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Complement regulates TLR4-mediated inflammatory responses during intestinal ischemia reperfusion[1]

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Running Title: TLR4 and Complement Regulation in Intestinal Damage

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ABSTRACT

Innate immune responses including TLR4 and complement activation are required for mesenteric ischemia/reperfusion (IR)-induced tissue damage. We examined the regulation of TLR4 and complement activation in a mouse model of intestinal IR. Intestinal IR induced C3 deposition in a TLR4 dependent manner. In addition, in wild-type but not TLR4 deficient mice, IR significantly increased C3 and Factor B (FB) mRNA expression within the intestine. To further examine the role of TLR4 and complement, we administered the complement inhibitor, CR2-Crry, to target local complement activation in wild-type C57Bl/10, and TLR4 deficient B10/ScN mice. TLR4 deficient mice sustained less damage and inflammation after IR than wild-type mice, but administration of CR2-Crry did not further reduce tissue damage. In contrast, CR2-Crry treatment of wild-type mice was accompanied by a reduction in complement activation and in C3 and FB transcription in response to IR. CR2-Crry also significantly decreased intestinal IL-6 and IL-12p40 production in both the wild-type and TLR4 deficient mice. These data indicate that TLR4 regulates extrahepatic complement production while complement regulates TLR4-mediated cytokine production during intestinal IR.

Key Words: TLR4, rodent, mucosa, complement, inflammation

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INTRODUCTION

Mesenteric thrombosis/embolism induces an ischemic event which may be followed by reperfusion of the blood flow (Collard et al., 1999; Eror et al., 1999; Kilgore et al., 1999). Although transient ischemia induces biological and chemical changes which leads to tissue damage (Zhang and Carroll, 2006), reperfusion magnifies intestinal damage (Austen et al., 1999). Reperfusion also generates numerous inflammatory mediators including complement activation products, cytokines and eicosanoids (reviewed in (Kilgore et al., 1999)). These mediators frequently lead to the inappropriate expression of adhesion molecules and neutrophil infiltration of local and remote tissues. The intense inflammatory response during mesenteric IR may lead to multiple organ failure, resulting in a mortality rate ranging from $60 - 80\%$ in humans (Clark and Coopersmith, 2007; Deitch, 2001; Leaphart and Tepas, 2007).

Complement is a significant component of the innate immune response and can be activated by multiple pathways to generate C3 opsonins, chemotactic peptides and the cytolytic terminal membrane attack complex on surface membranes. Natural inhibitors protect host cells and tissues from damage caused by excessive or inappropriate activation of the complement cascade. Natural human membrane-bound inhibitors include complement receptor 1 (CR1), decay accelerating factor (DAF; CD55) and membrane cofactor protein (Makrides, 1998; Makrides, 2000). These proteins differ somewhat in their mechanisms of action, but all three proteins inhibit all complement pathways at the C3 activation step (Makrides, 2000). In the mouse, complement receptor-related gene y (Crry) expresses an activity profile similar to that of CR1 in humans (Quigg et al., 1998). Administration of the recombinant soluble fusion proteins Crry-Ig (Rehrig et al., 2001) or CR2-Crry (Atkinson et al., 2005) attenuates intestinal IR-induced tissue

damage in wild-type mice. Crry-Ig systemically inhibits complement activation, whereas CR2-Crry inhibits complement activation locally with minimal systemic effect by targeting to C3 breakdown products deposited at sites of complement activation (Atkinson et al., 2005) However, it is unknown if CR2-Crry inhibits other components of the inflammatory response.

Reperfusion-induced tissue damage releases damage associated molecular patterns, which may be recognized by toll like receptors (TLR). Several reports suggest that TLR play a role in IR-induced organ damage and inflammation (reviewed in (Arumugam et al., 2009)). In multiple models of tissue damage, cellular debris and extracellular matrix degradation products including hyaluronic acid, fibronectin, fibrinogen and heparin sulfate induce "sterile inflammation" through the pattern recognition receptor, TLR4 (reviewed in (Mollen et al., 2006; Tsan and Gao, 2004)). We recently demonstrated that TLR4 expression is critical for Cox-2 induced prostaglandin E_2 (PGE_2) production during intestinal IR (Moses et al., 2009). In addition, stimulation of TLR4 on macrophages induces secretion of multiple cytokines including TNF, IL-1, IL-6 and IL-12 (Akira and Hemmi, 2003; Tsan and Gao, 2004). Expressed by both leukocytes and intestinal epithelial cells, TLR4 is critical to maintaining the intestinal epithelium's tolerance to commensal LPS (Fukata et al., 2006; Fukata et al., 2007; Fukata et al., 2005). Thus, multiple IR-induced inflammatory processes may be mediated by TLR4.

