Published in final edited form as:

Mol Immunol. 2010 February ; 47(5): 972. doi:10.1016/j.molimm.2009.11.022.

Early complement factors in the local tissue immunocomplex generated during intestinal ischemia/reperfusion injury

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Abstract

Recent work reveals that the innate immune system is able to recognize self targets and initiate an inflammatory response similar to that of pathogens. One novel example of this innate autoimmunity is ischemia/reperfusion (I/R) injury, in which reperfusion of the ischemic tissues elicits an acute inflammatory response activated by natural IgM (nIgM) binding to ischemia-specific self antigens, which are non-muscle myosin heavy chains type II (NMHC-II) subtype A and C. Subsequently, the complement lectin pathway is activated and eventually tissue injury occurs. Although earlier studies in the intestinal model showed that the classical complement pathway did not initiate I/R injury, C1q deposition was still observed in the local injured tissues by imaging analysis. Moreover, the involvement of the alternative complement pathway became unclear due to conflicting reports using different knockout mice. To explore the immediate downstream pathway following nIgM-ischemic antigen interaction, we isolated the nIgM-ischemic antigen immunocomplexes from the local tissue of animals treated in the intestinal I/R injury model, and examined the presence of initial molecules of three complement pathways. Our results showed that mannan-binding lectin (MBL), the early molecule of the lectin pathway, was present in the nIgM-ischemic Ag immunocomplex. In addition, C1q, the initial molecule of the classical pathway was also detected on the immunocomplex. However, Factor B, the early molecule in the alternative pathway, was not detected in the immunocomplex. To further examine the role of the alternative pathway in I/R injury, we utilized Factor B knockout mice in the intestinal model. Our results showed that Factor B knockout mice were not protected from local tissue injury, and their complement system was activated in the local tissues by nIgM during I/R. These results indicated that the lectin complement pathway operates immediately downstream of the nIgM-ischemic antigen interaction during intestinal I/R. Furthermore, the classical complement pathway also appears to interact with the of nIgM-ischemic antigen immunocomplex. Finally, the alternative complement pathway is not involved in I/R injury induction in the current intestinal model.

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Introduction

Ischemia/reperfusion (I/R) injury is a major complicating feature of many clinical disease entities. Intestinal I/R is a devastating syndrome. Approximately one-third of episodes are acute events and are responsible for most gastrointestinal ischemia-related deaths (mortality rate of 70–90%) (Brandt, 2003). Recent work reveals that the innate immune system is able to recognize self-targets and initiate an inflammatory response in a manner similar to that provoked by pathogens (Carroll and Holers, 2005; Zhang and Carroll, 2007a; Zhang and Carroll, 2007b). One novel example of innate autoimmunity occurs in I/R injury (Carroll and Holers, 2005; Zhang et al., 2008; Zhang et al., 2006a; Zhang et al., 2004; Zhang and Carroll, 2007a; Zhang and Carroll, 2007b; Zhang et al., 2006b; Zhang et al., 2006c). Studies of intestinal, skeletal muscle, and heart I/R models showed that reperfusion of ischemic tissues elicits an acute inflammatory response activated by natural IgM (nIgM) (Fleming et al., 2002; Reid et al., 2002; Williams et al., 1999; Zhang et al., 2006b; Zhang et al., 2006c). Newer reports further demonstrate that human nIgM, like that of mice, is capable of inducing I/R injury in the murine intestinal model, suggesting that innate autoimmunity may operate under pathogenic conditions in humans (Zhang et al., 2008). Isolation of a monoclonal natural IgM that initiates I/R injury (Zhang et al., 2004) has led to the identification in two different tissues of self-targets, which are nonmuscle myosin heavy chains type II (NMHC-II) subtype A and C, (Zhang et al., 2006a). A recent study also suggested that aggregation of the actin cytoskeleton during ischemia can lead to IgM-mediated tissue injury (Shi et al., 2008). New studies further suggest that the natural IgM-ischemic antigen complex provides a binding site for mannan-binding lectin (MBL), which subsequently leads to activation of complement and results in tissue injury (Zhang et al., 2004; Zhang and Carroll, 2007b; Zhang et al., 2006c).

