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Research Article **Immunology**

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Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection

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Previous studies indicate a pivotal role for complement in mediating both local and remote injury following ischemia and reperfusion of the intestine. Here, we report on the use of a mouse model of intestinal ischemia/reperfusion injury to investigate the strategy of targeting complement inhibition to sites of complement activation by linking an iC3b/C3dg-binding fragment of mouse complement receptor 2 (CR2) to a mouse complement-inhibitory protein, Crry. We show that the novel CR2-Crry fusion protein targets sites of local and remote (lung) complement activation following intestinal ischemia and reperfusion injury and that CR2-Crry requires a 10-fold lower dose than its systemic counterpart, Crry-Ig, to provide equivalent protection from both local and remote injury. CR2-Crry has a significantly shorter serum half-life than Crry-Ig and, unlike Crry-Ig, had no significant effect on serum complement activity at minimum effective therapeutic doses. Furthermore, the minimum effective dose of Crry-Ig significantly enhanced susceptibility to infection in a mouse model of acute septic peritonitis, whereas the effect of CR2-Crry on susceptibility to infection was indistinguishable from that of PBS control. Thus, compared with systemic inhibition, CR2-mediated targeting of a complement inhibitor of activation improved bioavailability, significantly enhanced efficacy, and maintained host resistance to infection.

Introduction

Intestinal ischemia/reperfusion injury (IRI) is a major complication associated with abdominal surgery, cardiopulmonary bypass, ruptured abdominal aneurysm, and cardiac arrest (1–5). Reduction of abdominal blood flow as a result of hemorrhagic shock also causes intestinal IRI, which commonly leads to bacterial translocation and sepsis. Intestinal IRI causes gut dysfunction that is characterized by impaired gut motility, increased intestinal permeability, and mucosal wall injury, all of which are thought to be mediated at least in part by complement activation and the infiltration of neutrophils (6–8). Complement activation products and tissue injury result in the induction of a systemic inflammatory response with the release of cytokines and chemokines, the upregulation of adhesion molecules, and the activation of leukocytes. The activation of a systemic proinflammatory state results in remote organ damage to which the lung is particularly susceptible (9–12).

Many studies have utilized rodent models of intestinal IRI to investigate the underlying pathophysiological mechanisms of IRI and to test potential therapeutic strategies. The pathogenesis of IRI is complex, but a series of elegant studies have shown that preexisting clonally specific IgM antibodies bind to neoantigens exposed by the ischemic insult and, following reperfusion, activate the complement system, which results in tissue damage (13–15). The role of antibodies in initiating IRI is further supported in other studies using *Cr2*$^{-/-}$ mice, which are protected from IRI due to a deficient natural antibody repertoire (8, 16). Pretreatment of these mice with IgM and IgG purified from wild-type mice showed that these Ig subclasses can each contribute separately to IRI (16), and it was recently shown that tissue injury can be restored in these mice by reconstitution with antibodies against negatively charged phospholipids or β2 glycoprotein 1 (17). These data indicate that multiple specificities may be involved in antibody interactions with ischemic antigens. The subsequent activation of complement and its role in IRI of various organs and tissues is supported by numerous studies using complement-deficient animals (18–22). Furthermore, studies with pharmacological agents that inhibit complement activation or block specific components of the complement system have been shown to be effective in ameliorating injury (23–30).

To date, all of the complement-inhibitory approaches used to protect from IRI in experimental models systemically inhibit the complement system. However, despite the therapeutic success of these approaches, there are potential hazards associated with systemically inhibiting complement since it plays important roles in host defense and immune homeostasis (31–36). Although these considerations may be of less significance for acute administration of complement inhibitors, there could be serious consequences if long-term therapy is required or if inhibition is required in immunocompromised patients undergoing a surgical procedure or with traumatic injury.

We recently described a strategy to specifically target complement inhibitors to sites of complement activation by linking human complement inhibitors to the C3-binding region of human com-

Nonstandard abbreviations used: CLP, cecal ligation and puncture; CR2, complement receptor 2; IRI, ischemia/reperfusion injury; sCrry, soluble Crry; sCR1; soluble CR1.

Conflict of interest: V.M. Holers is a cofounder of Taligen Therapeutics Inc., which develops complement inhibitors for therapeutic use. S. Tomlinson is a consultant for Taligen Therapeutics Inc.

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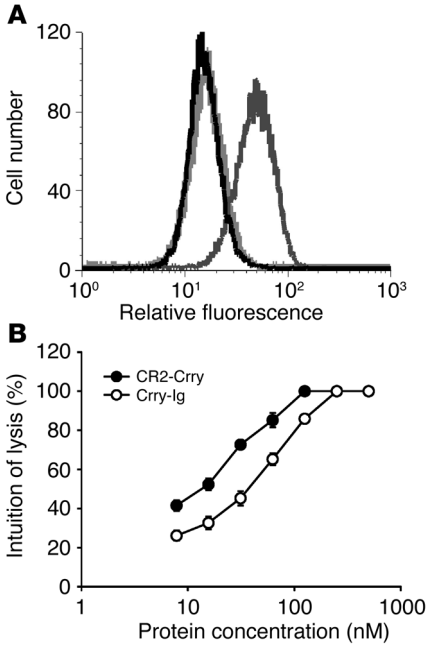

Figure 1
In vitro characterization of recombinant CR2-Crry and Crry-Ig. (**A**) Flow cytometric analysis of binding of recombinant fusion proteins to C3-opsonized CHO cells; Crry-Ig (light gray line), CR2-Crry (dark gray line) and PBS control (black line). Representative of 3 separate experiments. (**B**) Inhibition of complement-mediated lysis by recombinant fusion proteins. Antibody-sensitized CHO cells were incubated with 15% rat serum (which resulted in approximately 90% lysis in the absence of inhibitor), and lysis was determined after 1 hour at 37°C. Background lysis determined by incubating cells in heat-inactivated serum was less than 5% and was subtracted. Mean ± SD; $n = 4$.

plement receptor 2 (CR2) (37). CR2 is a member of the C3-binding protein family and is expressed predominantly on mature B cells and follicular dendritic cells (38, 39). Natural ligands for the CR2-targeting moiety are iC3b, C3dg, and C3d, cell-bound cleavage fragments of C3 that are present at sites of complement activation (40, 41). In vitro studies have shown that CR2-targeted complement inhibitors bind to C3-opsonized cells and are more effective than untargeted complement inhibitors at protecting target cells from complement deposition and lysis (37).