As two components of the innate immune response, TLR4 and the complement cascade have recently been shown to interact. The complement inhibitor DAF appears to bind LPS, and in conjunction with TLR4 and other proteins, make up the LPS receptor complex (El-Samalouti et al., 1999; Heine et al., 2001; Triantafilou et al., 2002). In the absence of DAF, C3aR and C5aR

activation up-regulates TLR4 mediated IL-1, IL-6, and TNFα production (Zhang et al., 2007).

In addition, in a model of *E.coli* infection, human granulocytes and monocytes respond

differentially to complement components or CD14, a component of the LPS receptor (Lappegard

et al., 2009). In contrast, C5a down-regulates IL-12 production in a TLR4 dependent manner

(Hawlisch et al., 2005; Zhang et al., 2007). Furthermore, complement regulates maturation and

recruitment of cells expressing TLR4 (Fang et al., 2009; Spirig et al., 2008). Treatment with

dextran sulfate, a complement inhibitor, prevented TLR4-mediated dendritic cell maturation,

while activation of both complement and TLR4 resulted in an infiltration of Th17 cells (Fang et

al., 2009; Spirig et al., 2008). Taken together, these data indicate that activation of either

complement or TLR4 directly affects the activation of the other innate immune components.

Since both TLR4 and complement play a role in IR-induced tissue damage, we examined the

cross-regulation between the two innate immune components during intestinal IR. Initial studies

demonstrated that TLR4 deficiency inhibits IR-induced complement production and activation as

well as intestinal damage. Importantly, administration of the complement inhibitor, CR2-Crry,

to wild-type or TLR4 deficient mice significantly reduced or eliminated complement activation

products and C3 and FB mRNA. However, CR2-Crry treatment reduced IR-induced intestinal

damage only in wild-type mice, not TLR4 deficient mice. Thus, a complex relationship exists

between TLR4 and complement.

MATERIALS AND METHODS

Mice: C57Bl/10 and B10/ScN mice were originally obtained from Jackson Laboratory and then bred and maintained in the Division of Biology at Kansas State University. Housed in a 12-hour light-to-dark, temperature-controlled room, mice were allowed food and water ad libitum. All mice were kept in specific pathogen free conditions (*Helicobacter* species, mouse hepatitis virus, minute virus of mice, mouse parvovirus, Sendai virus, murine norovirus, *Mycoplasma pulmonis*, Theiler's murine encephalomyelitis virus, and endo- and ecto-parasites). 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.

Ischemia/Reperfusion: Ischemia/reperfusion was performed on ketamine/xylazine anesthetized male 8-14 wk old mice (21-25 g) as described previously (Fleming et al., 2002). Briefly, a midline laparotamy was performed and the mice were allowed to equilibrate for 30 minutes, with body temperature maintained at 37°C using a water-circulating heat pad. Placing a warm, saline moistened piece of gauze over the abdominal cavity prevented peritoneal desiccation. The superior mesenteric artery was identified, isolated, and a small vascular clamp applied for 30 minutes and removed prior to 2 hours of reperfusion. Ischemia was noted by the intestine changing from a pink color to a gray color. Sham treated animals underwent the same procedure as the ischemic mice without occlusion of the superior mesenteric artery. After two hours of reperfusion, the mice were euthanized and sera, liver, spleen, and 2 cm sections of the small intestine, approximately 10 cm distal to the gastroduodenal junction, were collected for histological and other analyses. Mice treated with the targeted complement inhibitor, CR2-Crry, underwent the same procedures with i.v. administration of either 0.25mg CR2-Crry fusion

protein or PBS control 10 min after clamp removal. CR2-Crry was expressed and purified as described previously (Atkinson et al., 2005).

