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CR2\textsuperscript{+} Marginal Zone B Cell Production of Pathogenic Natural Antibodies Is C3 Independent

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Intestinal ischemia-reperfusion (IR)-induced damage requires complement receptor 2 (CR2) for generation of the appropriate natural Ab repertoire. Pathogenic Abs recognize neoantigens on the ischemic tissue, activate complement, and induce intestinal damage. Because C3 cleavage products act as ligands for CR2, we hypothesized that CR2\textsuperscript{hi} marginal zone B cells (MZBs) require C3 for generation of the pathogenic Abs. To explore the ability of splenic CR2\textsuperscript{+} B cells to generate the damaging Ab repertoire, we adoptively transferred either MZBs or follicular B cells (FOBs) from C57Bl/6 or Cr2\textsuperscript{−/−} mice into Rag-1\textsuperscript{−/−} mice. Adoptive transfer of wild type CR2\textsuperscript{hi} MZBs but not CR2\textsuperscript{lo} FOBs induced significant damage, C3 deposition, and inflammation in response to IR. In contrast, similarly treated Rag-1\textsuperscript{−/−} mice reconstituted with either Cr2\textsuperscript{−/−} MZB/B1 B cells (B1Bs) or FOBs lacked significant intestinal damage and displayed limited complement activation. To determine whether C3 cleavage products are critical in CR2-dependent Ab production, we evaluated the ability of the natural Ab repertoire of C3\textsuperscript{−/−} mice to induce damage in response to IR. Infusion of C3\textsuperscript{−/−} serum into Cr2\textsuperscript{−/−} mice restored IR-induced tissue damage. Furthermore, Rag-1\textsuperscript{−/−} mice sustained significant damage after infusion of Abs from C3\textsuperscript{−/−} but not Cr2\textsuperscript{−/−} mice. Finally, adoptive transfer of MZBs from C3\textsuperscript{−/−} mice into Rag-1\textsuperscript{−/−} mice resulted in significant tissue damage and inflammation. These data indicate that CR2 expression on MZBs is sufficient to induce the appropriate Abs required for IR-induced tissue damage and that C3 is not critical for generation of the pathogenic Abs. The Journal of Immunology, 2011, 186: 000–000.

Tissue damage that occurs in response to an ischemic event is significantly magnified by the return of blood flow (reperfusion). The inflammatory response mediates ischemia-reperfusion (IR)-induced tissue damage and results in amplified pathology in many clinical conditions, including myocardial infarction, stroke, and intestinal ischemia (1, 2). Although the precise mechanism of the magnified damage during reperfusion is unknown, cellular alterations which occur during ischemia appear to be recognized by the innate immune response. Because the local response frequently progresses to systemic inflammation and multiple organ failure, treatment of the IR-induced inflammatory response and subsequent tissue damage is the subject of intense investigation.

Tissue damage resulting from IR is proposed to be mediated in part by complement activation after natural Ab recognition of neoantigens present on the surface of ischemic tissue (2, 3). Primarily found as IgM or IgG3 isotypes, natural Abs appear to be produced by B-1 or MZB in the absence of immunization (4–9). Natural Abs may provide protection against bacterial (10) and viral pathogens (4, 11). However, many natural Abs also recognize self Ags and promote tissue damage in response to IR (12). Self-reactive Abs target several proteins expressed on ischemic tissue, including nonmuscle myosin heavy chains subtype A and C (13), β2-glycoprotein I (14), U1-ribonucleoprotein (15), and annexin IV (16). Ab recognition of the neoantigens leads to complement activation. However, the initiating complement pathway remains unresolved. Although there is strong evidence supporting the lectin-binding pathway (13, 17, 18), the presence of C1q deposition on ischemic tissue cannot completely rule out the contribution of the classical pathway (13). Regardless of the specific pathway, the process is Ab dependent with little known about the mechanism of autoreactive natural Ab selection.

