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Targeted Complement Inhibitors Protect against Posttransplant Cardiac Ischemia and Reperfusion Injury and Reveal an Important Role for the Alternative Pathway of Complement Activation

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Ischemia reperfusion injury (IRI) is an unavoidable event during solid organ transplantation and is a major contributor to early graft dysfunction and subsequent graft immunogenicity. In a therapeutic paradigm using targeted complement inhibitors, we investigated the role of complement, and specifically the alternative pathway of complement, in IRI to heart isografts. Mouse heterotopic isograft heart transplants were performed in C57BL/6 mice treated with a single injection of either CR2-Crry (inhibits all complement pathways) or CR2-H (inhibits alternative complement pathway) immediately posttransplantation. Transplanted hearts were harvested at 12 and 48 h for analysis. Both inhibitors resulted in a significant reduction in myocardial IRI, as measured by histology and serum cardiac troponin I levels. Furthermore, compared with untreated controls, both inhibitors reduced graft complement deposition, neutrophil and macrophage infiltration, adhesion molecule expression (P-selectin, E-selectin, and I-CAM-1), and proinflammatory cytokine expression (TNF-α, IL-1β, KC, and MCP-1). The reduction in myocardial damage and cellular infiltration was not significantly different between CR2-Crry– and CR2-H–treated mice, although adhesion molecule and cytokine levels were significantly lower in CR2-Crry–treated mice compared with CR2-H–treated mice. In conclusion, the alternative complement pathway plays a major contributing role in myocardial IRI after heart transplantation, and local (targeted) complement inhibition has the potential to provide an effective and safe therapeutic strategy to reduce graft injury. Although total complement blockade may be somewhat more efficacious in terms of reducing inflammation, specific blockade of the alternative pathway is likely to be less immunosuppressive in an already immunocompromised recipient.

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complemented recipient (16). In the current study, we characterize the effect of two targeted complement inhibitory proteins, CR2-Cry (inhibits all complement pathways) and CR2-fH (inhibits the alternative complement pathway), in an isograft model of cardiac transplantation. We have previously shown that these inhibitors target specifically to sites of complement activation and are therapeutically effective at doses that do not significantly affect systemic complement activity or susceptibility to infection (17). In the current study, our focus is on the inflammatory response after graft transplantation, and we use an isogenic pairing to minimize any potential differences in alloimmune responses and in particular any potential differences in alloantibody responses or titers.

Materials and Methods

Animals
Male adult C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) weighing 25–30 g were used for transplantation studies. Animals were housed under conventional conditions at the Medical University of South Carolina (Charleston, SC). All procedures were approved by the Medical University of South Carolina Committee for Animal Research in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Transplant surgery
C57BL/6 mouse hearts were transplanted into the abdomens of C57BL/6 recipients by anastomosis of the donor and recipient aortas and the donor pulmonary artery with recipient inferior vena cavae, as described previously (18). Donor hearts were removed, perfused, and stored in PBS at 4°C for 35 min prior to implantation. Anastomosis time was standardized to 30 min. Animals were randomized into three treatment groups: 1) PBS vehicle control, 2) 0.25 mg CR2-Cry, and 3) 0.4 mg CR2-fH. Animals were treated with inhibitor or control immediately following completion of surgery. Inhibitor dosage was based on previously published dose-response data in other models (17, 19, 20). The CR2-Cry and CR2-fH fusion proteins were produced and purified as described previously (17, 19, 20). Transplanted hearts were explanted 12 or 48 h postimplantation and divided into four cross-sections.

Histopathology
For histological examination, tissue blocks were placed in 10% buffered formaldehyde solution for 48 h before being embedded in paraffin. Four-micrometer heart sections were stained with H&E stain. Sections were scored by a pathologist (M.G.), who was blinded to the experimental group, using a semiquantitative histology scoring system. Sections were scored 0–3 in four categories for evidence of epicardial, myocardial, endocardial damage, and inflammatory cell infiltrate, where 0 = no damage, 1 = mild damage, 2 = moderate damage, and 3 = severe damage. The results are expressed as cumulative scores from 0 to 12. To further quantify evidence of cardiac damage, we measured cardiac enzyme troponin I activities as an index of cardiac damage, using a commercially available assay kit (Life Diagnostics, West Chester, PA).