Because of the species-selective activity of complement-inhibitory proteins, we decided to construct a novel recombinant protein consisting of a mouse CR2-targeting moiety linked to mouse soluble Crry (sCrry), an inhibitor of C3 activation which is a structural and functional analog of human soluble CR1 (sCR1). Here we investigate the efficacy of CR2-Crry and compare it with a systemically inhibitory counterpart, Crry-Ig, in a mouse model of intestinal IRI. We also explore the effects of targeted versus systemic complement inhibition on remote organ injury and on host susceptibility to infection using a cecal ligation and puncture (CLP) model of sepsis. This is a pertinent model in the context of intestinal IRI, as mice subjected to CLP die of fulminant bacterial release after necrosis and ischemia of the cecum, with sepsis developing as a consequence of bacterial translocation across the damaged mucosal barrier (42–44).

Results

In vitro comparison of CR2-Crry and Crry-Ig. Function of the CR2-targeting domain of mouse CR2-Crry was investigated in vitro by flow cytometry; CR2-Crry, but not the untargeted systemic inhibitor Crry-Ig, bound to mouse C3-opsonized CHO cells (Figure 1A). The complement-inhibitory activity of CR2-Crry and Crry-Ig was compared by measurement of the sensitivity of antibody-sensitized CHO cells to lysis by rat serum (Figure 1B). Mouse Crry is functionally active against rat serum (45), and rat serum was used for functional comparison of activities because isolated mouse serum has poor lytic activity in vitro (46, 47). CR2-Crry and Crry-Ig caused 50% inhibition of lysis at concentrations of 12 nM (0.84 μg/ml) and 40 nM (6.4 μg/ml), respectively, an approximately 3.5-fold difference. Of note, however, is that structural analysis of Crry-Ig predicts that the Crry antennae are relatively independent of the Fc fragment in solution and likely function independently (48), a prediction supported by the finding that monomeric sCrry and dimeric Crry-Ig have complement-inhibitory activities that are comparable on a Crry molar equivalent basis (49). On a Crry molar equivalent basis, CR2-Crry (70 kDa monomer) was approximately 7-fold more effective than Crry-Ig (160 kDa dimer) at providing 50% inhibition of CHO cell lysis. As the molecular weight of dimeric Crry-Ig is approximately twice that of monomeric CR2-Crry, a comparison of activities based on weight is also appropriate.

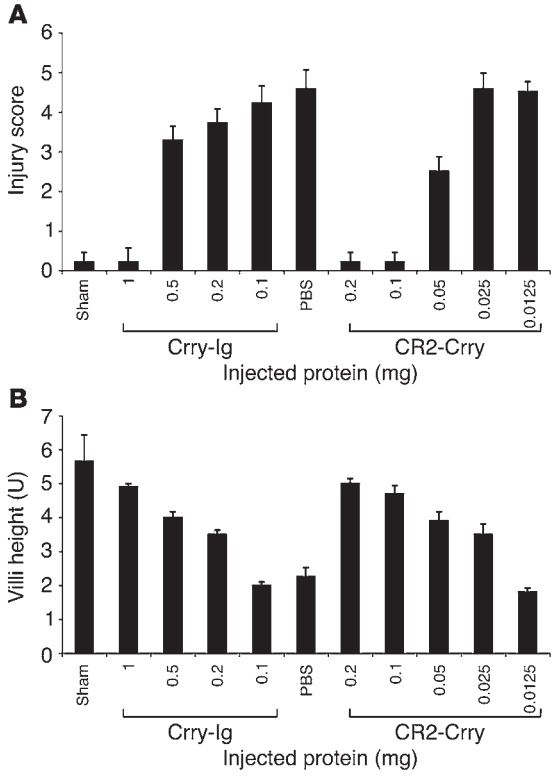

Figure 2
Quantitative evaluation of intestinal IRI with different doses of CR2-Crry and Crry-Ig. H&E-stained sections of intestine were assessed for mucosal injury score using the criteria described by Fleming et al. (46) (**A**) as well as for villi height (**B**). All measurements were obtained at ×200 magnification. Mean ± SD; $n = 4$–7 mice per group.

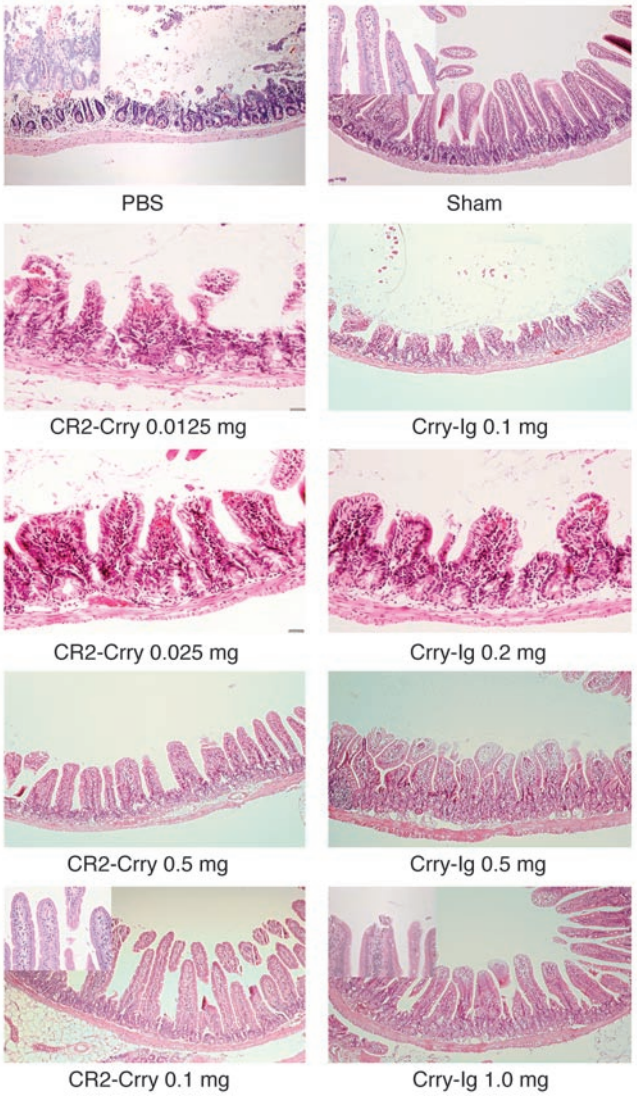

Figure 3
Representative sections of H&E-stained intestinal sections from BALB/c mice treated with different doses of CR2-Crry and Crry-Ig. Sham-operated and PBS controls are included for comparison. Photomicrograph magnification, ×200; high-power inserts, ×400. Each panel is representative of 4–7 mice per group.

