

Mice Deficient in Complement Receptors 1 and 2 Lack a Tissue Injury-Inducing Subset of the Natural Antibody Repertoire¹

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Intestinal ischemia-reperfusion (IR) injury is initiated when natural Abs recognize neoantigens that are revealed on ischemic cells. *Cr2*^{-/-} mice, deficient in complement receptors (CR)1 and CR2, demonstrate defects in T-dependent B-2 B cell responses to foreign Ags and have also been suggested to manifest abnormalities of the B-1 subset of B lymphocytes. To determine whether these CRs might play a role in the generation of the natural Abs that initiate intestinal IR injury, we performed experiments in *Cr2*^{-/-} and control *Cr2*^{+/+} mice. We found that *Cr2*^{-/-} mice did not demonstrate severe intestinal injury that was readily observed in control *Cr2*^{+/+} mice following IR, despite having identical serum levels of IgM and IgG. Pretreatment of *Cr2*^{-/-} mice before the ischemic phase with IgM and IgG purified from the serum of wild-type C57BL/6 mice reconstituted all key features of IR injury, demonstrating that the defect involves the failure to develop this subset of natural Abs. Pretreatment with IgM and IgG individually demonstrates that each contributes to unique features of IR injury. In sum, CR2/CR1 play an unanticipated but critical role in the development of a subset of the natural Ab repertoire that has particular importance in the pathogenesis of IR injury. *The Journal of Immunology*, 2002, 169: 2126–2133.

When organs such as the intestine are subjected to severe vascular ischemia that is then followed by the reperfusion of blood into the site, local as well as remote tissue inflammation and injury ensue (reviewed in Ref. 1). During the reperfusion phase, several important inflammatory mediators, including complement, cytokines, and adhesion molecules, are generated. These mediators amplify the ischemic injury beyond that associated with the lack of tissue perfusion alone. The relative roles and kinetics of expression of individual proinflammatory mediators in mediating the effector phases of ischemia-reperfusion (IR)³ injury are not well understood, although it appears that there is a variability of response when comparing different organs.

Evidence that complement activation is involved in the effector phases of intestinal IR injury has been provided by studies which showed that inhibition of the complement pathway at the point of

C3 or C5 activation can either prevent or substantially attenuate intestinal injury (2–7). In support of these findings, inflammatory mediators generated during complement pathway activation, such as the anaphylatoxin C5a and the membrane attack complex (MAC), are known to be able to directly cause cellular activation and injury (8–12).

The exact mechanism of complement activation during intestinal IR remains unclear, and there is relatively little information regarding the specific complement activation pathways (classical, alternative, or lectin) involved in tissue injury. However, two observations have strongly implicated the classical pathway in this process. The first is that intestinal IR injury is markedly attenuated in *RAG-1*^{-/-} mice, and this protective effect is lost after reconstitution of these Ig-deficient mice with purified IgM natural Ab to normal levels (6). The second is that mice with normal levels of natural Ab, but in which the gene encoding complement C4 is inactivated (*C4*^{-/-}), are protected from injury (6). The importance of natural IgM Ab and the classical complement pathway in mediating IR injury of skeletal muscle has also been shown using a similar experimental strategy with C3, C4, and Ig-deficient mice (13).

From these and other findings, it has been proposed that natural IgM Abs bind to neoantigens revealed on the surface membrane of cells injured by hypoxia in the intestinal or skeletal muscle vasculature during the ischemic phase and subsequently activate complement by recruiting C1 and then cleaving C4 (6). This is followed by the generation of complement C3 and C5 activation fragments as well as the MAC, with ensuing increases in adhesion molecule expression and release of a cascade of inflammatory mediators, including leukotriene B₄ (LTB₄) and others (3, 7).

Natural Abs are produced primarily by B-1 B cells, which in the adult mouse are found primarily in the peritoneum, and are frequently found to be polyreactive at low affinity with multiple self Ags (14–17). Natural Abs are an important part of the defense against infection and have been found to be protective against

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³ Abbreviations used in this paper: IR, ischemia-reperfusion; CR, complement receptor; LTB₄, leukotriene B₄; SMI, score of mucosal injury; TMB, 3,3',5,5'-tetramethylbenzidine; MAC, membrane attack complex.

challenge with bacterial (18) as well as viral (19, 20) pathogens and to play an important role in the clearance of endotoxin (21).

Complement receptor (CR) type 2 (CR2/CD21) is an important membrane receptor that greatly enhances B cell receptor (BCR)-mediated activation by binding complement iC3b/C3d-bound Ags and engaging the CR2/CD19/CD81 signaling complex (22–26). This recognition mechanism lowers the threshold for activation of B cells (27). In mice, CR2 is encoded along with the larger CR1 by the *Cr2* gene, which produces both proteins through alternative splicing of a common mRNA (28, 29). *CR2*^{-/-} mice demonstrate a substantial defect in the generation of IgG-switched isotype responses (30–32) as well as impaired B cell memory following immunization with T-dependent Ags (33, 34).