Complement deposition
Paraffin-embedded heart tissue sections (5 μm) were immunostained for the presence of C3d. Immunohistochemistry staining for C3d was performed using an Ab directed against C3d (R&D Systems, Volcano, CA). Ab binding was visualized using the Vector Laboratories Immunofluorescence detection kit and diaminobenzidine chromogen development (Vector Laboratories, Burlingame, CA). Specificity of staining was assessed by omission of primary Ab and the use of isotype controls.

Serum complement inhibition
Blood samples were taken at 12 and 48 h posttransplantation and serum was prepared to assay the effect of each targeted complement inhibitor on serum complement activity. Levels of serum complement inhibition were measured using a previously described method that uses flow cytometric analysis of C3 deposition on either zymosan A particles (Sigma-Aldrich, St. Louis, MO) for alternative pathway activity, or on Ab-sensitized CHO cells for classical pathway activity (17).

Immunohistochemistry

The presence of neutrophils (GR1; BD Pharmingen, San Jose, CA), macrophages (mac-3; BD Pharmingen), P-selectin, E-selectin, ICAM-1, and VCAM-1 (BD Pharmingen) was assessed by immunohistochemistry and scored semiquantitatively as described previously (21). In brief, sections were scored from 0 to 3, where 0 = no staining; 1 = weak diffuse endothelial staining < 25% of vessels; 2 = moderate staining involving <50% of vessels; and 3 = strong immunostaining > 50% of vessels are positive.

ELISA and cytokine array

Inflammatory cytokines MCP-1, KC, IL-1ß, TNF-α, and IL-6 were measured in heart grafts harvested at 12 and 48 h posttransplantation. Hearts were homogenized, and protein was extracted from tissues as described previously (22). Cytokine levels were measured using ELISA kits (R&D Systems) in accordance with the manufacturer’s recommendations.

Quantitative RT-PCR

Total RNA was extracted from hearts using Qiagen Fibrosis RNA extraction kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. cDNA was made from 1 μg total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time RT-PCR analysis was subsequently performed using the IQ SYBR Green Supermix kit (Bio-Rad) following the manufacturer’s protocols. Analyses were performed using My IQ Real-Time detection system (Bio-Rad) using intron-spanning primers specific for ICAM-1 (forward, 5’-GGCGTCCATGTTCTTCTCAA-3’; reverse, 5’-TTTGAGGGCAGAAAACAGG-3’), VCAM-1 (forward, 5’-CCCAACAG- GAGCCGAGTGT-3’; reverse, 5’-CAGGATTTTGAGCGTGTTG-3’), E-selectin (forward, 5’-AGCTACCATGAAACAGCAG-3’; reverse, 5’-C -GCAAATTCTCCAACGCT-3’), MCP-1 (forward, 5’-ATGTCTTGCTGCTGACTGTT-3’; reverse, 5’-CTTATGCAACAGTGAATC-3’), TNF (forward, 5’-ATGCTGCTGCTACGTGAACACT-3’; reverse, 5’-CTTATGCAACAGTGAATC-3’), and IL-1ß (forward, 5’-AGCTACCCATGGA- CACGAC-3’; reverse, 5’-CCGAAATCTTCTCCAGCTT-3’). All reactions were performed in triplicate, and the GAPDH gene was used as an internal control.

Statistics

Data are expressed as mean ± SD. Significant differences between groups were determined by ANOVA, with a Bonferroni correction for continuous variable and multiple groups. A two-tailed Student t test was used for the comparison of a normally distributed continuous variable between two groups, and nonparametric two-tailed statistical analyses were used for histological analyses using GraphPad Prism version 5.00 for Mac OS X, GraphPad Software (GraphPad, San Diego, CA).