Three pathways leading to activation of the complement system have been identified: the classical, the lectin, and the alternative pathways. Each is activated by different initiators but all converge on C3 activation, which is followed by a common cascade (Carroll, 1998). The classical pathway is initiated by antibody-antigen interaction followed by the activation of complement C1 and downstream components (C4, C2, and C3). The alternative pathway is activated by spontaneous hydrolysis of C3 which allows the binding of factor B (Janeway et al., 2004). Some recent studies also suggested that properdin (factor P) may directly activate the alternative pathway (Holt et al., 1990; Spitzer et al., 2007; Vuagnat et al., 2000). The lectin complement pathway is triggered by MBL recognizing certain patterns of carbohydrate structures (Gadjeva et al., 2004; Roos et al., 2003; Tsutsumi et al., 2005; Turner, 2003; Worthley et al., 2005). MBL naturally exists in a complex with the MBL-associated serine proteases (MASPs) (Matsushita and Fujita, 1992; Schwaebler et al., 2002; Stover et al., 1999; Takahashi et al., 1999; Thiel et al., 1997). The MASPs are activated when MBL binds to a fitting carbohydrate pattern, resulting in cleavage of the polypeptide chains of the MASPs (Vorup-Jensen et al., 2000). The activated MASPs further cleave relevant substrates, i.e. C4 and C2 for MASP-2; C3 and C2 for MASP-1 (Hajela et al., 2002).

Although the classical complement pathway was ruled out to initiate I/R injury in the intestinal model (Hart et al., 2005; Walsh et al., 2005; Zhang et al., 2006c), C1q deposition was still observed in the local injured tissues by imaging analysis (Zhang et al., 2006c). Whether or not C1q is involved in the nIgM–ischemic Ag immunocomplex is still unknown.

Studies of the role of the alternative pathway in I/R injury have generated conflicting reports. An earlier report using Factor D knockout animals by Stahl et al suggested that the alternative pathway plays an important role in intestinal I/R (Stahl et al., 2003). Later, the same group using double knockout mice of Factor B and complement C2 came up in the opposite finding (Hart et al., 2005).

To identify the immediate downstream pathway following nIgM-ischemic antigen interaction, we isolated the nIgM-ischemic antigen immunocomplexes from the local tissue of animals treated in an intestinal I/R injury model, and investigated the presence of initial molecules of the three complement pathways. To clarify the role of the complement alternative pathway in I/R injury, we used Factor B knockout mice to examine local tissue damage and nIgM-mediated complement activation in the intestinal model.

Materials and Methods

Animals and the intestinal model of ischemia reperfusion injury

Wild type BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Factor B knockout mice were generated by Dr. Harvey R. Colten (Matsumoto et al., 1997) and were kindly provided by Dr. Rick Wetsel (University of Texas). The Factor B^{-/-} strain has been bred onto the C57BL/6 background for at least 10 generations and maintained in the animal facilities of The Rockefeller University. The Factor B^{-/-} mice were genotyped in the Steinman laboratory at The Rockefeller University. The surgical protocol for I/R was performed as previously described (Zhang et al., 2004). Briefly, after anesthesia, a laparotomy was performed, and a microclip (125g pressure, Roboz, Gaithersburg, MD) was applied to the superior mesenteric artery. The ischemia-treated mice were kept in room temperature for 40 minutes. After the 40 minutes of ischemia the microclip was removed, and all animals were kept warm during reperfusion. At the end of the experiment, the ischemic segment of the intestine was harvested, washed with normal saline, and frozen for further analysis.

Isolation of nIgM-ischemic antigen immunocomplex and detection of initiating factors of three complement pathways

We have developed a quantitative immunoassay to isolate the nIgM-ischemic Ag complex and to detect the early complement factors in the immunocomplex (Fig.1). The intestine was thawed and homogenized by sonication for 5 minutes in 1ml saline on ice. Tissue lysate was centrifuged at 2000 g for 5 minutes and the supernatant was saved for subsequent analysis. Protein concentration in the supernatant was determined by Bradford Assay (Biorad, Hercules, CA).