CR2 and CR1 have been potentially linked to the regulation of B-1 B cell-derived natural Abs by the observation that there is an ~50% decrease in peritoneal B-1 B cell numbers in one strain of *Cr2*^{-/-} mice (30). *Cr2*^{-/-} mice made by our group did not demonstrate decreased B-1 cells at either an early (32) or later (see *Results*) backcross to C57BL/6, and, like the other strain of receptor-deficient mice, has also consistently demonstrated quantitatively normal levels of serum IgM. Nevertheless, based on these reported B-1 B cell findings, our previous demonstration that peritoneal B-1a and B-1b B cells express readily detectable levels of CR2 and CR1 (35), and the well-established role of CR1 and CR2 in T-dependent B-2 B cell responses, we sought to determine whether the complement system might be important not only in the effector phases of tissue injury, as discussed above, but also in the development of a pathogenic subset of natural Abs that recognize neoantigens on ischemic tissue and then initiate intestinal IR injury.

In the present study, we subjected mice to intestinal IR injury and found that *Cr2*^{-/-} mice were protected from the induction of tissue injury. The defect was shown to lie in an altered repertoire of natural Abs by the demonstration that IgM and IgG purified from the serum of wild-type C57BL/6 mice, when injected before the ischemic phase, were able to reconstitute IR-induced injury in *Cr2*^{-/-} mice. Of interest, transferred IgM and IgG each contributed to different aspects of tissue injury but together allowed the development of a complete injury phenotype. These findings demonstrate a previously unrecognized role for CRs in the development within the natural Ab repertoire of a tissue injury-inducing subset of Abs. We propose that these Abs have developed to serve a critical role in recognizing ischemic intestinal tissue and initiating processes that result in either repair or removal of the damaged regions.

Materials and Methods

Mice

Adult male and female *Cr2*^{-/-} and control *Cr2*^{+/+} mice were produced following seven generations of backcrossing to the C57BL/6 strain by intercrossing a single pair of *Cr2*^{+/+} mice to create *Cr2*^{-/-} and *Cr2*^{+/+} progeny, which were then bred and maintained as two sublines.

Experimental protocol

Mice were prepared for surgery, after at least a 7-day acclimation period following shipment, in the Uniformed Services University for the Health Sciences animal facility. Anesthesia was induced with ketamine (16 mg/kg) and xylazine (8 mg/kg) administered by i.m. injection. All procedures were performed with the animals breathing spontaneously and body temperature maintained at 37°C using a water-circulating heating pad. Experiments were performed according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals" (Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare publication no. 85-23).

Animals were subjected to IR as previously described (7). Briefly, a midline laparotomy was performed before a 30-min equilibration period.

The superior mesenteric artery was then identified and isolated, and a small nontraumatic vascular clamp (Roboz Surgical Instruments, Rockville, MD) was applied for 30 min. After this ischemic phase, the clamp was removed under direct visualization and the intestine was allowed to reperfuse for 2 h. In some experiments, 30 min before laparotomy animals were given 1 mg of purified IgG or IgM, or both in combination, by i.v. injection. Some mice received 1 mg IgA in an identical fashion while others received an equal volume of normal saline. Sham animals were subjected to the same surgical intervention, except they did not undergo superior mesenteric artery occlusion. To control for the effects of Ab injection, Igs were administered to sham-treated mice as well. The laparotomy incisions were sutured and the animals were monitored during the reperfusion period. Additional ketamine and xylazine was administered by i.m. injection as needed (total given was ~4 mg/kg ketamine and 2 mg/kg xylazine) and immediately before euthanasia. After euthanasia, the small intestine 10–20 cm distal to the gastroduodenal junction was removed for histologic and immunohistochemical analysis as well as for the measurement of inflammatory mediators as described below. There was no significant difference in survival between treatment and control groups.

Histology and immunohistochemistry

Immediately after euthanasia, segments of small intestine specimens were fixed in 10% buffered formalin, embedded in paraffin, sectioned transversely in 5- μ m sections, and stained with Giemsa. Score of mucosal injury (SMI) was graded on a six-tiered scale as described previously (3, 36). In addition, the villus height of at least 10 villi from the same section was measured using an ocular micrometer.

Additional tissue was fixed for 2 h in cold 4% paraformaldehyde in PBS before transfer to PBS for paraffin embedding and preparation of transverse sections. Following removal of paraffin from sections, nonspecific Ab binding sites were blocked by treatment with a solution of 20% rat serum in PBS for 30 min. After washing in PBS, the tissues were incubated with goat anti-mouse C3, IgG, or IgM (ICN Pharmaceuticals, Costa Mesa, CA) Ab overnight at 4°C. The tissue was then incubated with a biotinylated rabbit anti-goat secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by streptavidin conjugated to either fluorescein or 7-amino-4-methylcoumarin-3-acetic acid (Jackson ImmunoResearch Laboratories). The slides were then mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). A blinded observer examined the slides by fluorescent microscopy using a Leica DM RX/A fluorescent microscope (Leica Microsystems, Atlanta, GA) with SPOT diagnostic computer software (Diagnostic Instruments, Sterling Heights, MI).