Effect of CR2-Crry and Crry-Ig on intestinal IRI. Previous studies have shown that Crry-Ig, a systemic complement inhibitor of both the classical and alternative pathways, provides protection against intestinal IRI at doses of 2–3 mg when administered i.p. (50). To investigate the relative efficacies of mouse CR2-Crry and Crry-Ig at preventing intestinal IRI, we performed a dose-response study using an established BALB/c mouse model consisting of 30-minute ischemia followed by 2-hour reperfusion. Crry-Ig was administered i.v. 30 minutes after reperfusion at doses of 1–0.1 mg per mouse. Based on the increased complement-inhibitory activity of CR2-Crry compared with Crry-Ig in vitro (Figure 1B), CR2-Crry was administered at lower doses of 0.2–0.0125 mg per mouse.

Histological sections from intestines of BALB/c mice in each treatment group were assessed for IRI using criteria previously described (9, 50–52) and graded from 0 to 6. Sham-operated animals had no obvious intestinal damage, with the mucosal surface intact, villus height maintained, and an injury score of 0. Mice undergoing ischemia and reperfusion that received vehicle control (PBS) showed extensive damage to the intestinal wall with marked reduction in villus height and destruction of villus epithelial cells. Numerous villi were denuded, showing exposed lamina propria, frank hemorrhage, and capillary dilatation with an injury score of 4.6 ± 0.58 (Figures 2 and 3). Animals treated with Crry-Ig displayed a dose-dependent response to the intensity of IRI, with a minimum effective dose of 1.0 mg for complete protection; mice in this treatment group were completely protected from injury, with histological scores similar to those of sham-operated mice. Mice treated with 0.1 mg of Crry-Ig were similar to PBS controls in terms of damage to the villi and hemorrhage (Figure 2). The same dose-response phenomenon was observed in CR2-Crry–treated mice. However, only 0.1 mg of CR2-Crry was required for complete protection from injury; compared with the amount of Crry-Ig needed, this represented a 10-fold reduction (Figure 2A). The intestines from mice treated with either inhibitor showed a dose-dependent improvement in histological score (Figure 2A) and villi height (Figure 2B). Figure 3 highlights the improvements in the histological appearance of mucosal protection, indicating the different inhibitors and different doses. There was very little variation in injury scores and villi height within each treatment group.

We also subjected C57BL/6 mice to intestinal IRI using the same protocol, and treatment of these mice with PBS or the minimum effective dose of CR2-Crry or Crry-Ig produced results similar to those seen in BALB/c mice with regard to both local and remote injury (see "Assessment of remote organ injury") (data not shown). These C57BL/6 data are in agreement with a previous study, which showed that C57BL/6 and BALB/c mice have comparable levels of IRI and that systemic complement inhibition results in a similar degree of protection from tissue damage (53).

Assessment of remote organ injury. The lung is particularly susceptible to injury when ischemia is experienced in the intestine (9–11). This injury is characterized by expansion of the lung alveolar walls with acute inflammatory cell infiltration and, in extreme conditions, fibrin deposition and hemorrhage (52). Following intestinal ischemia and reperfusion, lung sections were analyzed by computerized image morphometry to assess the degree of airway wall expansion and reduction of alveolar spaces (Figure 4A). H&E staining revealed marked pulmonary neutrophil congestion and parenchymal expansion in mice treated with PBS. On the other hand, both Crry-Ig and CR2-Crry were protective against lung injury at doses that paralleled the extent of local injury (Figure 4B). There was a significant decrease in alveolar space in PBS-treated mice when compared with those treated with Crry-Ig (1.0 mg) and CR2-Crry (0.1 mg; $P = 0.001$, Mann-Whitney). However, a dose of 0.1 mg Crry-Ig had no significant protective effect ($P = 0.001$, Mann-Whitney).

Complement deposition. IRI has been shown to be complement dependent in numerous experimental models, and the demonstration of complement deposition within ischemic tissues would not be unexpected. However, in previous studies, whether or not C3 deposition could be demonstrated in rodent intestine and lung appeared to be dependent on species, length of ischemia, and time of reperfusion (12, 14, 17). In this study, we demonstrated the presence of C3 in both the intestine and the lung of PBS-treated mice. Complement deposition was noted on intestinal epithe-

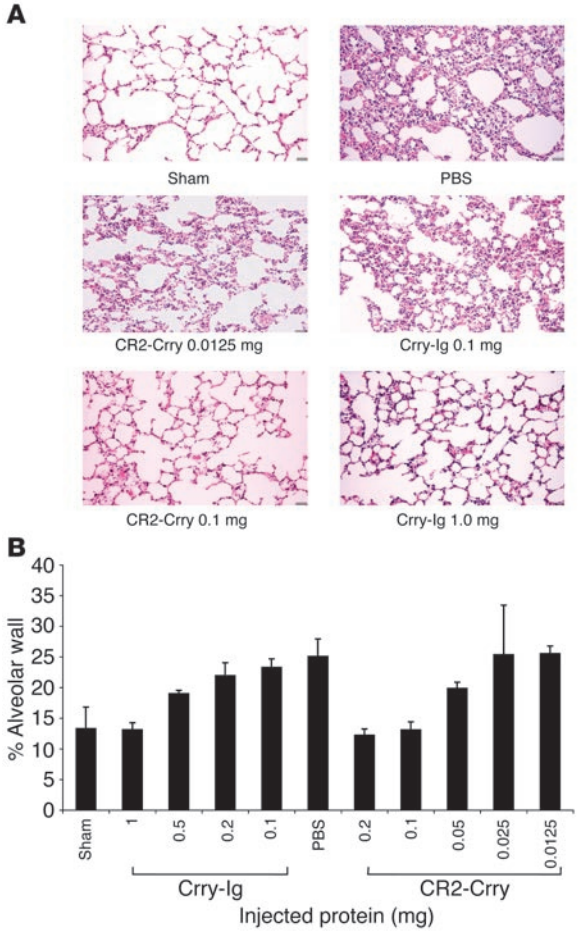

Figure 4
Evaluation of remote organ injury of the lung following intestinal IRI. (**A**) H&E-stained sections of lungs from CR2-Crry, Crry-Ig, sham-operated, and PBS groups. (**B**) Quantitative evaluation of the degree of alveolar wall expansion in the lungs measured as described in Methods. Mean ± SD; n = 4–7 mice per group.

lial and endothelial cells (Figure 5). In the lung, the staining was apparent on endothelial cells of the pulmonary vasculature. However, C3 staining could not be demonstrated in the intestine and lungs of mice that were treated with therapeutic doses of either CR2-Crry (0.1 mg) or Crry-Ig (1.0 mg). Some weak staining for C3 was apparent in intestine and lung sections from mice treated with lower, suboptimal amounts of CR2-Crry (0.012, 0.025, and 0.05 mg), but the intensity was significantly less than that seen in mice treated with PBS only (Figure 5).

