Inhibition of Polymorphonuclear Leukocyte–Mediated Graft Damage Synergizes With Short-Term Costimulatory Blockade to Prevent Cardiac Allograft Rejection

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Background—The early inflammatory response during reperfusion of cardiac allografts is initiated by the infiltration of polymorphonuclear leukocytes (PMNs) into the graft. The impact of early PMN infiltration on allograft rejection compared with long-term graft survival remains poorly understood.

Methods and Results—We tested the role of CXCR2, the receptor for 2 PMN attractant chemokines, KC/CXCL1 and MIP-2/CXCL2, on intragraft inflammation and vascularized cardiac allograft rejection in a murine model. Compared with allografts retrieved from control recipients, both PMN infiltration and intragraft proinflammatory cytokine expression were significantly attenuated in allografts from CXCR2-antisera–treated wild-type or from CXCR2/H11002/H11002 recipients. Adoptive transfer of alloantigen-primed T cells rapidly infiltrated and rejected allografts in control recipients, but T-cell infiltration was significantly decreased in recipients depleted of PMNs at transplantation. The influence of early PMN-mediated inflammation on the therapeutic efficacy of costimulatory blockade to prevent allograft rejection was tested. Short-term treatment of recipients with anti-CD154 mAb or CTLA-4 Ig induced modest prolongation of cardiac allograft survival. However, CD154 mAb or CTLA-4 Ig treatment, combined with either peritransplantation PMN depletion or antibodies specific for KC/CXCL1 plus MIP-2/CXCL2, prolonged cardiac allograft survival beyond 100 days.

Conclusions—Results suggest that strategies attenuating PMN-mediated tissue damage during reperfusion significantly improve the efficacy of short-term costimulatory blockade to prevent T-cell–mediated rejection of cardiac allografts. (Circulation. 2005;112:320-331.)

Key Words: endothelium • immunology • inflammation • lymphocytes • transplantation
ade and subsequent function of these T cells to reject the graft.

An intense early inflammatory response is initiated in solid-organ allografts by surgical tissue trauma and ischemia/reperfusion (I/R) injury imposed on the graft at the time of transplantation. Production of oxygen free radicals and chemokines and upregulated expression of adhesion and MHC molecules during reperfusion synergize to direct the recruitment of leukocytes, principally polymorphonuclear cells (PMNs), into the graft within hours after reperfusion. In addition to infiltration of ischemic tissues, chemokines stimulate PMN degranulation, causing parenchymal tissue damage. The impact of I/R injury and PMN-mediated tissue damage on cardiac allograft rejection is poorly understood and has not been directly tested. Furthermore, the use of therapeutic strategies attenuating this injury alone or in combination with blockade of T-cell activation on allograft outcome has not been investigated.

Potential consequences of PMN-mediated allograft tissue damage are structural matrix and cellular alterations of the allograft that do not fully heal to the pretransplantation state. This hypothesis proposes that PMN-mediated tissue injury permanently “marks” the allograft and can direct or facilitate the recruitment and infiltration of alloantigen-reactive T cells and other leukocytes. For example, alloreactive T cells that escape or are resistant to costimulatory blockade or those that may be activated when immunosuppression is withdrawn or tapered may be more effectively directed to the “marked” graft, where they are activated to mediate rejection. This model predicts that attenuation of early intragraft inflammation and tissue damage mediated by PMNs may result in a more effective strategy for prolongation of allograft function in recipients treated with costimulatory blockade. We have tested this hypothesis and demonstrate a novel strategy to achieve long-term allograft survival by first minimizing PMN-mediated intragraft inflammation after reperfusion coupled with a short course of costimulatory blockade. This therapy was administered for only 3 days after transplantation and prolonged the survival of complete MHC-mismatched cardiac allografts beyond 100 days with little to no cellular infiltration into the grafts.

Methods
Mice
A/J (H-2b), C57BL/6 (H-2b), BALB/c (H-2b), and SJL (H-2b) mice were obtained through Dr. C. Reeder at the National Cancer Institute (Frederick, Md). CXCR2-2 (H-2b) mice, obtained from the Jackson Laboratories (Bar Harbor, Maine), bred in the Biological Resources Unit of The Cleveland Clinic Foundation. Adult males of 8 to 12 weeks of age were used throughout this study.