Ig ELISA

Sera collected from 14 *Cr2*^{+/+} and 10 *CR2*^{-/-} age-matched mice were added in serial dilution to ELISA plates that had been coated overnight with goat anti-mouse κ (Southern Biotechnology Associates) or rat anti-mouse IgM (Southern Biotechnology Associates) and then blocked with 1% BSA/PBS. Plates were washed with PBS/0.1% Tween 20 and test serum samples or purified mouse Abs (IgG and IgM; Southern Biotechnology Associates) for standard curves were added and incubated overnight at 4°C. Following washing, plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-mouse IgM (Caltag Laboratories, Burlingame, CA), respectively. Plates were washed, substrate was added, and then the OD₄₀₅ was measured. The concentration of IgM and IgG was calculated using the standard curve.

Eicosanoid determination

The ex vivo generation of eicosanoids by small intestine tissue was determined as previously described (37). Briefly, fresh mid-jejunum sections were minced, washed, and resuspended in 37°C oxygenated Tyrode's buffer (Sigma-Aldrich, St. Louis, MO). After incubating for 20 min at 37°C, supernatants were collected and stored at -80°C until assayed. The concentration of LTB₄ was determined using an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). The tissue protein content was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) adapted for use with microtiter plates. LTB₄ levels were expressed per milligram of protein per 20 min.

Peroxidase activity

Supernatants generated for the eicosanoid assays were also used to determine peroxidase activity by measuring oxidation of 3,3',5,5'-tetramethylbenzidine as described previously (38). Briefly, supernatants were incubated with equal volumes of 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD) for 45 min. The reaction was stopped by the addition of 0.18 M sulfuric acid, and the OD₄₅₀ was

determined. The concentration of total peroxidase was determined using HRP (Sigma-Aldrich) as a standard and plotted as picograms of myeloperoxidase activity per milligram of tissue.

Ig preparation and purification

A total of 100 ml of serum from C57BL/6 mice was purchased from Charles River Breeding Laboratories (Wilmington, MA). For IgM purification, the serum was first fractionated by the addition of ammonium sulfate (50%). The precipitate was dialyzed into PBS and then passed through a Superdex HL-200 gel filtration column. The IgM containing fractions were pooled, concentrated, and dialyzed into sterile saline. The IgG fraction was obtained by passing the dialyzed supernatant of the 50% ammonium sulfate precipitate through a HiTrap DEAE column equilibrated in 50 mM Tris-HCL buffer (pH 8.6). A continuous gradient to 50 mM Tris-HCL (pH 8.6) and 1 M NaCl was used to elute the IgG material. Positive fractions were pooled, concentrated, and dialyzed into sterile saline. Purity of each preparation was assured by SDS-PAGE and isotype-specific Western blot analysis with either HRP-conjugated polyclonal anti-mouse IgG or anti-IgM. Purified IgA was purchased from Sigma-Aldrich. For some experiments, blood from $Cr2^{-/-}$ mice was collected by cardiac puncture, following which serum was obtained and pooled for injection into additional $Cr2^{-/-}$ mice that underwent intestinal IR injury.

Statistical analysis

Data are presented as mean \pm SEM and were compared by one-way analysis of variance with post hoc analysis using Newman-Keuls test (Instat; GraphPad, Philadelphia, PA). The difference between groups was considered significant when $p < 0.05$.

Results

$Cr2^{-/-}$ mice are protected from intestinal IR injury

We tested the hypothesis that the tissue damaging natural Ab repertoire is altered in $Cr2^{-/-}$ mice by subjecting these mice and their $Cr2^{+/+}$ controls to mesenteric IR. We first showed that the intestines of sham-treated $Cr2^{+/+}$ mice were macroscopically and microscopically normal (Fig. 1A), whereas those that had undergone mesenteric ischemia for 30 min followed by 2 h of reperfusion were swollen, edematous, and dusky, indicative of hemorrhage and severe injury (Fig. 1B). In addition, many villi were denuded with exposed lamina propria and hemorrhage. Tissue injury was also quantitatively scored on a scale of 0–6 as previously described to derive a SMI (3, 36). The SMI for $Cr2^{+/+}$ mice following IR was 3.3 ± 0.3 , significantly elevated when compared with sham-treated $Cr2^{+/+}$ mice (Fig. 2A). In addition, the villus height was significantly lower in mice with IR injury compared with sham-treated $Cr2^{+/+}$ mice (Fig. 2B), indicative of loss by sloughing of ischemic portions of the intestinal tissue.

Sham treatment of $Cr2^{-/-}$ mice also resulted in no injury (SMI = 0.6 ± 0.2), and the villus height ($43.4 \pm 1.5 \mu\text{m}$) remained unchanged when compared with sham-operated $Cr2^{+/+}$ mice (Figs. 1 and 2). In contrast to $Cr2^{+/+}$ mice, though, IR did not induce significant injury in $Cr2^{-/-}$ mice (Fig. 1D), with an injury score of 0.6 ± 0.2 and average villus height of $33.4 \pm 3.1 \mu\text{m}$ (Fig. 2). These results demonstrate that the presence of CR2 and CR1 is necessary for the induction of intestinal IR injury.