A 96-well flat-bottom immulon 2HB plate (Thermo, Waltham, MA) was coated with affinity purified rabbit IgG specifically recognizing NMHC-II A (1µg/ml, Covance, Princeton, NJ) and incubated overnight at 4°C. The plate was washed once and blocked with 1% BSA/0.05 Tween-20 (Calbiochem, San Diego, CA) in phosphate-buffered saline (PBS) for 2 hours at room temperature. The plate was washed once again. Intestinal lysate was added to the coated wells at a concentration of 12mg/ml, and incubated for 2 hours at room temperature to capture the nIgM-ischemic antigen complex (overnight incubation at 4°C also worked for this assay). The 12mg/ml concentration of intestinal lysates was determined as optimal by the results of pilot experiments, in which lysates with different concentrations were tested in the assays. The plate was then washed 3 times. To detect the bound natural IgM in the immunocomplexes, alkaline phosphatase (AP)-labeled rabbit anti-mouse IgM (0.33µg/ml, Rockland, Gilbertsville, PA) was added to the plate, and incubated for 1 hour at room temperature. The unbound antibodies were washed out with 0.05% Tween-20/PBS, and 1mg/ml phosphatase substrate (Sigma, St. Louis, MO) was added to develop color reaction. The optical density was determined in a Multiscan microplate reader (Thermo, Waltham, MA). To detect if early complement factors of any of the three complement pathways were to be found in the captured immunocomplexes, biotinylated antibodies specific for MBL (clone 131-1, (Jensenius et al., 2003); Hycult biotechnology, Canton, MA), and for C1q (Quidel, San Diego, CA), were used as the detection antibody followed by the addition of streptavidin-conjugated with alkaline phosphatase (0.19µg/ml, SouthernBiotech, Birmingham, AL). Horseradish peroxidase-

conjugated goat anti-human Bb (Bb fragment EIA kit, part# A9566, Quidel, San Diego, CA) was used as the detection antibody followed by the addition of the substrate.

A Pooled intestinal lysate from 13 I/R mice and from 11 sham treated mice was used as the internal standard. The standard curve was produced using the optical densities (OD) of the serially diluted the pooled lysates as x coordinates, and their protein concentrations (mg/ml) as y coordinates in the curve fitting. The OD of individual sample was converted to the relative amount according to the pooled standard lysates and then expressed as a ratio of per milligram per milliliter of protein in the tested sample lysates (Unit/mg.ml⁻¹). The presence of C3, properdin, and IgG was also examined in the ischemic Ag immunocomplexes captured by the above approach. To detect C3, goat anti-mouse C3 (0.2µg/ml, ICL, Newberg, OR) was used as the primary detection antibody, followed by the secondary detection antibody of AP-labeled rabbit anti-goat IgG (0.2µg/ml, Abcam, Cambridge, MA). To detect properdin, goat anti-human factor P (properdin) (ART, Tyler, TX) was used as the primary detection antibody to the captured ischemic Ag complex, followed by the secondary detection antibody of AP-labeled rabbit anti-goat IgG (0.2µg/ml, Abcam, Cambridge, MA). As for IgG detection in the captured immunocomplex, AP-labeled donkey anti-mIgG (0.12µg/ml, Jackson ImmunoResearch, West Grove, PA) was used as the detection antibody. The rest of the ELISA protocol followed the steps as described in the above section.

Binding specificity between the coating antibody and the self-antigen

To test for the specificity of the reaction of the coating antibody to the self-antigen, we carried out negative control experiments for the quantification of the IgM-ischemic Ag complex and detection of the initiating factors of the three complement pathways. Rabbit IgG against rat IgG (Vector laboratories, Burlingame, CA) was used as the coating antibody on the 96-well plates. The rest of experimental protocol followed the steps as described in the above section.

Histopathological and immunohistochemical analysis

Cryostat sections of intestinal tissues were stained with Hematoxylin and Eosin (H&E), blind-coded by a different person, and examined by light microscopy for mucosal damage. The pathology score was assessed based on a procedure developed by Zhang, which included a direct inspection of all microvilli over a 4-cm stretch of jejunum as described (Zhang et al., 2004).