In the mouse, complement receptor 2 (CR2) is an alternatively spliced, type I membrane glycoprotein expressed on mature B cells, follicular dendritic cells (FDCs) and epithelial cells providing a linkage between innate and adaptive immunity (19). Interestingly, B-1–like marginal zone B cells (MZBs) express higher levels of CR2 than do the B-2 follicular B cells (FOBs) (20). In conjunction with CD19 and CD81, CR2 composes part of the BCR complex, which enhances B cell signaling and activation (21). As a coreceptor, multiple ligands bind CR2, including the C3 cleavage products iC3b, C3dg, and C3d (22, 23), IFN-α (24), Epstein-Barr viral coat protein GP350/220 (23, 25, 26), and annexin (27). How the binding of these ligands specifically aids the ability of CR2 to promote an immune response is not known. However, evidence suggests that CR2 facilitates the presentation of Ags associated with complement-tagged structures to the BCR by binding complement fragments (28).

Previous studies showed that Cr2\textsuperscript{−/−} mice are resistant to IR-mediated tissue damage and that administering Abs from wild type mice restored damage (29). These studies suggested that Cr2\textsuperscript{−/−} mice do not generate the autoreactive natural Abs necessary for IR-induced mesenteric tissue damage (29). Moreover, these data suggest that CR2 may influence the selection of the natural Ab repertoire in such a way that it results in an autoreactive subpopulation. Because CR2 is required for generating pathogenic Abs, the CR2 ligands may also be required. Previous studies indicated that C3\textsuperscript{−/−} mice were also resistant to IR-in-
duced tissue damage (30). However, it is not clear whether C3 is required only for complement activation or for binding CR2 and initiating production of autoreactive natural Abs. We hypothesized that CR2-MZBs require C3 for generation of the pathogenic Abs. Our results show that similar to the peritoneal B-1 B cells, the CR2-MZBs produce the natural Ab repertoire necessary to induce tissue damage in response to IR. In addition, adoptive transfer of splenic B cells (either MZBs or FOBs) or administering serum from CR2-sufficient, C3−/− mice to the Ab-deficient Rag-1−/− mice induced normal levels of damage in response to IR. These data indicate that although CR2 is critical, the C3 ligands are not required for the production of pathogenic, autoreactive Abs.

Materials and Methods

Mice

Breeding pairs of C57BL/6 mice, C3−/− and Rag-1−/− mice were purchased from Jackson Laboratories and C2−/− mice (31) were obtained from Dr. Michael Holers (University of Colorado Denver, Denver, CO). All mice were bred and maintained in a 12-h light-dark, temperature-controlled room and allowed food and water ad libitum in the Division of Biology at Kansas State University. Mice were maintained under specific pathogen-free conditions (Helicobacter species, mouse hepatitis virus, minute virus of mice, mouse parvovirus, Sendai virus, murine norovirus, Mycoplasma pulmonis, Thielier’s murine encephalomyelitis virus, endoparasiotes, and ectoparasiotes). All research was approved by the Institutional Animal Care and Use Committee and conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations concerning animals.

Spleen cell and B cell reconstitution

Peritoneal exudate cells (PECs) were collected from euthanized mice by peritoneal lavage using normal saline, and RBCs were lysed prior to injection. Total splenic B cells were isolated using EasySep B cell negative selection (StemCell Technologies). The B220−, CD19+ MZB, Cr2−/−, and restored for 2 h. Some experiments reconstituted B220+ MZB, Cr2−/−, and restored damage, we isolated and adoptively transferred wild type MZB splenic cells also restored IR-induced intestinal injury in Rag-1−/− mice. However, the critical role of CR2-MZBs is not clear.

Ischemia-reperfusion

IR was performed on ketamine-xylazine-anesthetized mice. After a midline laparotomy, the mice were allowed to stabilize for 30 min while maintaining their body temperature using a water-circulating heat pad. Buprenorphine was administered locally for pain, and peritoneal desiccation was prevented by placing warm, saline moistened gauze over the abdominal cavity. The superior mesenteric artery was then identified and a small vascular clamp was applied. Ischemia was noted by intestinal blanching. Sham-treated animals underwent the same procedure as the ischemic mice without occlusion of the superior mesenteric artery. After 30 min of ischemia, the clamp was removed and the blood flow was restored for 2 h. Some experiments reconstituted Rag-1−/− mice with 200 μl serum or 100 μg Protein L purified Ab from C3−/− or Cr2−/− mice by i.v. injection 15 min prior to the resumption of blood flow. As described previously (34), when the lamina propria was exuding; finally, villi that displayed hemorrhage or were denuded were assigned a score of 6.