$Cr2^{-/-}$ mice have a qualitative but not quantitative difference in serum IgM and IgG

Because CR2 and CR1 are involved in B cell responses to foreign Ags, it was considered possible that these mice might manifest substantially decreased serum levels of IgM and IgG Abs. If this were the case, intestinal IR injury might be prevented in $Cr2^{-/-}$ mice simply because of a lower level of serum natural Abs that was then insufficient to initiate injury. However, as was found in the initial analysis of this strain of mice at the F₁ stage of breeding (32), both IgM and total IgG levels were not significantly different when comparing $Cr2^{+/+}$ to $Cr2^{-/-}$ mice (data not shown). These results indicate that the lack of intestinal IR injury in $Cr2^{-/-}$ mice

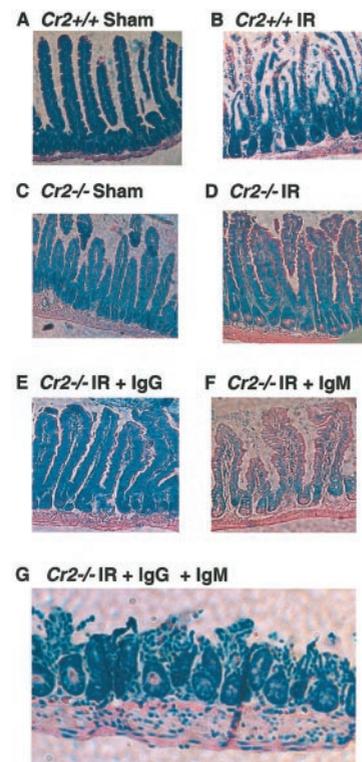


FIGURE 1. Histologic analysis of intestinal IR injury in the settings as shown demonstrating protection of $Cr2^{-/-}$ mice and reconstitution of injury following infusion of Ig as shown purified from wild-type C57BL/6 mice. Representative Giemsa-stained, formalin-fixed intestinal sections for each treatment group ($Cr2^{+/+}$ sham (A); $Cr2^{+/+}$ IR (B); $Cr2^{-/-}$ sham (C); $Cr2^{-/-}$ IR (D); $Cr2^{-/-}$ IR plus IgG (E); $Cr2^{-/-}$ IR plus IgM (F); $Cr2^{-/-}$ IR plus IgG plus IgM (G)) are shown. Magnification for all photomicrographs: $\times 200$.

is not due to a markedly lower level of serum IgM and IgG but rather to apparent differences in the composition of the natural Ab repertoire.

As part of this analysis, we have also re-examined $Cr2^{-/-}$ mice to determine the numbers of B-1 B cells in the spleen and peritoneum following backcrossing seven generations to the C57BL/6 strain. As in our first analysis of these mice (32), we were again unable to find substantial differences in the numbers of B-1a and B-1b B cells in the peritoneum and B-1a cells in the spleen of $Cr2^{+/+}$ and $Cr2^{-/-}$ mice, and the numbers of splenic B-2 B cells were also identical (data not shown). Therefore, although these data, as well as those presented below, indicate that there is a relative defect in B cell function, there are no apparent differences in the numbers of B-1 or B-2 B cells that develop in this strain of $Cr2^{-/-}$ mice.

In situ analysis of IgM, IgG, and complement C3 deposition

Previous studies have found that IgM and C3 are deposited in ischemic tissues (2–7). The deposition of these proteins as well as IgG in the intestines of mice subjected to IR was determined by immunohistochemistry. As expected, there was no significant deposition of IgM, IgG, or C3 in the intestines of sham-operated mice (Fig. 3). Similar to that seen by others, we also found that IgM, IgG, and C3 were deposited on the ischemic tissue of $Cr2^{+/+}$ mice after IR injury. However, consistent with the lack of injury, the intestines of $Cr2^{-/-}$ mice subjected to IR injury did not appear to have significant deposition of IgM, IgG, or C3. Importantly, we have shown that sera from $Cr2^{+/+}$ and $Cr2^{-/-}$ mice have equivalent ability to be activated by a heterologous IgG-opsonized target

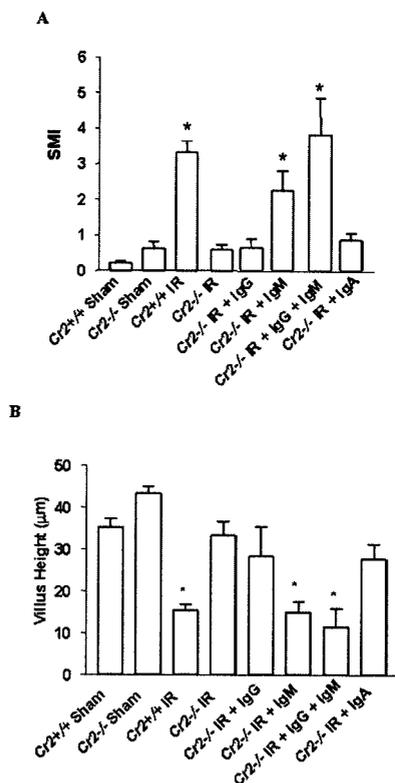


FIGURE 2. Quantitative analysis of intestinal IR injury. *A*, Giemsa-stained intestinal sections from each treatment group were scored for mucosal injury (0–6) as described in *Materials and Methods*. *B*, Villus height of individual villi was measured using an ocular micrometer. All measurements were obtained at a $\times 200$ magnification. Each bar is the average \pm SEM with six to eight mice per group. Using ANOVA with Newman-Keuls post hoc test, the asterisk indicates significant difference from sham treatment group ($p < 0.05$).