Heterotopic Cardiac Transplantation
Heterotopic cardiac transplantations were performed using the method of Corry and coworkers. Briefly, donor hearts were harvested and placed in chilled Ringer’s solution while the recipient mice were prepared. The donor heart was anastomosed to the recipient abdominal aorta and inferior vena cava using microsurgical techniques. On completion of the anastomoses and organ reperfusion, the heart grafts resumed spontaneous contraction. The strength and quality of cardiac graft impulses were monitored each day by palpation of the abdomen. Rejection of cardiac grafts was considered complete by cessation of impulse and was confirmed visually for each graft by laparotomy. Isografts were maintained in syngeneic recipients beyond 100 days.

Antibodies
Antibodies used for immunohistological analyses included OK1.5 (rat anti-mouse CD4) and 53-6.7 (rat anti-mouse CD8a) from BD Pharmingen and F4/80 (rat anti-mouse macrophage) from Serotec. Rat anti-Ly-6G mAb (RB6-8C5) was purified from culture supernatant using a protein G–sepharose column. Goat anti-mouse CXCR2 antisera, normal goat sera, rabbit anti-mouse KC/CXCL1, and rabbit anti-antibody MIP-2/CXCL2 antisera were prepared and characterized as previously described. Both anti-CD154 mAb and CTLA-4 Ig were purified from their respective cloned cell lines, MR1 and CTLA4 Ig-lg (ATCC) and purified using protein G–sepharose columns.

Immunohistology
A midventricular portion of the cardiac graft was embedded in OCT compound (Sakura Finetek USA) and immediately frozen in liquid nitrogen after harvest, and 8-μm-thick sections were prepared as previously described. Slides were immersed in PBS for 10 minutes and 0.1% H2O2 in PBS for 5 minutes at RT to eliminate endogenous peroxidase activity. Slides were stained with 10 μg/mL GK1.5 (for CD4+ cells), 53-6.7 (for CD8+ cells), F4/80 (for macrophages), or RB6-8C5 (for PMNs) in PBS with 1% BSA for 1 hour at room temperature and then with biotinylated rabbit anti-rat IgG (DAKO Corp) diluted 1:300 in PBS for 20 minutes at RT. The slides were developed with DAB for color change and counterstained by immersion in hematoxylin for 3 minutes. Images were captured and analyzed with Image-Pro Plus (Media Cybernetics). The number of positive cells was counted in a blinded fashion in 10 random fields per slide in 2 nonconsecutive sections per heart and for 3 hearts per time point at ×200 magnification. The counts were then normalized to the area of tissue and expressed in millimeters squared.

Leukocyte Labeling, Adoptive Transfer, and Immunofluorescence Analysis
Spleen cell suspensions were prepared on day 6 after transplantation from mice receiving A/J or SJL (allogeneic third party) cardiac allografts. After RBC lysis, the remaining cells were resuspended at 2×10^7/mL in 7 mL Hanks’ buffered salt solution (HBSS) and carefully layered above 7 mL Lympholyte-M (Cedarlane Laboratories), and the gradient was centrifuged at 1000 g for 30 minutes. Interface cells were collected, washed 3 times in HBSS and counted. The lymphocytes were resuspended in Diluent C (PKH26 Labeling Kit, Sigma) at a concentration of 10^7/mL and 5 μL of PKH26 dye was added for 2 minutes, followed by quenching with PBS 1% BSA for 1 minute. The cell suspension was washed 2 times in HBSS and resuspended at 1×10^7/mL in PBS, and 200 μL (eg, 2×10^7 cells) was injected intravenously to cardiac allograft recipients. Fluorescent images were captured and analyzed with Image-Pro Plus (Media Cybernetics). The fluorescence intensity in the PKH26 channel was extracted from 5 fields from 3 cardiac allografts per group, quantified by Image-Pro Plus software, and reported as mean fluorescence intensity ± SD.

