver donors. *Immunity* 2001, 14:369–376
32. Bishop DK, Wood SC, Eichwald EJ, Orosz CG: Immunobiology of allograft rejection in the absence of IFN- γ : CD8⁺ effector cells develop independently of CD4⁺ cells and CD40-CD40 ligand interactions. *J Immunol* 2001, 166:3248–3255
33. Baldwin III WM, Pruitt SK, Brauer RB, Daha MR, Sanfilippo F: Complement in organ transplantation: contribution to inflammation, injury and rejection. *Transplantation* 1995, 59:797–808
34. Karp CL, Grupe A, Schadt E, Ewart SL, Keane-Moore M, Cuomo PJ, Kohl J, Wahl L, Kuperman D, Germer S, Aud D, Peltz G, Wills-Karp M: Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. *Nat Immunol* 2000, 1:221–226
35. Farber JM: Mig and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol* 1997, 61:246–257