(data not shown), demonstrating that alterations in the endogenous complement system do not underlie the phenotypic differences. These results suggest that even though $Cr2^{-/-}$ mice have normal levels of serum IgM and total IgG there is a qualitative difference in the Ab produced in these mice manifested by the lack of reactivity with neopeptides on ischemic tissue.

Reconstitution of intestinal IR injury in $Cr2^{-/-}$ mice with IgM and IgG from wild-type mice

Prevention of injury in $Cr2^{-/-}$ mice could in principle be due to the lack of receptors on a cell population that are key to inducing local injury, rather than a lack of a particular subset of natural Abs. For example, CR1 and CR2 are expressed on activated murine polymorphonuclear cells (39). To determine whether this was the case, we asked whether infusion of $Cr2^{-/-}$ mice, before the ischemic phase, with IgM and IgG purified from the serum of wild-type C57BL/6 mice would result in intestinal IR injury. As a control, some mice were also pretreated with an identical amount of purified IgA. Additional controls included sham-treated animals treated with IgM, IgG, IgA, or IgM and IgG. As shown in Figs. 1 and 2, pretreating $Cr2^{-/-}$ mice with purified IgM alone induced edema and damage to the epithelial cells in the tips of villi, resulting in an injury score of 2.3 ± 0.05 . Pretreatment of $Cr2^{-/-}$ mice with purified IgG alone did not generate IR-induced mucosal injury. However, when $Cr2^{-/-}$ mice were treated with both IgM and IgG, intestinal IR injury was significant, with pronounced destruction of villi and a SMI of 3.8 ± 1 . The damage was not the

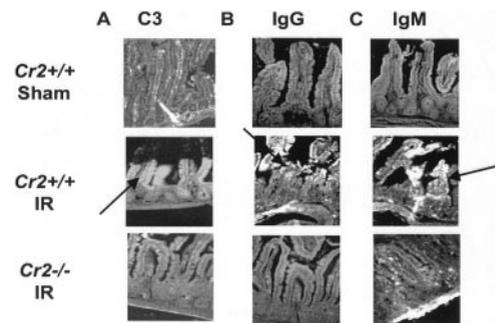


FIGURE 3. Deposition of C3, IgG, and IgM (arrows) in $Cr2^{+/+}$ but not $Cr2^{-/-}$ mice. Intestinal sections from $Cr2^{+/+}$ or $Cr2^{-/-}$ mice subjected to IR and $Cr2^{+/+}$ mice after sham treatment were stained for C3 (*A*), IgG (*B*), or IgM (*C*) deposition as described in *Materials and Methods*. Original magnification is $\times 200$. Data are representative of three individual experiments.

result of transfer of Abs alone, as sham-operated $Cr2^{+/+}$ or $Cr2^{-/-}$ mice that received IgM, IgG, or both IgM and IgG did not develop significant injury (data not shown). In addition, pretreatment with IgA did not result in the induction of intestinal IR injury (Fig. 2), suggesting that the injury-inducing Ig is found only in IgM and IgG isotypes. Finally, to determine whether $Cr2^{-/-}$ mice had only slightly lower levels of injury-inducing Abs, we pretreated $Cr2^{-/-}$ mice before the initiation of the ischemic phase with 400 μ l of pooled serum derived from other $Cr2^{-/-}$ mice containing ~ 250 μ g of IgM and 450 μ g of IgG. There was no significant injury in these animals, as indicated by an injury score of 0.2 ± 0.1 .

Cooperative effects of transferred IgM and IgG Ab in the induction of intestinal IR injury in $Cr2^{-/-}$ mice

To confirm that restoration of the mucosal injury in $Cr2^{-/-}$ mice treated with IgM and/or IgG activated complement locally in the intestine, intestinal tissues from $Cr2^{-/-}$ mice, which were treated with IgM or IgG, were analyzed for C3 deposition. Fig. 4 dem-

A $Cr2^{-/-}$ IR + IgG



B $Cr2^{-/-}$ IR + IgM

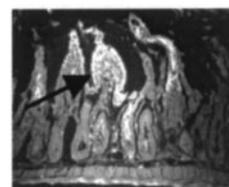


FIGURE 4. Deposition of C3 is IgM dependent. Intestinal sections from $Cr2^{-/-}$ mice reconstituted with normal IgG (*A*) or IgM (*B*) before being subjected to IR were stained for C3 deposition as described in *Materials and Methods*. Original magnification is $\times 200$. Data are representative of three individual experiments.

onstrates that C3 was deposited only in the intestines of mice subjected to IR and treated with IgM. Therefore, IgM is the most potent complement-fixing isotype in this model.

The partial injury phenotype observed with IgM pretreatment, coupled with the lack of injury with IgG alone, strongly suggested that there are additional mediators induced by IgG that cooperate with those induced by IgM to result in full injury. Previously, we showed that the neutrophil chemoattractant LTB₄ is rapidly produced in response to intestinal IR injury (3, 7). As shown in Fig. 5, when compared with sham-operated animals, *Cr2*^{+/+} mice subjected to IR demonstrated substantially elevated LTB₄ generation. Consistent with the lack of injury in *Cr2*^{-/-} mice, there was no increase in LTB₄ production following IR. Interestingly, in contrast to the lack of significant injury measured histologically, reconstitution of *Cr2*^{-/-} mice with wild-type IgG significantly increased LTB₄ production. In contrast, reconstitution with wild-type IgM did not result in increased LTB₄ generation. Reconstitution with both IgG and IgM also resulted in a significant increase of LTB₄ generation. These data suggest that the IgG natural Abs are critical for IR injury-induced LTB₄ production and provide one explanation for their additive effects with wild-type IgM.