Neutrophils. The role of neutrophils in IRI is controversial. Some studies show a positive correlation with neutrophil influx and tissue damage whereas interventional studies that block the production of C5a have demonstrated a neutrophil-independent mechanism of intestinal IRI (12, 15, 54). We therefore quantified neutrophil infiltration in both intestine and lung tissues of each treatment group following intestinal IRI. The number of neutrophils in both organs broadly correlated with injury scores. There was no difference in neutrophil count between sham-operated mice and mice that were treated with doses of inhibitor that were completely protective (Figure 6). Compared with sham-operated mice, however, PBS-treated mice showed significantly more neutrophils (P = 0.001, Kruskall-Wallis test).

CR2-Crry in vivo kinetics. The circulatory $t_{1/2}$ of Crry-Ig in BALB/c mice was previously shown to be 40 hours (30). We used the same procedure to determine the $t_{1/2}$ of CR2-Crry. BALB/c mice were injected i.v. with 0.1 mg CR2-Crry, and serum was prepared from blood samples removed at different time intervals thereafter. CR2-Crry serum concentration was assayed by ELISA and its $t_{1/2}$ was determined to be 8.7 hours (Figure 7).

Biodistribution of CR2-Crry and Crry-Ig. It has been reported that Crry-Ig, like other human systemic complement inhibitors, depends on a high level of systemic complement inhibition for efficacy (30, 55–57). To support the concept that the enhanced efficacy of CR2-Crry over Crry-Ig was due to its ability to target and localize at sites of complement activation, we performed a biodistribution study. The tissue distribution of ^{125}I-labeled CR2-Crry and ^{125}I-labeled Crry-Ig was determined in our model of intestinal IRI at 2 hours after reperfusion. In addition to being present in the blood, ^{125}I-labeled CR2-Crry localized primarily in the intestine and lung (known sites of C3 deposition following ischemia and reperfusion) and to a lesser extent the liver and kidney (Figure 8A). CR2-Crry binding in the kidney may be due to C3 deposition that occurs on normal mouse tubules, possibly caused by the presence of ammonium in the interstitium that can result in complement activation (58). In contrast, ^{125}I-labeled Crry-Ig was found to be localized only in the liver, and as expected, there was a high concentration in the blood. CR2-Crry binding to the intestine and lung was further confirmed by immunohistochemical staining by means of a mAb against mouse CR2. CR2-Crry was detected on epithelium and endothelial surfaces in the intestine and on endothelium and parenchymal wall in the lung in mice that were treated with 0.1 mg CR2-Crry (Figure 8, B–E). At lower doses that

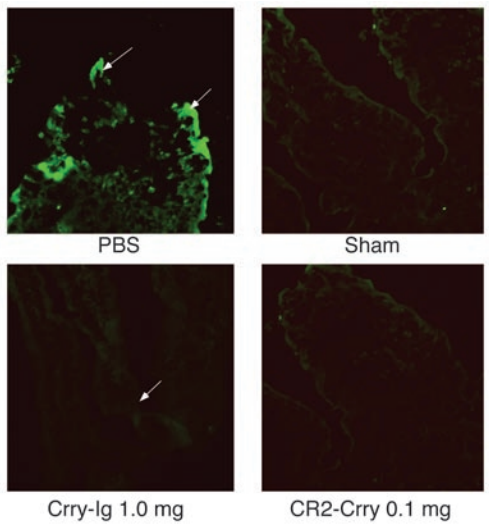

Figure 5
IRI-induced C3 deposition demonstrated by immunofluorescent staining. Intestinal sections were stained for C3 expression and visualized by confocal microscopy. C3 deposition is denoted by green fluorescence on apical epithelial cells (arrows). Each image is representative of 4–7 mice per group.

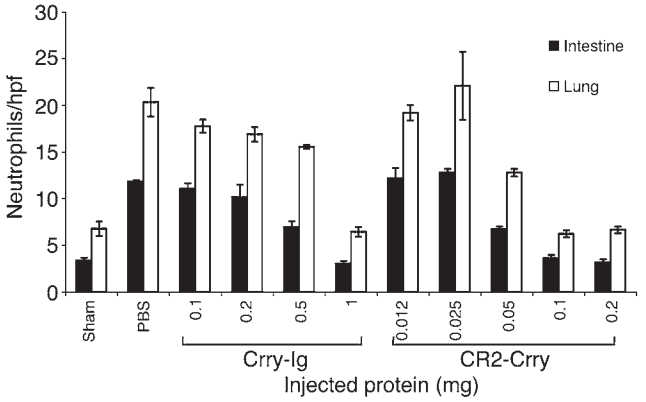

Figure 6
The dose-dependent effect of CR2-Crry and Crry-Ig on neutrophil infiltration into the intestine and lung. Neutrophil infiltration was assessed by immunohistochemical staining with mAb GR1. The number of neutrophils per high-power field (hpf) was counted in 3 fields per slide. Mean ± SD; n = 4–7 mice per group.

failed to provide complete local and remote protection from injury (0.0125 mg and 0.025 mg), only very weak staining was observed in the intestine and lung (data not shown), which correlated with increased neutrophil infiltration. No CR2-postive staining was seen on the epithelium or endothelium in sections from mice treated with Crry-Ig or PBS or in sham-operated controls, thus validating staining specificity. CR2 staining was, however, observed on B cells present within the lamina propria between the villi in Crry-Ig and PBS controls and in sham-operated mice. This was expected since B cells express CR2 on their surfaces.

Systemic complement inhibition. Serum complement-inhibitory activity of CR2-Crry and Crry-Ig injected at a range of doses was analyzed in serum collected from mice at 2 hours after reperfusion following a 30-minute period of ischemia. Analysis of serum complement-inhibitory activity was determined as described previously for Crry-Ig (30) using a zymosan A assay that measures alternative pathway activation. At 90 minutes after injection of inhibitor (i.e., 2 hours after reperfusion), Crry-Ig at minimum therapeutic dose (1 mg) reduced complement activity by 50% ± 9.2% (Figure 9). A similar level of reduction was found in serum from mice treated with a suboptimal dose of Crry-Ig (0.5 mg), but lower doses of Crry-Ig had lesser effects on serum complement activity. CR2-Crry, on the other hand, reduced serum complement activity by only 10.3% ± 0.08% at a therapeutic dose (0.1 mg), and this was not significantly different from results seen in PBS-treated mice and mice treated with lower concentrations of CR2-Crry. The approximate 10% reduction in serum complement activity in PBS-treated mice was presumably due to complement consumption that occurred following ischemia and reperfusion.