RNA Extraction, In Vitro Transcription, and Ribonuclease Protection Assay
After coculture, endothelial cells were washed in diluted trypsin to remove adherent PMNs. Endothelial cell total RNA was extracted using Trizol reagent as directed (Life Technologies). Cardiac allografts were retrieved and immersed in liquid nitrogen, pulverized with a mortar and pestle, and resuspended in Trizol reagent. Endothelial cell or cardiac allograft expression of genes found in mCK-3b or mCK-5c was quantified by ribonuclease protection assay (RPA) using RiboQuant RPA kits (BD Pharmingen) according to the manufacturer’s protocol. Total endothelial cell or cardiac allograft RNA was purified, quantified by absorbance at 260 nm, and prepared for use in RPA. The [3P]UTP-labeled anti-sense template set...
Antagonism or Deficiency of CXCR2 Prolongs Survival of Cardiac Allografts

Rapid intragraft production of the neutrophil chemoattractants KC/CXCL1 and MIP-2/CXCL2 and a corresponding PMN infiltration are observed in cardiac allografts within hours after transplantation.25 The receptor for both KC/CXCL1 and MIP-2/CXCL2, CXCR2, is constitutively expressed on murine PMNs but not on either CD4+ or CD8+ T cells.26,27 The role of CXCR2 in acute cardiac allograft rejection was tested with 2 complementary approaches. First, recipient C57BL/6 (H-2b) mice were treated with a CXCR2-specific goat antiserum after receiving complete MHC-mismatched heterotopically transplanted cardiac allografts from A/J (H-2a) donors. Whereas control-treated animals rejected cardiac allografts between days 7 and 8 after transplantation, allografts were maintained up to 20 days after transplantation in recipients treated with CXCR2-antisera (Figure 1A). Second, rejection of A/J cardiac allografts was tested in BALB/c (H-2d) CXCR2−/− recipients (Figure 1B). Similar to treatment with CXCR2-antisera, allografts were maintained up to 17 days after transplantation in CXCR2−/− recipients without any adjunctive treatment, whereas grafts were rejected by day 8 in wild-type recipients. Both approaches suggest a role for CXCR2 for the optimal rejection of cardiac allografts.

Attenuation of PMN Infiltration Results in Delayed T-Cell Graft Infiltration

Consistent with our previous studies,25 PMN infiltration in A/J cardiac allografts in C57BL/6 recipients was easily detected and reached peak levels at 24 hours after reperfusion (Figure 2A and 2B). However, PMN infiltration was virtually undetectable at 24 hours after reperfusion in CXCR2-deficient recipients and was significantly decreased in recipients treated with CXCR2 antisera. In conjunction with absent PMN graft infiltration, downstream T-cell infiltration into allografts was significantly delayed in CXCR2−/− recipients (Figure 2C and 2D). Specifically, CD4+ and CD8+ T cells were readily observed infiltrating the parenchyma of allografts harvested from control Ig-treated wild-type animals between days 4 and 5 after transplantation. At this time, low to undetectable numbers of either T-cell population were detected in allografts harvested from CXCR2−/− recipients. At the time of rejection, however, the T-cell infiltrate observed in grafts from control antisera–treated, CXCR2-antisera–treated wild-type, or CXCR2−/− recipients was equivalent (data not shown). These results suggest that inhibition of PMN infiltration into cardiac allografts may have a significant downstream impact on the efficacy of recipient T-cell responses to the allograft.
Intragraft Cytokine Expression Is Significantly Reduced When CXCR2 Is Antagonized

A potential mechanism for the delay in T-cell infiltration observed is a corresponding reduction in inflammatory and chemoattractant signals produced by the interaction of infiltrating PMNs and allograft tissues. To test the capacity of PMNs to amplify inflammation in cardiac allografts, grafts from control antisera–treated, CXCR2-antisera–treated wild-type BALB/c and CXCR2−/− recipients at day 5 after transplantation and stained with anti-CD8 mAb. CD8⁺ cell infiltrate into cardiac allograft parenchyma of CXCR2−/− recipients is minimal at this time. Identical results are observed with CD4⁺ cell infiltration into grafts from wild-type vs CXCR2−/− recipients (data not shown). D, Quantification of CD8⁺ T-cell infiltration in sections of cardiac allograft parenchyma harvested from CXCR2−/− recipients was low to absent at day 5 after transplantation compared with control allograft recipients. All results are displayed as mean and SD of 10 random myocardial fields from 2 nonconsecutive sections from at least 3 grafts per group counted twice in a blinded fashion. This value was normalized to graft parenchymal surface area and shown in millimeters squared. *P=0.021.
type, and CXCR2−/− recipients were tested for expression levels of proinflammatory cytokines at the peak of PMN infiltration, 24 hours after transplantation. Cytokine and chemokine expression analysis of allografts harvested from control animals demonstrated high levels of TNF-α, IL-6, LT-β, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, IP-10/CXCL10, and IFN-γ mRNA expression at 24 hours after transplantation (Figure 3A and 3B). Expression of these cytokines, except for MCP-1/CCL2 and IP-10/CXCL10, was significantly attenuated in PMN-depleted, CXCR2-antisera–treated, and CXCR2−/− recipients. These results provide further evidence linking the inhibition of PMNs with attenuated early posttransplantation cytokine expression in
the allografts harvested from CXCR2-antisera–treated wild-type and CXCR2−/− cardiac allograft recipients.