Based on its chemotactic effects, one relevant target of LTB₄ is the polymorphonuclear cell. As a measure of polymorphonuclear cell presence and/or prior degranulation, the number of PMN in the intestinal sections were counted and the level of peroxidase activity in the intestinal sections were counted and the level of peroxidase activity was quantitated in each setting. Fig. 6 demonstrates that, as expected, PMN infiltration and peroxidase activity were increased substantially in tissues of *Cr2*^{+/+} mice subjected to IR when compared with sham-operated mice. In contrast, tissue from *Cr2*^{-/-} mice subjected to IR did not demonstrate an increase in either PMN numbers or peroxidase activity. This is again consistent with the lack of injury induced in this setting. In *Cr2*^{-/-} mice reconstituted with IgG, there was a substantial increase in both PMN infiltration and peroxidase activity. Although reconstitution of *Cr2*^{-/-} mice with IgM also demonstrated a minor increase, the pronounced increase with IgG alone provides a further potential explanation for its additive effects in the induction of local injury.

Finally, we sought to determine whether a relevant adhesion molecule, one that would allow for efficient local polymorphonuclear cell recruitment, might be differentially regulated in the vascular endothelium by IgM and IgG in *Cr2*^{-/-} mice. To address this, the intestinal tissue was analyzed for ICAM-1 expression. In *Cr2*^{+/+} mice, IR induces ICAM expression on the vasculature of the intestine (Fig. 7B). In contrast, IR did not induce ICAM ex-

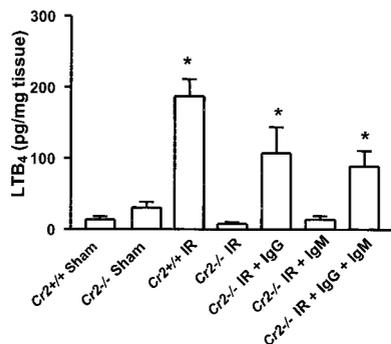


FIGURE 5. LTB₄ generation is IgG dependent. Eicosanoid production by tissue sections from each treatment group was determined by enzyme immunoassay as described in *Materials and Methods*. Each bar is average \pm SEM with four to six animals per group. Using ANOVA with Newman-Keuls post hoc test, the asterisk indicates significant difference from sham treatment group ($p < 0.05$).

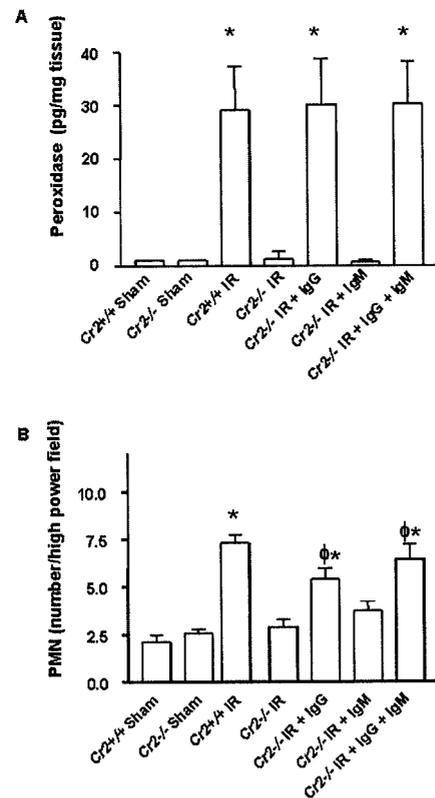


FIGURE 6. Peroxidase activity and polymorphonuclear presence are IgG dependent. Peroxidase levels were determined as described in *Materials and Methods* from in vitro incubation of intestinal sections obtained from mice after 2 h of reperfusion, and the number of PMN in three separate fields of Giemsa-stained sections was counted under high-power magnification. Each bar is the mean \pm SEM with four to six animals per group. Using ANOVA with Newman-Keuls post hoc test, the asterisk indicates significant difference from sham group ($p < 0.05$), and ϕ indicates significant difference from IR group ($p < 0.05$).

pression in tissues from *Cr2*^{-/-} mice (Fig. 7C). Of interest, *Cr2*^{-/-} mice reconstituted with IgM did express ICAM on the vascular endothelial cells after IR (Fig. 7E), while those treated with IgG did not (Fig. 7D). As expected, *Cr2*^{-/-} mice reconstituted with both IgG and IgM strongly expressed vascular endothelial cell ICAM-1 (Fig. 7F).