Immunity to infection. The CLP sepsis model results in abscess formation and passage of bacteria across the cecal mucosa, leading to blood sepsis (42, 43). Due to the nature of bacterial translocation, this model is pertinent to the study of potential deleterious side effects of complement inhibition in intestinal IRI. The model was used to evaluate the effect that targeted and systemic complement inhibition have on the host's ability to eliminate bacterial pathogens. Four study groups were used. Mice were subjected to CLP and treated with either a single injection of PBS, 1.0 mg Crry-Ig, or 0.1 mg CR2-Crry (the minimum effective doses of each inhibitor for protection against intestinal IRI) or multiple injections of 0.1 mg CR2-Crry. Mice receiving multiple injections of CR2-Crry received them at 0 hours and 8 hours, then every 12 hours thereafter to maintain the circulatory concentration of CR2-Crry based on its circulatory $t_{1/2}$ of 8.7 hours (see Figure 7). To our knowledge, this is the first formal study investigating the effect of a systemic complement inhibitor on mouse susceptibility to infection (as opposed to using complement-deficient mice). We found that all Crry-Ig–treated mice died within the first 48 hours after CLP. In stark contrast, mice subjected to CLP and treated with either a single dose or multiple doses of CR2-Crry survived significantly longer ($P < 0.001$; Figure 10). There was no significant difference in survival among PBS-treated groups and both CR2-Crry–treated groups. A second experiment was also performed and yielded the same result (data not shown). We used C57BL/6 mice for this study since the CLP model is well characterized in this strain. Although the therapeutic data on intestinal IRI presented in this report was obtained from BALB/c mice, both CR2-Crry and Crry-Ig were similarly therapeutic in BALB/c and C57BL/6 mice (see "Effect of CR2-Crry and Crry-Ig on intestinal IRI").

Discussion

Intestinal IRI is a serious clinical problem caused by a variety of insults to the abdomen and often results in remote organ damage and sepsis complications (1–5). Data obtained from complement-deficient animals clearly implicates complement in the pathogenesis of IRI, and in recent years, the development of therapeutic complement inhibitors has reinforced these data (9, 23–25, 59–61). The aim of this study was to investigate the efficacy of a novel targeted complement inhibitor, CR2-Crry, in a well-characterized experimental model that is clinically relevant. We show that CR2-mediated targeting of complement inhibition is not only significantly more effective than systemic complement inhibition, but does not affect host susceptibility to infection in a model of acute septic peritonitis.

Rehrig et al. (50) previously showed that Crry-Ig effectively reduced mucosal damage in a mouse model of IRI with the inhibitor being administered i.p. 30 minutes after reperfusion. The fact that complement-mediated damage could be inhibited 30 minutes after the initiation of damage is also relevant to the therapeutic window for the targeted inhibitor; the therapeutic dose of CR2-

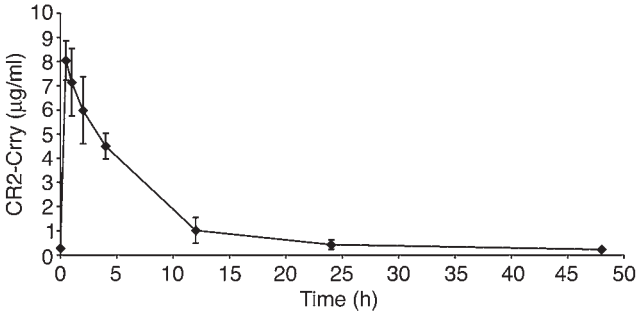

Figure 7
Mean serum circulatory levels of CR2-Crry. The serum levels of CR2-Crry were measured by ELISA in serum prepared from blood collected at indicated times following injection (0.1 mg). A $t_{1/2}$ of 8.7 hours was calculated. Mean ± SD; n = 4–7 mice per group.

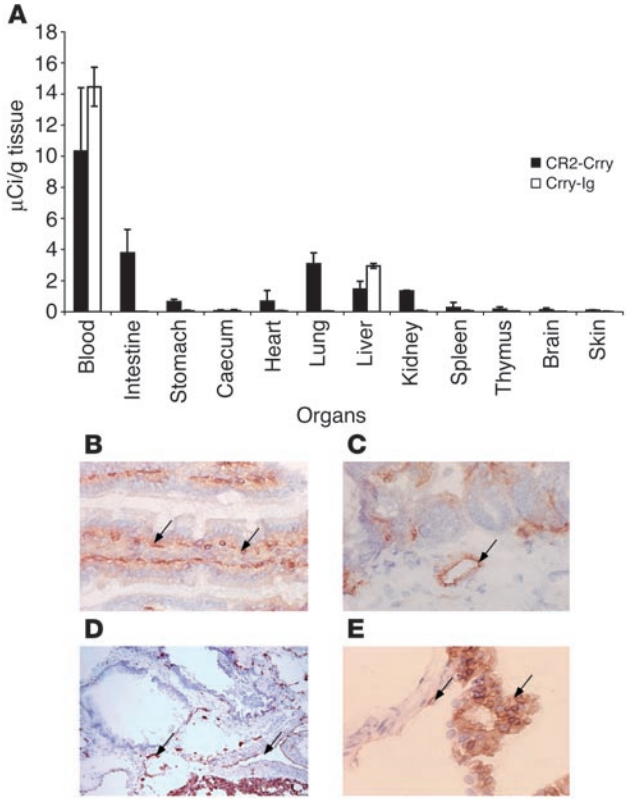

Figure 8
Biodistribution and localization of CR2-Crry and Crry-Ig following intestinal IRI. (**A**) ^{125}I-labeled CR2-Crry and ^{125}I-labeled Crry-Ig were injected 30 minutes after reperfusion; tissue distribution was assessed in all major organs 2 hours after reperfusion (mean ± SD; $n = 3$). (**B**–**E**) Representative anti-CR2 immunohistochemistry photomicrographs. (**B**) Intestine. Arrows indicate endothelial and epithelial CR2 immunostaining. (**C**) Intestine. Arrow indicates specific localization of CR2 immunostaining to endothelium of vessel beneath crypts of epithelial cells. (**D** and **E**) Lung. Arrows indicate endothelial and parenchymal wall CR2 immunostaining. There was no immunoreactivity with isotype control (data not shown). Magnification, ×200 (**B** and **D**); ×400 (**C** and **E**).

Crry resulted in only very low levels of systemic complement inhibition, indicating that, by 30 minutes after reperfusion, sufficient C3 fragments have been deposited on ischemic tissue to serve as target ligands for CR2-Crry. Such an interpretation is backed up by our biodistribution and immunohistochemistry data showing preferential binding of CR2-Crry to areas of local and remote injury.