**PMNs Amplify the Activation State of Endothelial Cells**

After reperfusion, PMNs are quickly recruited into ischemic tissues, including grafts, via the production of chemokines and upregulated adhesion molecule expression. The first allograft cell type encountered by responding PMNs are graft vascular endothelial cells. An in vitro cell coculture model was used to test the influence of PMNs on the activation state of endothelial cells as an indication of the posttransplantation inflammatory response observed in cardiac allografts in vivo. Control-treated endothelial cells expressed low to undetectable levels of cytokine and chemokine genes throughout the time course of the experiment (Figure 3C). Endothelial cells stimulated with a cocktail of TNF-α and IL-1β upregulated several cytokine and chemokine genes, including MIP-2/CXCL2 and IP-10/CXCL10, for 2 to 4 hours, and then expression quickly subsided. The addition of PMNs to cytokine-activated endothelial cells strongly upregulated MIP-2/CXCL2, MIP-1α/CCL3, and MIP-1β/CCL4 genes, far above the levels observed with TNF-α and IL-1β stimulation alone. Furthermore, this expression was extended beyond 12 hours after cytokine stimulation, in contrast to the short-term expression observed with TNF-α and IL-1β stimulation alone (data not shown). These data indicate that PMN–endothelial cell interactions amplify the proinflammatory state observed in endothelial cells and, in conjunction with the in vivo studies, suggest that PMN–vascular endothelium interactions amplify and sustain the inflammatory state observed in cardiac allografts shortly after transplantation.

![Figure 3](http://circ.ahajournals.org/)

**Delay of Allograft Rejection Is Not Due to a Delay in Priming**

Another mechanism that could participate in delayed rejection of the cardiac allografts is a corresponding delay in allospecific T-cell priming. To test the influence of CXCR2 deficiency on T-cell activation in allograft recipients, the number of alloantigen-specific IFN-γ-producing cells was quantified by ELISPOT assay to indicate the level of recipient alloreactive T-cell development to effector cells in response to the allograft. The ELISPOT assays were performed on splenocytes from wild-type and CXCR2−/− animals at 3 posttransplantation time.
microscopy and quantification of sections made from these repeated with fluorescently labeled T cells. Fluorescent transferred cells, the same experimental protocol was antibody staining of allograft sections were indeed the equivalent numbers of allospecific T cells producing IFN-γ (Figure 4). However, by day 5 and at day 7 after transplantation, results displayed are from single experiment and representative of 2 separate experi- ments performed on wild-type and CXCR2−/− spleenocytes har- vested and tested in parallel cocultures.

points. At day 4 after transplantation, few allospecific T cells were observed in either wild-type or CXCR2−/− recipients (Figure 4). However, by day 5 and at day 7 after transplantation, equivalent numbers of allospecific T cells producing IFN-γ were observed in both wild-type and CXCR2−/− recipients. This result indicates that allograft recipients suggested the ineffective infiltration of CXC2−/− recipients and further suggests that the delay in allograft rejection in CXCR2-deficient recipients is independent of an intrinsic T-cell dysfunction in allograft responses.