Results

Targeted complement inhibition with either CR2-Cry or CR2-fH confers myocardial protection

To investigate the effect of CR2-Cry and CR2-fH on posttransplant IRI, cardiac grafts were assessed for histological evidence of damage at 12 and 48 h after transplantation. Hearts from control-treated recipient mice exhibited key features associated with IRI, including myocyte damage in the epicardium, endocardium, and myocardium. Grafts from control-treated mice also showed evidence of inflammatory cell infiltration and endothelial activation, denoted by endothelial swelling. All of these features were markedly reduced in CR2-Cry– and CR2-fH–treated animals. To quantify these changes in inhibitor-treated mice, H&E-stained heart sections were scored using a semiquantitative cumulative histological scoring system (see Materials and Methods). Treatment with either complement inhibitor was associated with a significant decrease in histology score when compared with controls (Fig. 1A). The severity of histological injury increased significantly between 12 and 48 h in control grafts but not in complement-inhibited animals. The release of the cardiac enzyme cardiac troponin I as an index of cardiac cell damage was measured in serum prepared from blood collected at 48 h posttransplantation. There was a significant increase in cardiac troponin I in control-treated animals compared with sham controls. However, both...
Complement inhibition reduces inflammatory cytokine expression

Previous studies have demonstrated that graft cytokine expression levels change dramatically after transplantation, with different cytokines peaking at different times posttransplant (27). We analyzed TNF-α, IL-1β, MCP-1, and KC in protein extracted from grafts isolated at 12 and 48 h posttransplant. All cytokine expression levels were normalized by total protein concentrations. At 12 h posttransplant, there was a significant increase in all measured cytokines in grafts from control-treated recipients. However, compared with control-treated mice, levels of all cytokines were significantly reduced in both CR2-Crry- and CR2-fH-treated mice (Fig. 5). Expression levels of TNF-α and IL-1β were significantly lower in CR2-Crry–treated mice compared with CR2-fH–treated mice. By 48 h after transplantation, cytokine levels in all groups were not significantly different to sham baseline levels (Fig. 5).

Complement inhibition reduces inflammatory cytokine and adhesion molecule gene transcription

To corroborate protein analysis of adhesion molecule and cytokine expression, we also analyzed transcript expression in grafts harvested at 12 and 48 h after transplant. There was some divergence between protein and gene expression data for ICAM-1 at 12 h and between protein and gene expression data for ICAM-1 at 12 h and for E-selectin at 48 h posttransplant in CR2-Crry–treated recipients, but otherwise, gene expression data were in keeping with protein data (Fig. 6). IL-1β and TNF-α can both exert transcriptional activation of adhesion molecules through an NF-κB–dependent axis and can also promote inflammation and direct myocyte death. Compared with controls, both CR2-Crry and CR2-fH significantly reduced IL-1β and TNF-α gene expression in grafts at both 12 and 48 h posttransplant (Fig. 6). These data correspond to cytokine protein levels seen 12 h posttransplant, although differences in cytokine protein levels between sham- and inhibitor-treated mice did not reach significance at 48 h posttransplant. Neither inhibitor impacted IL-6 gene expression.

Therapeutic doses of CR2-Crry and CR2-fH have no significant effect on serum complement activity

The complement system plays an important role in host immunity, and systemic complement inhibition has been shown to increase susceptibility to infection, a potential limitation for the use of complement inhibition in immunocompromised transplant recipients. We therefore assessed the effect of CR2-Crry and CR2-fH on
serum complement activity in recipient mice. We determined alternative and classical pathway activity by measuring C3 deposition on zymosan particles and Ab-sensitized CHO cells, respectively. At the doses used in the above experiments that provided protection from myocardial IRI, neither inhibitor had any measurable effect on serum complement activity in recipient mice at 12 or 48 h posttransplant (Fig. 7). We have previously shown that the circulatory half life of both CR2-Crry (17) and CR2-fH (20) is ∼8.5 h and that both inhibitors target to sites of complement activation in vivo.