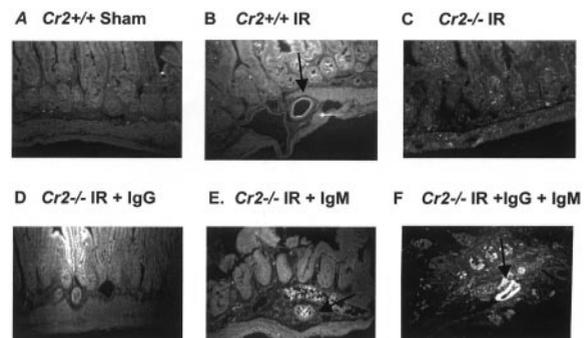


FIGURE 7. ICAM expression is IgM dependent. Intestinal sections were stained for ICAM expression as described in *Materials and Methods*. Stained sections from each group (*Cr2*^{+/+} sham (A); *Cr2*^{+/+} IR (B); *Cr2*^{-/-} IR (C); *Cr2*^{-/-} IR plus IgG (D); *Cr2*^{-/-} IR plus IgM (E); *Cr2*^{-/-} IR plus IgG plus IgM (F)) are shown. Each photomicrograph is representative of three separate experiments. Original magnification is $\times 400$.

Discussion

In these studies we have shown that $Cr2^{-/-}$ mice lack a subset of natural Abs that are capable of recognizing an as-yet-unknown neoantigen or neoantigen complex revealed to the immune system during the ischemic phase of tissue injury. These natural Abs are responsible for activating complement, generating inflammatory mediators such as LTB_4 , and recruiting activated polymorphonuclear cells. The natural Abs that are lacking in $Cr2^{-/-}$ mice are derived from both IgM and IgG isotypes. Interestingly, each Ig isotype appears to provide a unique contribution to the overall phenotype of intestinal IR injury. This observation provides potential explanations for the requirement for pretreatment with both IgM and IgG to generate a fully competent injury phenotype.

There are several major findings that support these conclusions. First, $Cr2^{-/-}$ mice do not develop intestinal IR injury despite having levels of serum IgM and total IgG that are indistinguishable from $Cr2^{+/+}$ mice. Second, the lack of intestinal IR injury in $Cr2^{-/-}$ mice is associated with a lack of local complement activation, generation of LTB_4 , and recruitment of polymorphonuclear cells as measured by tissue peroxidase levels. Third, transfer of purified IgM and IgG obtained from wild-type C57BL/6 mice together into $Cr2^{-/-}$ mice before the ischemic phase restores the development of intestinal IR injury in these mice. Transfer of serum from $Cr2^{-/-}$ mice does not reconstitute injury. Finally, pretreatment of $Cr2^{-/-}$ mice with wild-type IgM and IgG individually results in a pattern of isotype-specific local activation of complement as well as the generation of LTB_4 and the recruitment of polymorphonuclear cells.

Several additional issues relevant to this mouse strain were also explored in these studies. The numbers of B-1 (B-1a and B-1b) as well as B-2 B cells were found to be essentially identical in $Cr2^{+/+}$ and $Cr2^{-/-}$ mice following seven generations of backcrossing to C57BL/6, which is the same result as that originally reported for the F_1 analysis of this CR2/CR1-deficient strain (32). In addition, the lack of expression of either CR2 or CR1 in this strain was verified by flow cytometry, and a functional deficiency was shown by the inability to flux Ca_i^{2+} in response to co-cross-linking of the B cell receptor with CR2/CR1 (Ref. 40 and data not shown). Finally, $Cr2^{-/-}$ mice at this backcross also continue to manifest the original phenotype that consists of a severe defect in the switched IgG response to T-dependent Ags (41).

In our studies we have not yet determined whether the lack of generation of a pathogenic subset of natural Abs is due to a defect in B-1 or B-2 B cells, as both contribute to the serum pool of IgM and IgG (15, 16, 19). In support of the concept that B-1 B cells generate this subset is the extensive previous analysis of these cells demonstrating that they produce a large repertoire of low-affinity self-reactive Abs that result from positive selection during B-1 cell development (14, 42–44). If CR2/CR1 coreceptor function on B-1 B cells is required for the efficient recognition of a critical self Ag that is either identical to, or antigenically related to, the neoantigen revealed on ischemic tissue, this could, in principle, explain our findings relative to the lack of development of this subset of Abs. A previous review of the role of CR2/CR1 in B-1 cell selection has also suggested this possibility (45, 46).

It is important to point out that our results do not exclude a role for B-2 B cells in this phenotype. B-2 B cells generate a portion of the IgM natural Ab repertoire as well as the great majority of the natural IgG repertoire, the latter in likely response to environmental Ag exposure (44). In particular, marginal zone B cells are responsible for the immediate IgM response to particulate Ags and, like B-1 cells, are responsible for a substantial portion of the IgG3 response (15, 47, 48). It is apparent from our studies that both IgM

and IgG must be given to $Cr2^{-/-}$ mice to generate a full injury phenotype. It is also intriguing that marginal zone B cells in $Cr2^{-/-}$ mice, while present in apparently normal numbers (Ref. 49 and data not shown), do not appear to trap blood-borne, complement activating, T-independent Ags appropriately (50). Therefore, it is possible that B-2 B cells recognize a foreign environmental Ag and generate a response that cross-reacts with the neoantigen(s) revealed on ischemic intestinal tissue. The lack of efficient T-dependent follicular and marginal zone B-2 B cell responses in $Cr2^{-/-}$ mice would be consistent with this finding. Of course, it is also possible that both B cell subsets are necessary in this complex phenotype. Also not explored yet are two additional possibilities. One is the potential role of CR2 acting as a target for the alternative pathway of complement (51), and the other the role of intestinal mast cell CR1 and CR2 (52) in potentiating injury. Although the IgM and IgG transfer experiments rule out a primary role for either of these mechanisms in this phenotype, each may play an enhancing role.