Transferred Alloantigen-Primed T Cells Require PMN-Mediated Damage to Infiltrate and Rapidly Reject Cardiac Allografts

Because alloreactive T-cell priming was unaffected by the absence of CXCR2, the delayed allograft infiltration of T cells in CXCR2−/− recipients suggested the ineffective infiltration of alloantigen-primed T cells into allografts in the absence of PMN-mediated inflammation. An adoptive transfer model was used to directly test the influence of PMN-mediated parenchymal and endothelial damage on the capacity of T cells to infiltrate the allograft. Donor alloantigen-primed T lymphocytes were transferred into control-treated or PMN-depleted cardiac allograft recipients 1 day after transplantation, and allograft infiltration of the transferred T cells was assessed. Adoptively transferred alloantigen-activated T cells quickly infiltrated the cardiac allografts in control-treated animals. In contrast, a striking decrease in T-cell infiltration into the allograft was observed in animals treated with PMN-depleting antibodies at the time of transplantation (Figure 5A and 5B). This attenuated infiltration was observed at days 2, 3, and 4 after transplantation, a time when endogenous allograft recipient T-cell infiltration into the graft remains low to undetectable.

To verify that the CD4+ and CD8+ cells observed via antibody staining of allograft sections were indeed the transferred cells, the same experimental protocol was repeated with fluorescently labeled T cells. Fluorescent microscopy and quantification of sections made from these allografts yielded results identical to those obtained by staining graft sections by immunohistochemistry to detect graft-infiltrating CD4+ and CD8+ T cells (Figure 5C). Low to undetectable infiltration of T cells was observed in allografts harvested from animals receiving either naïve allogeneic lymphocytes or lymphocytes primed to a third allogeneic party. These results indicate the direct influence of early PMN-mediated events in cardiac allografts that facilitate the migration and infiltration of alloantigen-primed T cells into the grafts.

Costimulatory Blockade During a “Window Period” of Reduced PMN-Mediated Inflammation Results in Long-Term Graft Survival

The reduced ability of alloantigen-primed T cells to infiltrate cardiac allografts when PMN-mediated tissue damage is inhibited suggested that delayed or suboptimal alloreactive T-cell priming in synergy with attenuation of the early posttransplantation inflammation may improve allograft survival. To test the influence of early inflammation on the effectiveness of costimulatory blockade, recipients of complete MHC-mismatched cardiac allografts were treated with a short course of anti-CD154 mAb during a peritransplantation period of PMN depletion. Recipient treatment with antibodies to deplete circulating PMNs resulted in the prolongation of cardiac allograft survival to day 18 to 21 (Figure 6A). With 1 exception, recipients treated with either anti-CD154 mAb (days 0, 1, 2) or CTLA-4 Ig (days 2, 4, 6) alone rejected the cardiac allografts by day 30 after transplantation (Figure 6A and 6B). Combination treatment with anti-PMN plus anti-CD154 antibodies, however, resulted in complete MHC-mismatched allograft survival beyond 100 days in 80% of recipients, and PMN depletion plus CTLA-4 Ig promoted long-term survival in 60% of the recipients.

Cardiac allografts from recipients treated with anti-CD40L mAb, CTLA-4 Ig, or anti-PMN mAb alone were heavily infiltrated with CD4+ and CD8+ T cells, macrophages, and PMNs at the time of rejection (not all data shown). Histological analyses demonstrated minimal cellular infiltration in the long-term-surviving cardiac allografts retrieved from recipients treated with anti-PMN mAb plus costimulation blockade (Figure 6C). However, elastic van Gieson staining demonstrated histological evidence of collagen deposition, intimal thickening, and smooth muscle proliferation in these cardiac allografts. This indicates that despite long-term survival and minimal cellular infiltration, the development of chronic transplant-associated vasculopathy is not entirely prevented (Figure 6D).