To detect IgM deposition in the local tissues, cryosections were pretreated with 4% paraformaldehyde (PFA) (J.T. Baker, Phillipsburg, NJ) /PBS and incubated with 2.5µg biotin-labeled goat anti-mouse IgM (SouthernBiotech, Birmingham, AL) per ml of 1% BSA/0.01 Tween-20 /PBS for 2 hours, followed by staining with 4µg streptavidin-Alexa-568 (Molecular Probes, Carlsbad, CA) per ml of 1% BSA/0.01 Tween-20 /PBS for 1 hour. The deposition of C3 was detected by using 66µg FITC-labeled anti-C3 (Dako, Carpinteria, CA) per ml of 1% BSA/0.01 Tween-20 /PBS. Sections were counterstained and mounted in ProLong Gold Antifade Mounting Medium with 4', 6-diamidinio-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). Fluorescent images were made with an Olympus digital imaging system (Olympus, Center Valley, PA).

Statistical analysis

All quantitative data were entered into a Microsoft Excel database and a two-sided student t-test was used to analyze the statistical differences between the groups. The data were expressed as mean ± standard error of the mean (SEM) and p<0.05 was considered to be statistically significant.

Results

To identify the presence of early complement factors in the nIgM-ischemic antigen complex, we developed an immunoassay to isolate the immunocomplexes from WT mice subjected to intestinal I/R (Fig.1). Histopathological analyses confirmed that local tissues from I/R treated WT mice had significant injury compared with those of sham controls (pathology score of I/R group=19±2.8, n=27; pathology score of sham group=5±0.9, n=26; p=0.00003) (Fig.2a and 2b). Intestinal tissues from WT mice treated in the intestinal I/R model were homogenized to release the potential immunocomplexes in the supernatant of the lysates. The ischemic self antigen from the lysates, NMHC-II, was captured by the pre-coated anti-NMHC antibody, and after washout of unbound components, the nIgM that bound to NMHC-II during the I/R treatment was detected by anti-IgM antibody. Compared with samples from the sham control mice, local tissues from I/R treated animals had significantly more nIgM bound to the NMHC-II (Sham=14±1.6 Unit/mg.ml⁻¹, n=21 vs. I/R=40±5.7 Unit/mg.ml⁻¹, n=16, p=0.0005) (Fig. 3a).

To detect early complement factors in the nIgM-ischemic antigen complex, we modified the above immunoassay by replacing the anti-IgM detection antibody with antibodies specific to either C1q, MBL, or Factor B, which are the initial factors in each of the three complement pathways. As expected, MBL, the early molecule of the lectin pathway, was detected in the immunocomplex and significantly elevated in the intestinal tissues of I/R- treated mice compared to those of sham-treated animals (Sham=10±1.6 Unit/mg.ml⁻¹, n=21 vs. I/R=21±3.3 Unit/mg.ml⁻¹, n=16, p=0.006) (Fig.3b). In addition, C1q, the initial molecule of the classical pathway was also detected in the nIgM-ischemic antigen complex formed in local tissues of I/R treated mice (Sham=10±3.2 Unit/mg.ml⁻¹, n=21 vs. I/R=25±6.1 Unit/mg.ml⁻¹, n=16, p=0.042) (Fig.3c). However, there is no significant increase of Factor B, the early molecule in the alternative pathway, present in the nIgM-ischemic Ag immunocomplex (Sham=10±3.2 Unit/mg.ml⁻¹, n=6 vs. I/R=6±2.0 Unit/mg.ml⁻¹, n=6, p=0.243) (Fig.3d).

We also carried out negative control experiments for the above quantification assays. The coating antibody against NMHC was replaced with a nonspecific antibody of the same immunoglobulin isotype (IgG), which was generated from the same animal species (rabbit). The rest of the experimental protocol remained the same. The assays using the nonspecific antibody did not distinguish between the sham-operated vs. the I/R treated groups (Fig.4). Thus, these results provide support for the validity of our quantitative immunoassay for the natural IgM- ischemic Ag complex.

To further investigate the involvement of the complement alternative pathway in intestinal I/R injury induction, we examined complement C3 and properdin (factor P) in the immunocomplexes. Spontaneous hydrolysis of C3 was reported as an activator for Factor B and formation of C3(H₂O)Bb, the fluid phase C3 convertase, further cleave more C3 for activation. Properdin functions in the alternative path by stabilizing the fluid phase C3 convertase. Recent studies indicate that properdin can directly activate the alternative pathway by binding sulfated glycoconjugates (Holt et al., 1990) and specific target surfaces (Spitzer et al., 2007; Vuagnat et al., 2000). C3 was detected in the immunocomplex in the intestinal tissues of I/R-treated mice (I/R=105±14 Unit/mg.ml⁻¹, n=8, vs. Sham=45±19 Unit/mg.ml⁻¹, n=8, p<0.05) (Fig.5a). In contrast, no specific binding of factor P to the immunocomplex was detected (Fig.5b).