Discussion
IRI occurs in all solid organ transplants, with the length of ischemia correlated to graft outcome (14, 28). The process of transplantation exposes the donor organ to periods of cold ischemia, incurred during organ transportation, and a period of warm ischemia associated with organ implantation into the recipient. The complement system has been implicated in the pathogenesis of IRI in the nontransplant setting, and clinical cardiac transplantation studies have highlighted a significant correlation between complement deposition early post transplant and subsequent graft rejection (24). Given these correlations, treatments directed at inhibiting IRI, and specifically at inhibiting complement activation, have attracted interest as potential therapeutic interventions. A key challenge is the application of a therapeutic strategy that minimizes any impact on host immune or homeostatic functions. In this study, we have used a clinically relevant cardiac transplant model that incorporates cold and warm ischemia to investigate whether the complement system plays a role in posttransplant IRI and whether the application of targeted therapeutics can...
significantly reduce damage while maintaining normal systemic complement function.

Pharmacological blockade of complement has been shown to reduce IRI following organ transplantation in various rodent models, but only a single study has addressed myocardial IRI post-transplantation. In this previous study, systemic inhibition with an anti-C5 minobody reduced myocardial IRI in a rat isograft model (16). In another study, pretreatment of the donor heart with University of Wisconsin solution containing small interfering RNA targeting TNF-α, Fas, and C3 resulted in improved graft outcome after prolonged cold storage, but given the triple therapeutic approach, it is not possible to assess the relative contribution of C3 silencing in this model (16). Myocardial IRI in a nontransplant setting with warm ischemia, however, has been more extensively studied with regard to the role of complement. In a seminal study, soluble CR1 was shown to significantly reduce inflammation and infarct size in a rat model of IRI (29). CR1, like Crry, promotes decay acceleration of the classical and alternative pathway C3 convertases and acts as a cofactor for factor I-mediated proteolysis of C3b and C4b. Each of these activities serves to block the subsequent activation of new C3 molecules (fH acts by promoting decay acceleration of the alternative pathway C3 convertase and as a cofactor for factor I-mediated proteolysis of C3b). Subsequent studies using C6-deficient animals or animals treated with anti-C5 Ab or C5a receptor antagonist indicate an important role for the terminal complement activation products C5a and/or the MAC in warm myocardial IRI (30–33). More recently, it has been shown that myocardial IRI in a nontransplant setting is dependent on IgM and the lectin pathway (34), and mice deficient in the classical or alternative pathway are not protected from myocardial IRI (35–37). In a transplant setting, however, and under therapeutic conditions of complement inhibition (as opposed to deficiency), we show in this paper an important role for the alternative complement pathway in myocardial IRI. Inhibiting all complement activation pathways or specifically inhibiting the alternative pathway was similarly effective at reducing posttransplant myocardial injury, although CR2-

**FIGURE 5.** Effect of complement inhibition on intragraft cytokine levels. Grafts transplanted into PBS-, CR2-Crry–, and CR2-fH–treated mice were harvested at 12 or 48 h posttransplantation. Intragraft expression of TNF-α (A), IL-1β (B), KC (C), and MCP-1 (D) was examined by ELISA. Note that intragraft cytokine levels decline to baseline levels by 48 h. Results are expressed as mean ± SD; n = 7–8 for all groups. #p < 0.001; @p < 0.01 CR2-Crry versus CR2-fH.

**FIGURE 6.** Effect of complement inhibition on transcript levels of adhesion molecules and cytokines in grafts. mRNA levels were determined in tissue samples from hearts isolated at 12 h (A) or 48 h (B) posttransplantation by quantitative RT-PCR. Results are expressed as mean ± SD; n = 6 for all groups. #p < 0.01 versus vehicle control group; *p < 0.01 CR2-Crry versus CR2-fH.