Because PMN depletion is not a viable clinical option, KC/CXCL1- and MIP-2/CXCL2-specific antibodies were administered to antagonize PMN recruitment into the allografts. These antisera do not deplete or bind any cell popu- lation, and a similar agent would be more amenable for use in human patients. Previous studies indicated the ability of these antibodies to prolong AJ cardiac allograft survival in C57BL/6 recipients to 21 days after transplantation.23 These antibodies in synergy with costimulatory blockade similarly prolonged the survival of cardiac allografts beyond 100 days after transplantation (Figure 6A). Collectively, these results indicate that inhibiting the development of intragraft inflam-
Figure 5. Transfer of alloantigen-primed T cells 24 hours after transplantation infiltrates cardiac allografts at delayed rate in recipients depleted of PMNs at time of transplantation. A, Representative images (×200) of sections stained with anti-CD8 mAb to detect presence of T cells in cardiac allografts harvested from control- and anti-PMN–treated recipients on days 3 and 4 after transplantation. CD8⁺ T-cell infiltrate is markedly attenuated in PMN-depleted recipients vs control-treated recipients on both days. B, Quantification of cardiac allograft sections stained on days 2, 3, and 4 after transplantation (days 1, 2, and 3 after alloantigen-primed T-cell transfer) shows marked attenuation of both CD8⁺ and CD4⁺ (data not shown) T-cell infiltration when recipients are treated with 3 peritransplantation doses of anti-Ly-6G mAb. Of note, cardiac allografts are rejected in accelerated fashion in control-treated recipients (by day 4 or 5 after transplantation), whereas cardiac allografts continue to beat well in anti-PMN–treated recipients at this time. All results are displayed as mean of 10 random myocardial fields from 2 nonconsecutive sections from 3 grafts per group counted twice in blinded fashion. This value was normalized to graft parenchymal surface area and shown in millimeters squared. *P<0.0057, **P<0.014. C, Fluorescence images (×200) and quantification of sections prepared from cardiac allografts harvested from control- and anti-PMN–treated recipients on day 3 after transplantation and day 2 after transfer of PKH26-labeled alloantigen-primed T cells. Images and quantification show marked attenuation in cell infiltration in PMN-depleted recipients vs control-treated recipients. In addition, cardiac allografts harvested from recipients of PKH26-labeled T cells from naïve animals or PKH26-labeled third-party alloantigen-primed (SJL, H-2b) T cells show minimal to no labeled cell infiltration. *P<0.002.
Figure 6. Long-term survival of complete MHC-mismatched cardiac allografts after short-term antagonism of early post-transplantation inflammatory response and T-cell costimulatory blockade. A, Control-treated C57BL/6 (H-2b) recipients reject A/J cardiac allografts (H-2a) on days 7 to 9 after transplantation (\( P < 0.0001 \)). C57BL/6 recipients treated with 150 \( \mu g \) anti-PMN mAbs on days 1, 0, and 1 after transplantation rejected A/J cardiac allografts on days 19 to 21 after transplantation (\( P < 0.0001 \)). C57BL/6 recipients treated with 200 \( \mu g \) anti-CD154 mAbs on days 0, 1, and 2 after transplantation rejected A/J cardiac allografts on days 19 to 54 (mean, 24 days) after transplantation (\( P < 0.0001 \)). However, C57BL/6 recipients treated with combination therapy of 150 \( \mu g \) anti-PMN antibodies on days 1, 0, and 1 plus 200 \( \mu g \) anti-CD154 antibodies on days 0, 1, and 2 after transplantation similarly prolonged survival of allografts beyond 100 days after transplantation (\( P < 0.0001 \)). n = 5 to 8 mice per group. B, C57BL/6 recipients treated with 200 \( \mu g \) CTLA-4 Ig on days 2, 4, and 6 after transplantation rejected A/J cardiac allografts on days 18 to 27 (mean, 23 days) after transplantation (\( P < 0.0001 \)). C57BL/6 recipients treated with combination therapy of 150 \( \mu g \) anti-PMN antibodies on days −1, 0, and 1 plus 200 \( \mu g \) anti-CD154 antibodies on days 0, 1, and 2 after transplantation inhibited rejection of A/J cardiac allografts (\( P < 0.0001 \)). Administration of anti-KC and anti-MIP-2 antibodies on days −1, 0, and 1 and 200 \( \mu g \) anti-CD154 antibodies on days 0, 1, and 2 after transplantation similarly prolonged survival of allografts (\( P < 0.0001 \)). n = 4 to 6 mice per group. C, Representative images (\( \times 200 \)) of sections of cardiac allografts retrieved from MR1 mAb–treated recipients at time of rejection and anti-PMN mAb plus MR1 mAb–treated recipients at day 100 after transplantation. Sections were stained with primary antibodies against Ly-6G (PMNs), F4/80 (macrophages), CD4 (CD4\(^+\) T cells), and CD8\(^\alpha\) (CD8\(^\alpha\) T cells). In MR1–treated recipients, PMNs, macrophages, and T cells heavily infiltrate cardiac allograft at time of rejection. However, low to absent infiltration by PMNs, macrophages, and T cells is observed in anti-PMN plus MR1 mAb–treated recipients at day 100 after transplantation. D, Representative images (\( \times 200 \)) of elastic van Gieson–stained sections of cardiac allografts retrieved from anti-PMN mAb plus MR1 mAb–treated recipients and syngeneic cardiac grafts harvested on day 100 after transplantation. Collagen deposition and intimal thickening are observed in treated group; isografts are without significant pathology.
mation at early times after transplantation via PMN antagonism may provide a “window” for a more effective costimulatory blockade, resulting in long-term allograft survival.