Because IgG natural antibodies are known to activate the alternative pathway of complement (Lutz, 2007), we investigated whether IgG was present in the immunocomplexes. Our results showed no specific presence of IgG in the ischemic Ag immunocomplex (I/R=77±15 Unit/mg.ml⁻¹, n=8 vs., Sham=47±13 Unit/mg.ml⁻¹, n=8, p=0.16) (Fig.5c).

The role of Factor B was further studied in vivo by using Factor B-deficient (FB^{-/-}) mice in the intestinal I/R model. Morphological analysis showed that there was no significant reduction of tissue injury in the FB^{-/-} mice compared to the WT littermate FB^{+/+} controls (pathology score of FB^{-/-} group=10±3.1, n=11; pathology score of WT littermate group=16±6.4, n=5; p=0.454) (Fig.6b). Immunohistochemical analysis further demonstrated that there were IgM and C3 depositions in the I/R treated intestinal tissues of both FB^{-/-} knockout mice and WT littermate controls (Fig.7).

Discussion

Our results show that nIgM-ischemic antigen complex formed under I/R conditions (Fig. 3a), and also indicate that early complement factors from the lectin and classical pathways, namely MBL and C1q, were present in these nIgM-ischemic antigen immunocomplexes (Fig.3b and 3c).

The detection of C1q in the nIgM-ischemic antigen complex in this study complicated the hypothesis of the initial mechanism of I/R injury induction. Earlier reports, including ours, have shown that C1q knockout mice were not protected from I/R injury in intestinal models (Hart et al., 2005; Zhang et al., 2006c), suggesting that the classical pathway of complement does not play a dominant role in I/R injury induction. Nevertheless, in our earlier report, we indeed noticed that C1q deposited and colocalized with IgM in the local tissues of I/R injured WT mice by immunohistochemical staining (Zhang et al., 2006c), although no further study was done at that time to identify if C1q was present in the immunocomplex. One possibility is that C1q joins the I/R immunocomplex after MBL has been recruited to the nIgM-ischemic antigen complex, thus acting as an auxiliary component in the amplification loop of downstream complement activation.

Our study shows that the early component of the alternative complement pathway, Factor B, was not detected in the ischemic antigen complex (Fig.3d). The ELISA assay for Bb fragment of Factor B in this study used an antibody from an established commercial source (Quidel, CA) which could detect Bb level as low as 0.018ug/ml. The ELISA system used in this study was designed to capture the ischemic Ag immunocomplex formed in vivo, which has enabled us to successfully detect complements from classical and lectin pathways, proving such an ELISA system worked as expected. Thus, Factor B is unlikely to be actively involved in the formation of the ischemic Ag immunocomplex (or if it might participate in the complex formation, the level was too low and far beyond the lower limit of detection).

Our study further examined the presence of early molecules in the alternative pathway. A recent view is that there are two models for alternative pathway: 1) the standard model by C3 hydrolysis, and 2) the properdin-directed model (Holt et al., 1990; Spitzer et al., 2007; Vuagnat et al., 2000). The first model also needs properdin (Factor P) to stabilize the fluid phase C3 convertase, C3(H₂O)Bb.

Our results showed that complement C3 was indeed present in the ischemic Ag complex, but properdin was not. This suggests that properdin is not part of the ischemic Ag complex. Our interpretation for C3 in the complex is that it is activated following the classical or lectin pathways because C3 is known to be a common factor of all three complement pathways. The possibility that the deposition of C3 in the ischemic Ag immunocomplex following the common pathway may activate the alternative complement pathway in a second phase can not be ruled out in this study.

Because IgG can potentially activate the alternative pathway through the formation of C3b2-IgG complexes (Banda et al., 2008; Boxx et al., 2009; Lutz and Jelezarova, 2006; Otten et al., 2009; Xiao et al., 2007), we also examined the presence of IgG in the ischemic Ag complex.