**FIGURE 7.** Effect of targeted complement inhibitors on serum complement activity. Classical and alternative pathway complement activity was assessed in serum samples obtained at 12 or 48 h posttransplantation in control and complement inhibited animals. A, C3 deposition on Chinese hamster ovary cells assessing classical pathway activation. B, C3 deposition on zymosan particles assessing alternative pathway activation. Neither CR2-Cry nor CR2-fH significantly effects C3 deposition on either CHO cells or zymosan particles indicating neither inhibitor has any effect on classical or alternative pathway serum activity (n = 6 in all groups).
Crry was more effective than CR2-fH at reducing the expression of certain inflammatory markers. This finding is in line with the increasing recognition that the alternative pathway plays a key role in causing tissue injury in a variety of inflammatory and ischemic conditions, even when there is dependence on the classical or lectin pathways for initiating complement activation (20, 38).

Clinical and animal studies indicate an important role for adhesion molecule expression in the infiltration of both innate immune cells and lymphocytes (1, 39, 40), and although in the current study both inhibitors reduced adhesion molecule expression, CR2-Crry had a more profound effect than CR2-fH. Although this difference did not appear to impact innate cellular infiltrate, it is possible that lymphocyte infiltration and immune priming may be differently impacted in an allograft. In this context, the absence of allograft ICAM-1 has been shown to attenuate alloantigen-specific T cell priming with an associated delay in the onset of rejection (39). Similar correlations have also been reported for P-selectin expression, with deficiency associated with reduced acute rejection, although deficiency appears to exacerbate vasculopathy (25, 41).

In our isograft model, both complement inhibitors resulted in a marked reduction in myocyte cytotoxic cytokines TNF-α and IL-1β and in neutrophil and macrophage chemokines KC and MCP-1. However, similar to our findings with adhesion molecule expression, CR2-Crry treatment resulted in a more pronounced reduction in the level of these molecules compared with CR2-fH treatment. TNF-α and IL-1β can influence adhesion molecule expression via NF-κB activation, and the difference between CR2-Crry and CR2-fH treatment on adhesion molecule expression may be mediated via the effect of the inhibitors on cytokine expression. However, complement activation products can also directly modulate adhesion molecule expression, and complement inhibition likely effects adhesion molecule expression via both direct and indirect complement-dependent mechanisms.

A potential problem in the translation of a complement inhibitory strategy to the clinic is the immunosuppressive effect of systemic complement inhibition in an already immunocompromised recipient. Although infection is not currently a concern in transplant recipients, current immunosuppressive drugs inhibit adaptive T cell responses, and the effect of additionally inhibiting an important innate immune mechanism (i.e., complement) is not known. Strategies investigated to alleviate potential concerns of systemic complement inhibition include treatment of the donor organ with a complement inhibitor prior to transplantation and inhibition of the terminal complement pathway with anti-C5 Ab therapy. However, although anti-C5 therapy (blocks C5a and the MAC) is effective at reducing myocardial IRI following transplantation, C5a has important roles in host defense, and C3 deposition in the graft was undetectable. Whether the greater reduction in inflammatory markers seen with CR2-Crry compared with CR2-fH translates to an enhanced reduction in alloimmunity and graft rejection remains to be determined. Further studies in an allograft model of heart transplantation would address this and, together with the current data, would provide further information on the contribution of complement-dependent myocardial IRI to graft immunogenicity and rejection.

In conclusion, we demonstrate the efficacy of two targeted complement inhibitors at protecting cardiac isografts from IRI and further demonstrate a key role for the alternative complement pathway in posttransplant IRI under therapeutic conditions. Total complement blockade (CR2-Crry) was more effective than alternative pathway blockade (CR2-fH) at reducing the expression of certain inflammatory molecules, but neither inhibitor had any effect on systemic complement activity 12 or 48 h after transplant, a potential benefit in an already immunocompromised patient.

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Disclosures
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