Discussion

Ischemia and reperfusion of a harvested allograft are intrinsic steps of solid-organ transplantation. The ensuing I/R injury imposed on transplanted organs initiates an intense intraallograft inflammatory state after transplantation. The histological hallmark of this early innate immune response is a PMN-dominant infiltrate that contributes to parenchymal tissue damage, stimulates the upregulation of adhesion and MHC molecules on the vascular endothelium, and induces production of cytokines and chemokines that promote leukocyte trafficking to the allograft. In addition, this intraallograft proinflammatory milieu stimulates the maturation and emigration of donor-derived antigen-presenting cells from the graft to draining lymphoid tissue, where they prime naïve recipient alloreactive T cells.

The contribution and impact of specific components of early inflammation in directing leukocyte trafficking into allografts remain unclear. Despite intriguing evidence, relatively limited attention has been paid to the role of the early inflammatory response or the influence of the PMN on the rejection process. The present study demonstrates that antagonizing CXCR2 or its ligands provide a window period of low PMN-mediated inflammation in allografts after transplantation. CXCR2 antagonism reduced the number of infiltrating PMNs observed within the graft parenchyma at 24 hours after reperfusion and delayed graft infiltration by alloantigen-primed T cells. The levels of intraallograft cytokine and chemokine expression after reperfusion were also attenuated, indicating an important aspect of inflammation that infiltrating PMNs observed within the graft parenchyma at 24 hours after reperfusion and delayed graft infiltration by alloantigen-primed T cells. The requirement for costimulatory interactions during T-cell activation has suggested these molecules as targets for therapeutic intervention in antirejection protocols. Such strategies, aimed at inhibiting alloantigen-specific T-cell priming, have typically attempted to block costimulation signals at the time of transplantation, which is a period of intense inflammation. Bingaman and colleagues demonstrated that T cells adoptively transferred to immunodeficient animals 7 weeks after cardiac allograft transplantation were capable of trafficking to and rejecting the allografts. These results were interpreted as indicating that T cells can be directed to and infiltrate allografts in the absence of the ongoing inflammation associated with I/R injury. In light of our present study indicating a role for PMN-mediated tissue damage, it is possible that early parenchymal and endothelial cell damage permanently “marks” the cardiac allograft and would direct transferred T cells to the graft at later times after transplantation when the allograft is considered healed. Results of the present study predict that attenuation of early PMN-mediated inflammation would protect the allograft from parenchymal tissue damage at the time of transplantation and attenuate the recruitment of the transferred T cells to the allograft.

The requirement for costimulatory interactions during T-cell activation has suggested these molecules as targets for therapeutic intervention in antirejection protocols. Such strategies, aimed at inhibiting alloantigen-specific T-cell priming, have typically attempted to block costimulation signals at the time of transplantation, which is a period of intense inflammation in the allograft. Despite great hopes for its potential, no costimulatory blockade protocol has been universally effective or clinically applicable. During the course of these experiments, we hypothesized that an impediment to achieving robust and long-lasting allograft survival with costimulatory blockade was the observed early inflammation and parenchymal tissue damage that may permanently mark the graft and direct the recruitment of T cells that escape costimulatory blockade and are activated to alloantigens. A combination strategy that antagonizes these early inflammatory events and their effects would be expected to protect the graft from PMN-mediated damage and to improve the efficacy of costimulatory blockade as a strategy for allograft acceptance. A therapy that first depleted PMNs at the time of transplantation to establish a window period of low inflammation in combination with a short course of costimulatory blockade resulted in long-term allograft survival (>100 days). This prolonged survival was accompanied by low to no cellular infiltration into the allograft at day 100, indicating the absence of rejection. These results support the concept of allograft marking in which early PMN-mediated inflammation damages allograft parenchyma and endothelium and...
directs allograft immune responses (despite costimulatory blockade) to the graft. A recent case report by Spitzer and colleagues34 may provide a clinical correlate to the present findings. They reported that a patient with end-stage renal disease secondary to multiple myeloma received bone marrow and renal transplants. Of note, this patient was neutropenic for the initial 11 days after transplantation and was able to discontinue all immunosuppressive therapy after 10 weeks without evidence of renal dysfunction through at least 6 months after transplantation.