We also demonstrated protection from remote organ damage of the lungs. Similar to the relative effects of CR2-Crry and Crry-Ig on local injury, we found that the effective dose of CR2-Crry was significantly lower (10-fold) than the effective dose of Crry-Ig for providing protection from lung injury. In the study by Rehrig et al. (50), the authors noted that C3 deposition within the intestine was absent at the time of sacrifice (2 hours) and concluded that C3 deposition was an early event in the course of IRI. This is in contrast with the data presented here and by others, as we show deposition of C3 in PBS-treated control animals 2 hours after reperfusion (12, 14, 18). C3 was also detected, albeit in lower concentrations, in CR2-Crry– and Crry-Ig–treated mice receiving subprotective doses. Thus it appears that therapeutic doses of CR2-Crry and Crry-Ig inhibit complement-mediated damage and prevent further C3 deposition. Our inability to demonstrate significant C3 deposition within the lungs and intestines of animals treated with therapeutic doses of CR2-Crry is noteworthy since the targeting ligands for CR2 are iC3d, C3dg, and C3d. This finding may attest to the potent intrinsic protection provided by this inhibitor or may possibly be the result of bound CR2 interfering with the binding site of the anti-mouse C3 antibody. The specificity of CR2-Crry for ischemic and injured tissue is supported by the lack of binding in other areas of the gastrointestinal tract, such as the stomach and cecum. CR2 immunohistochemistry detected CR2-Crry binding to the epithelial and endothelial surfaces of the intestine and the endothelial surfaces of the lung. Biodistribution studies using Crry-Ig showed that the systemic inhibitor was present largely in the blood and showed no specific affinity for ischemic tissue. Taken together, these data confirm that CR2-Crry binds to sites of complement activation and injury and that C3 deposition and cleavage is an early event after reperfusion.

Traditionally, neutrophils have been attributed with a central role in the initiation of tissue damage (57, 61–63). The influx of neutrophils into ischemic tissue results in degranulation and superoxide production, which damages tissue and subsequently amplifies the neutrophil response (9, 61–63). Complement activation results in the release of chemoattractants (C3a and C5a), which can directly or, through the activation of endothelial cells, indirectly recruit neutrophils to sites of complement activation (64). In this study, intestinal and pulmonary accumulation of neutrophils was inhibited by the administration of CR2-Crry and Crry-Ig in a dose-dependent manner. Although this result is consistent with previous data indicating a link between neutrophils and tissue injury, it does not alone distinguish between neutrophil infiltration as a cause or a consequence of intestinal damage. Other studies of intestinal IRI in the mouse, however, have indicated that local intestinal injury is a neutrophil-independent event (54, 65). In these previous studies, anti-C5 antibody or Crry-Ig was administered 30 minutes after reperfusion and was protective against mucosal damage, although there was significant

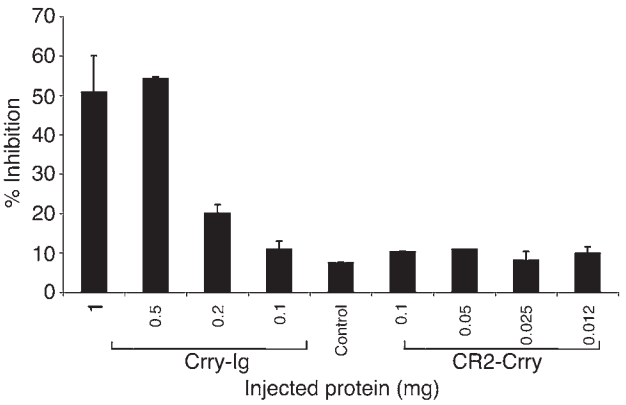

Figure 9
Effect of administration of CR2-Crry and Crry-Ig at different doses on systemic complement inhibition. Serum was isolated from animals following intestinal IRI and complement activity assessed using zymosan particles and flow cytometry to detect surface-bound C3. Mean ± SD; $n = 3$ mice per group.

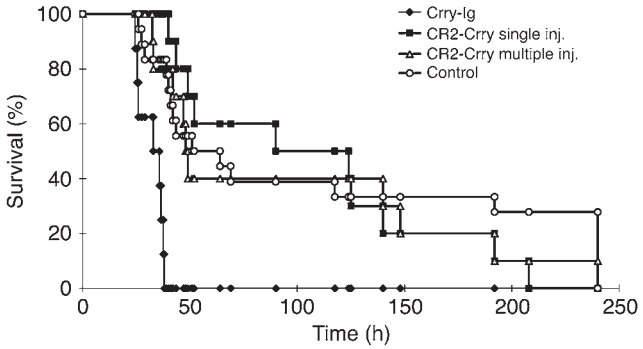

Figure 10
Survival analysis in CLP model of sepsis. Mice were administered a single dose of PBS (n = 18), Crry-Ig (1.0 mg; n = 8), or CR2-Crry (0.1 mg; n = 10) or multiple doses of CR2-Crry (0.1 mg; n = 10) and monitored. Inj., injection.

neutrophil infiltration (54, 65). The authors concluded that neutrophil infiltration was an early event and that complement regulation could downregulate neutrophil-mediated damage (50, 54). The complement-activation products C3a and C5a have direct chemoattractant properties and, together with other complement-activation products, can induce the expression of various chemokines and adhesion molecules that are important for the recruitment of neutrophils (66–68). There is also evidence that intestinal IRI in mice is mediated solely by the membrane attack complex (65). One possible reason for the differences between our neutrophil data and that of others (50, 54) may be related to the route of inhibitor administration. In this study, inhibitors were administered i.v. as opposed to i.p., and the kinetics and bioavailability of complement-inhibitory activity may have been altered.

We show that a benefit of targeted complement inhibition is that it has only a minimal effect on serum complement activity at a therapeutic dose, indicating that complement-dependent innate immune function will be largely preserved. To test this hypothesis, we investigated the effects of CR2-Crry and Crry-Ig in a mouse model of sepsis. We employed a mouse CLP model that is relevant in terms of intestinal IRI, as pathogenic mucosal damage may lead to translocation of bacteria and subsequent sepsis (42, 43). Administration of a single therapeutic dose of Crry-Ig led to death within 48 hours of surgery in all cases. This finding reinforces data obtained with complement-knockout mice, which indicate that complement plays a crucial role in immunity to infection. In most cases, complement-dependent host immunity involves the opsonization of pathogens with C3b, iC3b, and C3d ligands and subsequent destruction by immune effector cells bearing C3 receptors (31–36). In this regard, however, it is likely that C3-opsonized bacteria will also act as targets for CR2-Crry. Thus, although a therapeutic dose of CR2-Crry only minimally affects serum complement activity, there remains the potential for CR2-Crry to directly and effectively interfere with bacterial clearance by binding to opsonized bacterial surfaces. However, CR2-Crry–treated mice were no more susceptible to infection than PBS-treated controls (in contrast to Crry-Ig–treated animals). Why CR2-Crry did not affect bacterial clearance is unclear but may be related to its short $t_{1/2}$ and its rate of clearance relative to increase in bacterial load. To investigate this, we performed an additional experiment in which mice were given repeated doses of CR2-Crry following CLP in order to maintain serum concentrations of CR2-Crry. Somewhat surprisingly, these mice were not significantly more susceptible to infection following CLP than mice treated with either PBS or a single dose of CR2-Crry. One possible explanation is different thresholds of complement opsonization necessary for causing tissue damage versus bacterial killing, which may in turn relate to effector mechanism. For example, cell-mediated killing via C3 receptors is known to be critical for bacterial clearance whereas the membrane attack complex, formation of which is controlled at a second level on host tissue, may play a predominant role in intestinal IRI. Further mechanistic studies investigating this phenomenon are clearly warranted.