C3 deposition

After sham treatment or IR, a 2-cm midjejunal section was frozen in OCT freezing medium and stored at −80°C until used. Intestinal cryosections (8 μm) were fixed in cold acetone, and nonspecific binding was blocked using 10% donkey serum in PBS. Tissues were stained for C3 deposition using a rat–anti-mouse C3 Ab (Hyclut Biotechnologies) followed by a Texas Red-conjugated secondary Ab (Jackson ImmunoResearch). Serial sections stained with isotype control Abs were used as background. A blinded observer examined the slides by fluorescent microscopy using a Nikon 80i fluorescent microscope and acquired images using a CoolSnapCF camera (Photometrics) and MetaVue Imaging software (Molecular Devices).

PGE2 determination

Immediately after collection, a 2-cm intestinal section was minced and washed in freshly oxygenated Tyrode’s buffer at 4°C (Sigma-Aldrich). The tissue was then incubated at 37°C for 20 min, and the supernatants were collected. PGE2 concentrations were determined using enzyme immunoassay kits (Cayman Chemical) and standardized to the total tissue protein content as determined by BCA assay (Pierce).

Statistical analysis

Data are presented as mean ± SEM and were compared by unpaired t test or one-way ANOVA with post hoc analysis using Newman-Keuls test (GraphPad/Instat Software). The difference between groups was considered significant at p < 0.05.

Results

CR2+ splenic cells restore IR-induced tissue damage to Rag-1−/− mice

Previous studies indicated that peritoneal B1Bs produce naturally occurring Abs required for IR-induced tissue damage (36). Because CR2+CD23+ splenic MZB are similar to B1Bs (37), we hypothesized that MZBs may also restore damage to IR-resistant Rag-1−/− mice. To test this hypothesis, we adoptively transferred either PECs or splenic cells from C57BL/6 mice into Rag-1−/− mice, 8–12 wk old, which were injected i.v. with 1–2 mg Ab from C3−/− or Cr2−/− mice by i.v. injection 15 min prior to the resumption of blood flow. As described previously (34), when the lamina propria was exuding; finally, villi that displayed hemorrhage or were denuded were assigned a score of 6.

CR2-MEDIATED NATURAL Abs ARE C3 INDEPENDENT

Serum was collected by cardiac puncture following IR or sham treatment and euthanasia. Aliquots were isolated and analyzed per the manufacturer’s protocol using a mouse Isotyping Milliplex kit on a Luminex SD200 (Millipore).

Histology and injury scoring

A 2-cm intestinal section was immediately fixed in 10% buffered formalin and embedded in paraffin for H&E staining of 8-μm transverse sections. Macosal injury was graded on a six-tiered scale adapted from Chiu et al. (35) as described previously (29). The average injury score (0–6) of 75–150 villi per midjejunal tissue section was determined in a blinded manner. Normal villi were assigned a score of zero; villi with tip distortion were assigned a score of 1; a score of 2 was assigned when Guggenheim’s spaces (lifting of the epithelium from the lamina propria to form a space) were present; villi with patchy disruption of the epithelial cells were assigned a score of 3; a score of 4 was assigned to villi with exposed but intact lamina propria with epithelial sloughing; a score of 5 was assigned when the lamina propria was exuding; finally, villi that displayed hemorrhage or were denuded were assigned a score of 6.

Ab isotypes

Statistical analysis

Data are presented as mean ± SEM and were compared by unpaired t test or one-way ANOVA with post hoc analysis using Newman-Keuls test (GraphPad/Instat Software). The difference between groups was considered significant at p < 0.05.

Results

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Adaptive transfer of marginal zone B cells restores IR-induced damage and complement activation to Rag-1−/− mice

To clarify which splenic cell population required CR2 expression and restored damage, we isolated and adoptively transferred wild
These data suggest that the presence of CR2+ MZBs is sufficient to or MZB/B1Bs from prior to sham or IR treatment. Additional controls included sham or IR treatment of C57BL/6, Cr2−/−, and Rag-1−/− mice; ~75–150 villi per H&E-stained intestinal section were scored for injury as described in Materials and Methods. Injury scores for the sham-treated mice for all experimental conditions were pooled. Each bar represents the group average ± SEM with four to eight mice per group. Using ANOVA: ∗significant difference (p < 0.05) from pooled sham treatment; †significant difference from C57BL/6 IR; ‡significant difference from Rag-1−/− IR.