A recent report from our laboratory elucidated a novel immune mechanism that amplifies PMN infiltration into cardiac allografts within hours after transplantation.35 In the absence of recipient CD8+ T cells or the ability to produce IFN-γ, the intensity of PMN infiltration and tissue necrosis is reduced in the allograft. The induction of similar allogeneic memory CD8+ T cells has been shown to be induced by viral infections (ie, heterologous immunity), and such T-cell populations are likely to be ubiquitously present in most if not all transplant recipients.36,37 With regard to the present studies, it is unclear whether the activity of these allogeneic memory CD8+ T cells is dependent on the prior low-level PMN infiltration into the allograft that is also observed in isografts. However, the decreased PMN infiltration and proinflammatory cytokine expression observed in allografts in CXCR2−/− recipients at 24 hours after transplantation suggest that prior PMN activity in the allografts is required to induce the function of the memory CD8+ T cells. This requirement may also underlie the ability to promote long-term survival of the allografts by interfering with PMN activity and the activation of naïve donor-reactive T cells without therapy directed at these memory CD8+ T cells. Alternatively, mechanisms mediating the vasculopathy observed in the long-term-surviving allografts are undefined at this point but may involve the activity of the memory CD8+ T cells.

The early inflammation mediating graft marking could be at the level of the parenchyma or at the vascular endothelium. Early parenchymal damage could potentially lead to scarring and fibrosis that establishes a chronic inflammatory state and antigen leak, providing alloantigen and additional recruiting signals to alloantigen-reactive T cells. These events would, in turn, promote the migration and infiltration of T cells that escape or are resistant to the use of costimulation blockade at early times. Alternatively, PMN-mediated damage to the graft vascular endothelium may disrupt the extracellular matrix and/or vascular monolayer, leading to improper healing. Improperly healed endothelium may be permanent and direct the infiltration of circulating antigen-presenting cells into the graft. Additionally, improperly remodeled matrix and/or damaged endothelium may also direct the infiltration of primed allogeneic T cells, despite costimulatory therapies, into the marked graft. This is the first demonstration of a strategy using intervention aimed at both early inflammation and T-cell activation for the protection and prolongation of allograft survival. Importantly, short-term antibody administration to interrupt or block PMN trafficking to and infiltration into the graft would be a clinically feasible strategy. The long-term effects and influence of the early inflammatory response on allograft function remain unclear. The potential of combination therapy of short-term PMN antagonism with costimulatory blockade may obviate the need for chronic or long-term immunosuppression and avoid the deleterious effects of currently used immunosuppressive strategies.

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**CLINICAL PERSPECTIVE**

The present report indicates that polymorphonuclear leukocyte (PMN)-mediated damage is associated with upregulated expression of proinflammatory cytokines and adhesion molecules and facilitates alloantigen-primed T-cell infiltration into the allograft. Rejection episodes indicate both graft-reactive T-cell activation and the ability of the activated T cells to traffic to and infiltrate the cardiac allograft. Factors directing activated T cells to a seemingly healthy graft several months to years after the transplantation remain poorly understood. The results of this study indicate the impact of I/R injury and PMN-mediated inflammation on directing alloantigen-primed T-cell trafficking to the cardiac allograft. Results suggest that strategies directed at attenuating these early inflammatory effects might further improve the efficacy of immunosuppressive regimens directed at preventing T-cell activation.
Inhibition of Polymorphonuclear Leukocyte–Mediated Graft Damage Synergizes With Short-Term Costimulatory Blockade to Prevent Cardiac Allograft Rejection

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