While it is acknowledged that systemic complement inhibition may be of more serious consequence if long-term inhibition is required, there are situations in which even short-term inhibition is not optimal, such as when the host is immunocompromised or has experienced traumatic injury. Although serious adverse side effects have not generally been noted for sCR1 and anti-C5 mAb and their derivatives in clinical trials, infectious complications can occur during short-term treatment. (The FDA requires vaccination against meningococcus for anticomplement therapies.) Also, whether these systemic inhibitory strategies can be used in diseases in which the risk of infection is higher remains an important and unresolved question. Finally, clinical trials with complement-inhibitory drugs have failed to reach study-designated primary endpoints, and there remains a need for improved modalities of complement-inhibitory treatment. Thus, the 10-fold increase in therapeutic efficacy obtained with the targeting strategy described here appears to represent a significant advance in the development of improved and safer complement-inhibitory therapeutics.

Methods

Construction of expression plasmids, protein expression, and protein purification. cDNA constructs of the recombinant fusion protein were prepared by joining the mouse CR2 sequence encoding the 4 N-terminal short-consensus repeat units (residues 1–257 of mature protein, GenBank accession number M35684) to sequences encoding extracellular regions of mouse Crry. The Crry sequence used encoded residues 1–319 of the mature protein (GenBank accession number NM013499). To join CR2 to Crry, linking sequences encoding $(GGGGS)_2$ were used. Gene constructs were prepared by standard PCR methods. All cloning steps were performed in the PBM vector, which was also used for protein expression (30). For expression, plasmids were transfected into NSO cells using a Bio-Rad Gene Pulser Electroporator (Bio-Rad Laboratories) with a pulse of 1500 volts, 3 uFd to the cuvette, and a wait of 1 minute before applying a second pulse using the same settings. Stably transfected clones were selected by limiting dilution as described (30), and protein expression of clones was quantified by ELISA. Tissue culture supernatants were collected, filtered through a 0.22-μm filter (Pall Corp.), and then concentrated using a 10,000 MW cellulose concentrator (Millipore). Following concentration, supernatant was passed through a HiTrap N-hydroxysuccinimide column (Amersham Biosciences) that had been previously coupled with a rat anti-mouse Crry mAb (clone 5D5) (69). Protein was eluted with glycine pH 2.7 and fractions collected into 1 M Tris hydrochloric acid. Fractions were further concentrated and buffer exchanged using 10,000 MW centrifuge concentrators (Millipore). Purified CR2-Crry was aliquoted and frozen at –80°C until use. Crry-Ig was prepared as previously described (30).

In vitro characterizations of recombinant proteins. Binding of recombinant fusion proteins to C3-opsonized cells was determined by flow cytometry. CHO cells were incubated in 10% anti-CHO antiserum (70) (30 minutes

at 4°C), washed, and incubated in 10% mouse serum (30 minutes at 37°C). These conditions resulted in C3 opsonization with less than 5% lysis of CHO cells. The C3-opsonized cells were then washed and incubated with 1 μM recombinant protein (60 minutes at 4°C). After washing, cells were incubated with 10 μg/ml of anti-mCrry Ab 5D5 (30 minutes at 4°C) followed by FITC-conjugated secondary antibody (1:100, 30 minutes at 4°C). Cells were then washed, suspended in PBS containing propidium iodide (2 μg/ml), and analyzed using a FACScan flow cytometer (BD Biosciences — Immunocytometry Systems). All incubations and washes were performed in DMEM. Complement-mediated lysis of sensitized CHO cells was determined in the presence or absence of recombinant complement inhibitors by trypan blue exclusion as previously described (37). In these assays, 15% (final concentration) rat serum was used as complement source, which resulted in approximately 90% lysis of unprotected CHO cells. Percentage of inhibition of lysis was calculated as $(A - B)/A$, where A is equal to the percentage of cell lysis in 15% normal rat serum and B is equal to the percentage of cell lysis in 15% normal rat serum with recombinant protein.

Intestinal IRI. Adult male BALB/c and C57BL/6 mice (National Cancer Institute) aged 8 weeks and weighing 20–25 g were anesthetized with 10 mg/kg ketamine and 6 mg/kg xylazine by i.p. injection. Animals were breathing spontaneously, and body temperature for the entire experiment was maintained with a heat mat. A medial laparotomy was performed, and the intestines were carefully moved, allowing access to the superior mesenteric artery. The superior mesenteric artery was clamped using a microsurgical clamp (Fine Surgical Instruments Inc.). Ischemia was confirmed by pallor of the small intestine. Sham-operated mice underwent laparotomy without clamping of the superior mesenteric artery. After 30 minutes of ischemia, the arterial clamp was removed, allowing reperfusion of the mesenteric vasculature. Animals were sutured using 6.0 ethicon suture and allowed to reperfuse for 2 hours. Animals were randomized into CR2-Crry– and Crry-Ig–treatment groups and given 0.012, 0.025, 0.05, 0.1, or 0.2 mg CR2-Crry and 0.1, 0.2, 0.5, or 1.0 mg Crry-Ig (n = 4–7). Inhibitors were administered i.v. 30 minutes after reperfusion, and animals were sacrificed 90 minutes later following 2 hours of reperfusion. Animal procedures were approved by the Medical University of South Carolina Animal Care and Use Committee.