type splenic FOBs and MZBs into Rag-1−/− mice. Wild type B220+ B cells were isolated and sorted using differential staining and FACS based on CD23 and Cr2, with MZBs expressing CR2+CD23lo and FOBs expressing CR2−CD23hi. Reconstitution of each animal was confirmed by flow cytometry (data not shown) and further verified by serum IgG1, IgG2b, IgG3, and IgM concentrations (Table I). Both MZBs and FOBs produced IgG1and IgG2b as indicated in Table I. Adoptive transfer of wild type MZBs resulted in significantly more IgM than did transfer of wild type FOBs. Importantly, after adoptive transfer of wild type MZBs, IR induced significant damage in Rag-1−/− mice (Fig. 2A, 2C, 2D, 2F). Although transfer of wild type FOBs slightly elevated the IR-induced injury score, the injury score was not significantly different from Rag-1−/− without adaptively transferred cells and was significantly different from Rag-1−/− with MZBs. Because MZBs are CR2+ and CR2, expression is required for production of the pathogenic natural Abs, MZB/B1Bs (IgM6, IgD6), and FOBs (IgM6, IgD6) from Cr2−/− mice were adoptively transferred as a control. Despite equivalent spleen cell numbers, adoptive transfer of FOBs or MZB/B1Bs from Cr2−/− mice resulted in significantly less IgG3 and IgM production compared with adoptive transfer of respective wild type cells (Table I). In addition, adoptive transfer of Cr2−/− MZB/B1Bs to Rag-1−/− mice failed to restore tissue damage to wild type levels in response to IR and did not significantly enhance injury compared with untreated Rag-1−/− after IR (Fig. 2A, 2B, 2C, 2G). Similar to wild type FOBs, IR induced no significant damage after adoptive transfer of FOBs from Cr2−/− mice (Fig. 2A–2E). These data suggest that the presence of Cr2−/− MZBs is sufficient to induce tissue damage in response to IR.

Previous studies indicated that IR-induced tissue damage requires complement activation (30, 38, 39). After adoptive transfer, we assessed complement activation by C3 deposition on intestinal tissue sections after sham or IR treatment. Immunohistochemistry of intestinal tissues from wild type C57BL/6 mice after IR (Fig. 3B) but not sham (Fig. 3A) treatment showed significant C3 deposition on the basolateral side of the epithelium along the majority of the villi. As expected, no C3 deposits were detected on Rag-1−/− and Cr2−/− mice (Fig. 3E, 3F) after IR. Tissue derived from IR-treated Rag-1−/− mice reconstituted with C57BL/6 MZBs exhibited patterns of C3 deposition similar to C57BL/6 mice (Fig. 3C). Adoptive transfer of control Cr2−/− MZB/B1B into Rag-1−/− resulted in limited intestinal C3 deposition, which was visible only at the tips of the villi (Fig. 3G). In contrast, C3 staining of the tissue from Rag-1−/− mice reconstituted with FOBs from either C57BL/6 or Cr2−/− mice showed minimal C3 deposition (Fig. 3D, 3H). C3 deposition suggested decreased complement activation and correlated with the lower injury scores shown in Fig. 2. Thus, the Ab repertoire of Rag-1−/− mice after adoptive transfer with wild type MZBs but not Cr2−/− MZB/B1B activated complement and caused tissue damage similar to that in wild type mice in response to IR. These data suggest that Cr2+ MZBs are sufficient to generate the autoreactive Ab repertoire required for complement-mediated, IR-induced tissue damage.