Two additional points regarding the mechanism of tissue injury deserve further comment. First, LTB_4 is generated by arachidonic acid metabolism via the 5-lipoxygenase pathway in the intestinal mucosa in response to reperfusion and complement activation (52–55). The specific cells that mediate the generation of LTB_4 are likely to be phagocytic cells (either resident macrophages or polymorphonuclear cells) that are present in the intestinal muscle and mucosa (56). Second, our studies themselves do not rule out a role for FcR engagement and cell activation, especially because it is clear that IgG Abs are required for the full intestinal injury phenotype. However, studies by ourselves and others have shown that complement blockade alone completely ameliorates intestinal IR injury. Therefore, the major effect of IgG deposition in this setting may be polymorphonuclear cell activation, consistent with our findings herein that pretreatment of $Cr2^{-/-}$ mice with purified wild-type IgG results in local infiltration by these cells. In contrast, the role of polymorphonuclear cells in intestinal IR injury is unclear, as previous studies have provided evidence both for and against an important role of these cells (57, 58). Therefore, although the recruitment of polymorphonuclear cells is IgG dependent, we do not know whether this is a critical cell type for generating tissue injury in the setting of intestinal IR injury.

An additional question one must consider is why CRs would be necessary for the generation of this subset of IgM and IgG natural Abs. We believe that this is consistent with the major evolutionary role of complement, and of natural Ab itself, which is self protection for the organism. Certainly in a much less medically advanced, presurgical era the human species was entirely dependent for survival upon endogenous innate and adaptive immune responses. Tissue injuries, often of a severe nature, would have been a common event, and for survival it would be necessary to rapidly identify injured tissue and then isolate and remove it before overwhelming infection sets in. The evolution of a system by which natural Ab would recognize neoantigens revealed on ischemic tissue and activate complement, another ancient member of innate immunity (59), is an elegant solution to this problem. In addition, given the known requirement for the CR2/CD19/CD81 coreceptor complex for efficient cellular activation (24, 25), one would anticipate that B cells would use these same coreceptor functions to generate this subset of “protective” natural Abs. Therefore, the finding that $Cr2^{-/-}$ mice lack this particular subset would not be unexpected.

These findings also suggest the possibility that complement is activated on the Ag that selects this B cell reactivity, resulting in the generation of covalently bound C3 or C4 ligands for CR2/CR1.

In this regard, if the relevant Ags for generating intestinal neoantigen reactivity are natural Ags derived from apoptotic cells, and the B cell subset that generates them is the B-1 subset, this would be consistent with the findings that apoptotic cell membranes bind complement C1 and activate complement directly (60). Thus, CR2/CR1 may be involved in positive selection of this subset of B-1 cells. In contrast, if the relevant Ags are foreign in origin, perhaps infectious organisms, and the B-2 B cell subset is recruited, then it is already well established that CRs are important coreceptors for their function (24, 25). Recently Nielsen et al. (53) found that blocking the CR2 binding site with Ab decreased both C3 and MAC deposition by inhibiting the alternative complement pathway. Therefore, another possibility is that the Abs are forming immune complexes and activating the alternative complement pathway via CR2.

These studies also suggest an interesting dichotomy related to the function of CR2/CR1 in B-2 vs B-1 B cells. As noted above, our data suggest that CR2/CR1 may function in positive selection of B-1 cells to complement-bound Ags. In contrast, several other studies have suggested that CR2/CR1 may function in negative selection of B-2 B cells. That hypothesis is supported by studies demonstrating that B6/*lpr* *Cr2*^{-/-} mice manifest accelerated autoimmunity (61) and that *Cr2* itself is a New Zealand White-derived lupus susceptibility gene in the NZM2410 murine model of systemic lupus erythematosus (62, 63). In those particular studies, the protein encoded by the *Sle1c* allele was found to contain an additional glycosylation site that interferes with receptor binding of its C3d ligand and subsequent signal transduction (63). Thus, while the role of CR2/CR1 on peripheral lymphoid follicular and marginal zone B-2 B cells is to enhance responses to foreign Ags (26), during early B-2 cell development CR2/CR1 may be required to impart a negative signal to immature or transitional B cells that are self reactive. In sum, the biologic outcome of ligating CR2/CR1 appears to greatly depend on the lineage and stage of development of the particular B cell.

Future studies will be focused on determining whether B-1, B-2, or both B cell subsets are required for the generation of these Abs. In addition, while the nature of the neoantigen(s) that are recognized by natural Abs is still obscure, the availability of *Cr2*^{-/-} mice and the demonstration of their defect in the generation of this subset of Abs should be helpful in identifying the relevant Ag-Ab reactivity that initiates injury. Identification of relevant target Ags is very important for the development of therapeutic strategies that seek to interrupt the development of tissue injury by interfering with the binding of natural Ab to ischemic tissues before the generation of inflammatory mediators.

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