Histology. Tissue samples for histological staining were taken from the intestine and either fixed in 10% formalin at 4°C overnight and subsequently processed to paraffin or frozen in liquid nitrogen for immunofluorescence analysis. Lungs were inflated via the bronchia at 15 mm Hg with formalin and allowed to fix. Sections of intestine from each animal were stained with H&E and scored for mucosal damage and villi height as previously described (51). A score of 0 was assigned to normal villi; villi with tip distortion were scored as 1; villi lacking goblet cells and containing Gugenheim spaces (oedematous spaces within the epithelial cells) were scored as 2; villi with patchy disruption of the epithelial cells were scored as 3; villi with exposed but intact lamina propria and epithelial cell sloughing were scored as 4; villi in which lamina propria were exuding were scored as 5; and finally, denuded villi or villi displaying hemorrhage were scored as 6. All histological evaluations were carried out in a blinded fashion.

Lung sections were cut and stained with H&E. The extent of remote lung damage was assessed by the expansion of the parenchymal wall by the infiltration of inflammatory cells as previously described (71). The degree of parenchymal wall expansion was measured using the Leica QWin image analysis system. In brief, slides were screened in a blinded fashion, and 6 images per lung recorded in fields not containing bronchi or major pulmonary vasculature. For the area of the captured image, first, the field area was calculated; second, the area occupied by alveolar wall was calculated by thresholding analysis using the Leica QWin image analysis system. The percentage of alveolar wall per 6 random fields was then calculated and expressed as a mean percentage per unit area ± SD.

Immunofluorescence staining for complement component C3. Briefly, sections were fixed in cold acetone for 5 minutes and then washed in running water and then PBS. Sections were incubated for 1 hour at room temperature with anti-mouse C3 FITC and then washed in 3 changes of PBS. Sections were then coverslipped with Fluoromount-G (SouthernBiotech) and analyzed using a Zeiss LSM5 confocal microscope.

In vivo kinetics of CR2-Crry. BALB/c mice were injected i.v. with 0.1 mg CR2-Crry in sterile PBS. Blood was collected before CR2-Crry injection and then at various time points as indicated in Figure 7. Serum samples were prepared as previously described and stored at –80°C for later analysis (30). Serum concentrations of CR2-Crry were assessed by ELISA. Dynatech Immulon II (Dynatech Laboratories) 96-well plates were coated and kept overnight with 1 μg/well of IgG-purified rat anti-mouse Crry mAb (clone 5D5). Plates were then washed 4 times with PBS containing 0.05% Tween-20, and blocked for 1 hour at room temperature with PBS containing 1% BSA (Sigma-Aldrich). The plates were then washed 4 times with PBS containing 0.05% Tween-20 before the addition of biotinylated anti-mouse CR2 (clone 7G6) (72). Plates were washed with PBS containing 0.05% Tween-20 and incubated with streptavidin peroxidase for 2 hours before development with O-phenylenediamine dihydrochloride reagent (Sigma-Aldrich), and readout was performed at 492 nm.

^{125}I radiolabeling biodistribution. Radiolabeling was conducted using ^{125}I (New England Nuclear Corp.) by the IODO-GEN method (Pierce Biotechnology Inc.). We used 5 mCi to label 100 μg of CR2-Crry and Crry-Ig protein. Free iodine was removed from the mixture after labeling by anion exchange resin. Iodine incorporation was in the 50–80% range. Radiolabeled protein was injected i.v. 30 minutes after reperfusion as outlined previously. After 2 hours of reperfusion, mice were sacrificed (n = 3 per group) and blood removed by perfusion with PBS before thymus, heart, lung, liver, kidney, spleen, skin, intestine, cecum, and stomach were removed. Tissues were rinsed in RPMI (Gibco; Invitrogen Corp.) and counted with a Hewlett-Packard 5780 γ counter at the ^{125}I window with appropriate corrections for count decay.

Immunohistochemistry. Frozen sections of intestine and lung were cut and fixed in cold acetone. The presence of neutrophils and CR2 was assessed using antibodies directed against rat anti-GR1 (neutrophils) (BD Biosciences) and rat anti-mouse CR2 mAb 7G6 (65). Antibodies were detected using a standard streptavidin biotin detection system (Vector Laboratories) and visualized with 3,3 diaminobenzidine. Sections immunostained with CR2 mAb were analyzed using an Olympus OX40 microscope for the presence or absence of specific staining. The number of neutrophils stained in lung and intestine sections was analyzed by 2 independent observers. Three random high-power fields were assessed for the presence of neutrophils and the number observed recorded in each field in animals from each group. The number of neutrophils was then expressed as the average per high-power field.

Systemic complement inhibition. Blood samples were taken from mice subjected to intestinal IRI prior to operation (to assess baseline complement levels) and at the completion of the experiment from mice receiving either CR2-Crry (0.1, 0.05, 0.025, and 0.0125 mg) or Crry-Ig (1.0, 0.5, 0.2, and 0.1 mg) to assay the effect of the complement inhibitors on serum complement function. Complement inhibition was measured in both baseline and post-IRI samples using a previously described method that utilizes flow cytometric analysis of C3 deposition on zymosan A particles (Sigma-Aldrich) (30). The extent of systemic complement inhibition seen in each animal was normalized by the subtraction of post-IRI complement deposition levels from their baseline levels.

CLP sepsis model. Before surgery, pathogen-free mice were kept for at least 2 weeks in the animal facility to recover after transport. CLP was performed as outlined by Maier et al. (42). Briefly, adult male C57BL/6 mice (National Cancer Institute) aged 8 weeks and weighing 20–25 g were anesthetized i.p. with 10 mg/kg ketamine and 6 mg/kg xylazine. Animals were breathing spontaneously, and body temperature was maintained using a heat mat for the entire experiment. The abdominal wall was opened with a 1-cm midline incision and the cecum exposed. The cecum was ligated about 15 mm proximal to the cecal pole with a 5/0 prolene thread (Ethicon) without stricture of the ileocecal valve. The cecum was punctured once with an 18-gauge needle (Terumo Medical Corp.), then slightly compressed until a small drop of stool appeared. The cecum was then replaced and the animal's peritoneal cavity rinsed with sterile saline solution before closure of the abdominal wall. Animals were randomized into 4 groups, and all surgical procedures were performed in a matched experimental setting. Immediately following surgery, mice in group 1 received an i.v. injection of PBS, those in group 2 received an i.v. injection of CR2-Crry (0.1 mg), those in group 3 received an i.v. injection of Crry-Ig (1 mg), and those in group 4 received i.v. injections of CR2-Crry (0.1 mg) at 0 hours and 8 hours, then every 12 hours thereafter (up to 6 injections in surviving mice). Survival after surgery was assessed every 4 hours within the first 48 hours and then every 8 hours for the next 10 days. No antibiotic therapy was administered.

Statistical analysis. Data are presented as means ± SD. All data were subjected to statistical analysis using Statview Statistical Analysis Software (version 5; SAS Institute Inc.). A P value less than 0.05 was considered significant.

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