Our results showed that IgG was not detected in the complexes. Fleming et al have previously showed that natural IgG by itself does not initiate the I/R injury but could augment the natural IgM mediated injury (Fleming et al., 2002). The absence of IgG in the ischemic Ag complex in our current study confirms the previous view of IgG in I/R injury. Nevertheless, it is still possible that natural IgG may bind to other self targets exposed by the initial attack through the IgM mechanism (Fleming et al., 2004).

This study has provided in vivo evidence that knockout of Factor B neither significantly protected the animals from intestinal I/R injury (Fig.6a and 6b), nor blocked the nIgM-mediated complement activation (Fig.7). Factor B is the critical factor in the alternative pathway either initiated by C3, properdin, or IgG. Thus, this study clarifies previous conflicting reports (Hart et al., 2005; Stahl et al., 2003) and suggests that the alternative complement pathway does not play an important role in intestinal I/R injury induction.

The publication claiming that Factor D knockout mice were protected from intestinal I/R injury indeed generated controversy (Stahl et al., 2003), not only against findings by us and others (Fleming, 2006), but even the later result from the same group using C2/fb knockout mice (Hart et al., 2005). Careful examination of the study using Factor D knockout revealed some drawbacks in the data which weaken the conclusion of that paper. First, the histological protection of FD knockout mice was only demonstrated in 1 small area of the H&E staining section with less than 20 microvilli, and no overall pathology scores were given to estimate the degree of injury (the later C2/fb paper by the same group did include impression scores, probably recognizing this flaw in the earlier report). We typically examine 200-400 microvilli of a 4cm intestinal segment, and have developed a pathologic scoring system representing both the severity and total number of injured microvilli (Zhang et al., 2006a; Zhang et al., 2004). Second, the fluorescent staining of C3 in the FD knockout paper showed green fluorescent staining over all types of cells in the positive control duodenum, which appeared to be non-specific. We typically saw specific C3 deposition within the microvilli of jejunum as demonstrated in our current study (Fig.7). Fluorescence on the outer epithelium of the intestine particularly tends to be non-specific as food components often cause auto-fluorescent background. The choice of the duodenum for C3 staining in FD paper is also very questionable as the same group has been using the surgical protocol for jejunum ischemia and showed H&E analysis in the jejunum (Stahl et al., 2003). It is known that the duodenum does not give consistent pathology in the I/R model. Therefore, these drawbacks in the FD study severely weaken the conclusion claimed in the FD study. It is unlikely that different genetic backgrounds caused the controversy as they were using mice from C57BL/6J background in both the FD knockout study and C2/fb knockout experiments. Our FB deficient mice also have C57BL/6 backgrounds.

However, our results do not exclude the possibility that the alternative complement pathway might participate in a later phase of inflammatory response during intestinal I/R. For instance, a recent publication indicated that although intact Factor H, an inhibitor of the alternative pathway that competes with Factor B for C3b binding, was not able to protect against intestinal I/R injury, a fusion protein of Factor H and complement receptor 2 could reduce local injury and complement activation (Huang et al., 2008). Moreover, the complement alternative pathway may be involved in I/R injury in other tissues. For example, some studies find that in the kidney I/R injury the complement system is exclusively activated via the alternative pathway, and selective inhibition of this pathway could protect the kidneys from ischemic acute renal failure (Thurman and Holers, 2006; Thurman et al., 2003; Thurman et al., 2006). However, others found that the MBL pathway is also involved in tissue injury in a kidney I/R model (Moller-Kristensen et al., 2005). Thus, I/R injury may operate through multiple mechanisms and different results in current literatures may reflect the complexity regarding the models and strategies chosen for analysis.

In summary, our study confirms the important role of the complement lectin pathway in intestinal I/R injury induction, and suggests that the classical pathway may play an auxiliary role in the current model. Our study further indicates that the alternative pathway plays only a marginal role in intestinal I/R injury induction.

Acknowledgments

The authors would like to thank Drs. James Cottrell and Ira Kass for continued support, and Benjamin Barbash and Hyein Koh for valuable assistance. We also thank Dr. Ralph M. Steinman for helpful discussions and critical comments. This research was supported in part by SUNY-Downstate Dean's Award for Pilot Project (MZ) and NIH grant 1R21HL088527 (MZ).