Abs from C3−/− or C4−/− mice restore IR-induced damage to Rag-1−/− mice

Previous studies indicated that IR-induced complement-mediated damage was attenuated in C3−/− mice, presumably owing to a lack of complement activation; however, the specific role of C3 in pathogenic Ab production was not determined (30, 36). Because CR2 is required for IR-induced damage and C3 cleavage products are CR2 ligands, we examined the role of the C3 in generation of the autoreactive Abs. To determine whether the CR2+ B cells found in C3−/− mice produce the damaging Ab repertoire, serum or Abs purified from C3−/− mice were injected into Cr2−/− or Rag-1−/− mice prior to IR or sham treatment. As expected, untreated Rag-1−/−, C3−/−, and Cr2−/− mice did not sustain IR-induced tissue damage (Fig. 4A, 4C). After infusion of C3−/− serum or purified Abs into either Rag-1−/− (Fig. 4A, 4E, 4F) or Cr2−/− (data not shown) mice, significant intestinal damage was observed in response to IR. As CR2 also binds C4b, it was possible that C4 was required for CR2 induction of Ab. However, similar to C3−/− serum, infusion of serum from C4−/− mice into Rag-1−/− induced significant injury (2.03 ± 0.3 injury score). PGE2 expression was analyzed as evidence of an inflammatory response. Correlating with intestinal damage, infusion of Rag-1−/− mice with Abs or serum from C3−/− mice significantly increased intestinal PGE2 expression in response to IR (Fig. 4B). In contrast, injection of Cr2−/− serum or Abs into Rag-1−/− mice resulted in minimal IR-induced tissue damage and background levels of intestinal PGE2.
FIGURE 2. Adoptive transfer of MZBs or PECs from C57BL/6 mice restored IR-induced tissue damage in Rag-1−/− mice. A, FOBs, MZBs, PECs (1.5–2 × 10⁶) from either C57BL/6 or Cr2−/− mice were adoptively transferred to Rag-1−/− mice 8–9 wk prior to sham or IR treatment. C57BL/6, Cr2−/−, and Rag-1−/− mice were subjected to sham or IR treatment as positive and negative controls. H&E-stained intestinal sections were scored for injury as described in Materials and Methods. B–G, Representative H&E-stained, formalin-fixed intestinal sections from each treatment group are shown: C57BL/6 IR (B), Rag-1−/− IR (C), Rag-1−/− + C57BL/6 FOB (D), Rag-1−/− + Cr2−/− FOB (E), Rag-1−/− + C57BL/6 MZB (F), and Rag-1−/− + Cr2−/− MZB/B1B (G). Each bar represents the group average ± SEM with seven to nine mice per group. Using ANOVA: *significant difference (p < 0.05) from pooled sham treatment; ‡significant difference from C57BL/6 IR; §significant difference from Rag-1−/− IR. Injury scores for the sham-treated mice for all experimental conditions were pooled. Original magnification ×100 for all photomicrographs; scale bar, 0.8 μm.

To confirm that Abs from CR2+ B cells are sufficient for IR-induced intestinal damage, C3-deficient mice produce the appropriate Ab repertoire to induce complement activation in response to IR.

Ab-deficient, C3-sufficient serum restores IR-induced damage to C3−/− mice

To confirm that Abs from CR2+ B cells are sufficient for IR-induced damage in the absence of C3, C3−/− mice were infused with fresh Rag-1−/− serum prior to IR or sham treatment. After infusion of freshly obtained, C3-containing serum into C3−/− mice, the injury score of sham-treated mice was unchanged compared with sham-treated C3−/− mice. In contrast, infusion of C3+ Rag-1−/− serum into C3−/− mice prior to IR induced significant injury, although the injury was attenuated when compared with wild type mice (Fig. 6A, 6C). The attenuated damage is likely due to dilution of C3. In addition, serum reconstitution of C3−/− mice significantly increased PGE2 production compared with sham or IR treatment of C3−/− mice (Fig. 6B). To verify that the C3−/− MZBs (CD23lo CD21/35lo) produce sufficient Abs for IR-induced intestinal damage, C3−/− MZBs (CD23lo CD21/35lo) and FOBs (CD23lo CD21/35lo) were adoptively transferred to Rag-1−/− mice 2 mo prior to IR. As expected, MZBs from C3−/− mice restored IR-induced intestinal damage, PGE2 production and complement activation in Rag-1−/− mice (Fig. 6, data not shown).

FIGURE 3. C3 deposition was restored in Rag-1−/− mice after adoptive transfer of C57BL/6 MZBs. Intestinal sections from Rag-1−/− mice without transfer (E) or after adoptive transfer of MZBs, MZB/B1Bs, or FOBs from C57BL/6 (B) or Cr2−/− mice (C, D, G, H) were stained for C3 deposition after IR treatment. Similarly stained tissue sections from C57BL/6 sham (A), IR (B), or Cr2−/− IR (F) treated mice are shown as controls. Photomicrographs are representative of six photos of each intestinal section with three to five animals per treatment group. Original magnification ×200.
Surprisingly, C3−/− FOBs (CD23hi CD21/35hi) induced significant intestinal injury and PGE2 production at a level similar to C3−/− MZBs (CD23hi CD21/35hi; Fig. 6). In addition, adoptive transfer of either C3−/− cell type induced significant Ab production (Table I). These data indicate that C3 is required for complement activation, but is not required as a CR2 ligand in the generation of pathogenic Abs.

Discussion

Previous studies indicated that IR-induced, complement-mediated tissue damage requires CR2 for generation of the pathogenic Abs (12, 29, 30, 40). In addition, IR-induced injury required C3, but the exact role of C3 in the production of pathogenic Abs was unclear (30). As a central component in the complement cascade, C3 is required for complement activation during complement-dependent tissue injury. However, C3 cleavage products, iC3b, C3d, and C3dg, also bind CR2 resulting in enhanced Ab production. In this study, we demonstrate that although C3 is required for complement activation, it is not required for generation of the pathogenic Abs essential for IR-induced tissue damage. Furthermore, we established that CR2+ splenic MZBs are sufficient for IR-induced tissue damage. Thus, the MZB production of pathogenic Abs required for IR-induced tissue damage is CR2 dependent and C3 independent.

The role of complement, and therefore C3, in IR-mediated tissue damage is well established (41). However, the requirement of C3 cleavage fragments for production of the appropriate natural Ab repertoire to initiate complement activation was not known. We demonstrated that injection of purified Abs or serum from C3−/− mice into Rag-1−/− or Cr2−/− mice restored IR-mediated mesenteric damage and inflammation. In addition, adoptive transfer of C3−/− but not Cr2−/− MZB to Rag-1−/− mice resulted in IR-induced damage. Therefore, the CR2-dependent generation of the pathogenic Ab repertoire does not require C3 cleavage fragments. The generation of CR2-dependent pathogenic Abs in the absence of C3 has been demonstrated previously, because the CR2-mediated Ab response to Streptococcus pneumoniae is C3 independent but CD19 dependent (42). Although the specific ligand of CR2 has not been elucidated, IFN-α is a strong candidate.

A recent study showed that IFN-α enhances MZB production of pathogenic autoantibodies (43). Finally, the current studies do not rule out enhanced Ab production owing to C3 fragments, as was previously shown to occur in the absence of CR2 (44).

Because C3 degradation products are the primary ligand for CR2 during Ab production, we expected that C3- and CR2-deficient mice would produce similar Ab repertoires. Surprisingly, transfer of C3−/− sera or Ig purified from C3−/− mice resulted in significant IR-induced tissue damage. In addition, adoptive transfer of C3−/− FOB significantly increased Ab production. Thus, in the absence of C3, both cell types produce pathogenic Abs, which induce intestinal damage in response to IR and IR-induced inflammation. Because C3 degradation products are an important ligand for CR2, and CR2-deficient B cells are not tolerized to soluble hen egg lysozyme (45), it is possible that C3 is

FIGURE 4. Ig from C3−/− mice induces injury and PGE2 production in Rag-1−/− mice after IR. A, Mucosal injury was determined from H&E-stained intestinal sections from Rag-1−/− or Cr2−/− mice that received either protein L-purified Ig or serum from C3−/− or Cr2−/− mice by i.v. injection 15 min prior to reperfusion. B, PGE2 was analyzed from supernatants of intestinal tissues as described in Materials and Methods. Each bar represents the mean ± SEM with four to six animals per treatment group. C–F, Representative H&E-stain of intestinal sections from Rag-1−/− mice of each treatment group. Original magnification ×100 for all photomicrographs; scale bar, 0.8 μm.

FIGURE 5. Ig from C3−/− but not Cr2−/− mice induces complement activation in Rag-1−/− mice. Intestinal sections from C57BL/6 (B6) (A) or Rag-1−/− mice without (B) or with infusion of C3−/− (C) or Cr2−/− serum (D) were subjected to IR and then stained for C3 deposition. Photomicrographs are representative of six photos of each intestinal section from three to five animals per treatment group. Original magnification ×200.
critical to the induction of tolerance of the CR2<sub>b</sub> FOB. This hypothesis is supported by recent data indicating that C3 deficiency upregulates CR3 expression on splenic B cells (46), and CR3 and iC3b are critical for TGF-β and IL-10 production, which lead to tolerance (47). In contrast, excessive CR2 decreases auto-Ab production (48). Thus, in the absence of the CR2 ligand (C3<sup>-/-</sup> mice), both FOBs and MZBs produce increased autoantibodies. It is also possible that C3<sup>-/-</sup> mice have more B cell precursors or B1Bs, which contaminated the sorted cells. However, the adoptively transferred, wild type and C3<sup>-/-</sup> B cells appeared similar when stained for B cell markers after IR (data not shown).