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Figure 1.

Schematic diagram of the immunoassay for the analytical isolation of the nIgM-ischemic Ag complexes and the detection of the early complement factors in the immunocomplex. Plates were coated with rabbit anti-NMHC-II A and intestinal lysates from I/R-treated or sham control mice were added to the plates. To detect the nIgM in the immunocomplex, AP-labeled anti-IgM antibody was used. To detect bound complement factors deposited on the immunocomplex, specific antibodies for MBL, C1q, or Factor B were used instead of the anti-IgM antibody.

Figure 2.

Tissue injury following I/R. Local tissues from I/R-treated WT mice had significant injury compared with those of sham controls. *a*) Representative intestinal sections from sham-treated and I/R-treated WT mice were stained with H&E. Arrows indicate typical pathology features of microvilli injury. Original magnification= x200. *b*) Pathology scores were assigned based on the degree of microvilli injury as described in Methods. Results are mean $\pm$ standard error of the mean (SEM), and asterisks indicate statistical significance (Student's *t* test; $p < 0.01$).

Figure 3.

Isolation of nIgM-ischemic Ag immunocomplex and detection of initiating factors of three complement pathways. The nIgM-ischemic Ag complexes were captured in antibody-coated microtiter wells as described in Figure 1. *a*) Detection of nIgM in the captured immunocomplexes from intestinal tissues. *b*) Detection of MBL. *c*) Detection of C1q. *d*) Detection of Bb fragment of Factor B. Asterisks indicate statistical significance (Student's *t* test; * $p < 0.05$ ** $p < 0.01$). Error bars indicate SEM.

Figure 4.

Negative control experiments for the quantification of the IgM-ischemic Ag complex and detection of the initiating factors of the three complement pathways. The anti-NMHC-II antibody was replaced with rabbit antibody against rat IgG as the coating antibody on the 96-well plates. The rest of the experimental protocol followed the steps as described in Figure 3. Statistical analysis showed no significant differences between sham vs. I/R treated groups (Student's *t* test; $p>0.05$). Error bars indicate SEM.

Figure 5.

Detection of early complement factors of the alternative pathway and IgG natural antibody. The nIgM-ischemic Ag complexes were captured in antibody-coated microtiter wells as described in Figure 1. *a*) Detection of C3 in the captured immunocomplexes from intestinal tissues. *b*) Detection of Factor P. *c*) Detection of IgG. Asterisks indicate statistical significance (Student's *t* test; * $p < 0.05$). Error bars indicate SEM.

Figure 6.

Tissue injury in factor B KO mice. No significant reduction of tissue injury was seen in the FB^{-/-} mice compared to the WT littermate controls (FB^{+/+}). *a*) Representative intestinal sections from I/R-treated FB^{-/-} and FB^{+/+} mice were stained with H&E. Arrows indicate typical pathology features of microvilli injury (x200 magnification). *b*). Pathology scores and statistical significance were analyzed as described in Methods. Error bars indicate SEM.

Figure 7.

IgM and C3 in tissue following I/R. $FB^{-/-}$ mice were not protected from intestinal reperfusion injury. IgM and complement C3 were present within the microvilli of I/R-treated $FB^{+/+}$ and $FB^{-/-}$ mice. Sections *i*, *ii*, and *iii* were representative images from sham-treated $FB^{+/+}$ mice. Sections *iv*, *v*, and *vi* were representative images from sham-treated $FB^{-/-}$ mice. Sections *vii*, *viii*, and *ix* were representative images from I/R-treated $FB^{+/+}$ mice. Sections *x*, *xi*, and *xii* were representative images from I/R-treated $FB^{-/-}$ mice. Sections *i*, *iv*, *vii*, and *x* were stained with anti-IgM-biotin followed by streptavidin-Alexa-568 (red). Sections *ii*, *v*, *viii*, and *xi* were stained with anti-C3-FITC (green). Sections *iii*, *vi*, *ix*, and *xii* were merged images of IgM and C3 to demonstrate the colocalization (yellow). All sections were counterstained with DAPI (violet). Original magnification=x400.