Innate-like, peritoneal B1Bs produce natural Abs, which restore IR-induced injury (49). Both B1Bs and MZBs produce IgM and IgG3 isotypes, recognize microbial and self Ags, and participate in T-independent Ag responses (37). A recent study suggested that both MZBs and B1Bs use a similar mechanism for production of the pathogenic natural Abs (7). The use of either phosphoinositide 3-kinase p110δ-deficient mice or a specific inhibitor of p110δ inhibited production of cardiac myosin Abs by both MZBs and B1Bs (7). In this study, adoptive transfer of either PEC-B1Bs or MZBs to Rag-1<sup>-/-</sup> mice restored IR-induced intestinal damage and inflammation as identified by increased complement activation and PGE2 production. Thus, both innate-like B cell populations produce the pathogenic Abs sufficient for IR-induced tissue damage, possibly via a distinct molecular mechanism from B-2 B cells.

Previous results indicated that CR2<sup>-/-</sup> mice have normal total Ig concentrations despite expressing significantly less IgM and IgG to annexin IV, a primary IR-induced Ag (16, 50). In this study, adoptive transfer of CR2<sup>-/-</sup> cells also resulted in dramatically lower serum IgM and IgG3 titers compared with adoptive transfer of wild type cells. Because the total B cell number recovered from the spleens after reconstitution was not different, the decreased Ab titer reflects the role of CR2 in Ab production. Similar to these results, recent evidence indicates that, compared with wild type mice, CR2<sup>-/-</sup> mice expressed less IgM and IgG3 in response to T-independent type 2 Ags (42). The decreased production of Ab was also complement independent (42). Although the exact function of CR2 in production of these pathogenic Abs remains unknown, Ab generation is C3 independent.

Although previous studies indicate that CR2 is required for intestinal IR-induced tissue damage, the specific cell types that require CR2 are not clear. MZBs express high CR2 levels, but other host cells, including the FDCs (51) and T cells (52), also express CR2. Adoptive transfer of MZBs from CR2<sup>-/-</sup> mice to CR2-sufficient Rag-1<sup>-/-</sup> mice did not restore IR-induced damage. Despite attenuated tissue damage, immunohistochemistry detected limited C3 deposition at the villi tips in tissue from Rag-1<sup>-/-</sup> mice reconstituted with CR2<sup>-/-</sup> MZBs. In contrast, no C3 deposition was detectable on intestinal tissue of IR-treated CR2<sup>-/-</sup> mice. It is possible that CR2 expression on the host cells induces production of small quantities of pathogenic Abs by the CR2<sup>-/-</sup> MZBs. This hypothesis is supported by previous studies of CR2 and germinal center formation. Although optimal germinal center formation requires CR2 on both B cells and FDCs, CR2 expression on either B cells or FDCs is sufficient for germinal center formation (51, 53, 54). Another possibility is that CR2<sup>+</sup> B cells contribute to the IR-mediated intestinal damage in an Ab-independent manner. B cell depletion of wild type mice prior to IR resulted in attenuated intestinal damage, suggesting an additional role for CR2<sup>+</sup> B cells (55). Therefore, although other cell types may also require CR2, MZBs require CR2 expression to provide the natural Ab repertoire necessary for IR-induced mesenteric tissue damage.

In summary, we demonstrate that similar to B1Bs, the CR2<sup>+</sup> MZBs also produce Abs, which induce pathogenicity in response to IR. Importantly, although CR2 expression is required, the generation of these pathogenic Abs is C3 independent. Further investigation is required to elucidate the exact role of CR2 in the generation of the natural Ab repertoire and the specific CR2 ligand inducing these pathogenic Abs.
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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Verification of MZB phenotype after reconstitution. Wildtype splenic B cells were sorted with CR2 and CD23 (upper panels) or IgM and IgD (lower panels) to collect MZB. A. The sorted cells were further stained for MZB and B1B markers: CD9, CD43, and CD5. B. Two months after adoptive transfer, the splenic cells were reanalyzed for CR2/CD23. Each dot plot is representative of 3 animals.