Histology and Injury Scoring: A 2 cm mid-jejunum tissue section was immediately fixed in 10% buffered formalin, embedded in paraffin, and 8μm sections were cut transversely and H+E stained. Mucosal injury was graded on a six-tiered scale adapted from Chiu et al. (Chiu et al., 1970) as described previously (Fleming et al., 2002). Briefly, the average damage score of the intestinal section (75-150 villi) was determined after grading each villus from 0-6. 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 Guggenheims' spaces 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; last, villi that displayed hemorrhage or were denuded were assigned a score of 6.

Ex vivo eicosanoid generation: Immediately after collection, a 2 cm intestinal section was minced, washed, resuspended in 37°C oxygenated Tyrode's buffer (Sigma-Aldrich), incubated at 37°C for 20 minutes and the supernatants collected. PGE_2 concentrations were determined using enzyme immunoassay kits (Cayman Chemical). IL-6, IL-12p40, and IL-12p70 concentrations were determined using a Milliplex MAP immunoassay kit (Millipore) and read on a Luminex 200 and analyzed with Milliplex Analyst software (Millipore). All PGE_2 and cytokine concentrations were standardized to the total tissue protein content determined by BCA assay (Pierce) adapted to microtiter plates.

Immunohistochemistry: After euthanasia, a 2 cm intestinal section was immediately snap frozen in O.C.T. freezing medium and 8 μm sections were placed on slides for immunohistochemistry. The C3 and C3d deposition on the tissue sections was detected by staining with either a rat-anti-mouse C3 or C3d monoclonal antibody (Hycult Biotechnologies) followed by a texas-red conjugated secondary antibody (Jackson Immunoresearch). Additional tissue sections were stained with appropriate isotype controls. Images were obtained using a Nikon eclipse 80i microscope equipped with a CoolSnap CF camera (Photometrics) and analyzed using Metavue software (Molecular Devices). C3 deposition was quantified using ImageJ software (NIH) by dividing the C3 antibody fluorescence area by the isotype control fluorescence area.

C5a ELISA: Sera C5a concentrations were determined by capture ELISA (BD Biosciences). Briefly, NUNC-immulon plates (Thermo Fisher) were coated overnight at 4°C with rat-anti-mouse C5a (BD Biosciences). After blocking with 10% FBS for 1 hr at room temperature, ice cold sera was added in duplicate prior to addition of the Biotin detection antibody (BD Biosciences). After addition of Avidin-HRP (BD Biosciences) and TMB (KPL), the ELISA was analyzed spectrophotometrically. All samples were compared to a standard curve of purified mouse C5a.

Real time PCR: Total RNA was isolated from the jejunum and liver using TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA integrity and genomic DNA contamination was assessed using a BioAnalyzer (Agilent) and quantity determined by

Nanodrop evaluation. Only samples with no DNA contamination and RIN values greater than 7.0 were used for cDNA synthesis. Total RNA (2 ug) was reverse transcribed using RevertAid first strand cDNA synthesis kit (Fermentas) using random primers. Quantitative real time PCR was performed in 25 ul volumes using a Mini-Opticon real time thermal cycler (Bio-Rad) and B-R SYBR Green Supermix for iQ reagent (Quanta Biosciences) using the following protocol: 3m at 95°C; 50 cycles of 10s at 95°C, 20s at Tm (Table 1), 10s at 72°C; melt curve starting at 65°C, increasing 0.5°C every 5s up to 95°C. After amplification, TLR4, C3 and FB Ct values were normalized to 18s rRNA and then ΔΔCt fold change relative to Sham-treated wild-type mice was determined as described previously (Zhao et al., 2008). Melt-curve analysis of the PCR products ensured amplification of a single product.

Statistical Analysis: Data are presented as mean ± 95% confidence interval (CI) and were compared by unpaired T test using the Mann-Whitney test (GraphPad Software).

RESULTS

TLR4 is required for IR-induced complement activation

Mesenteric IR induces intestinal damage and PGE_2 production in wild-type mice which are attenuated but not totally absent in TLR4 deficient mice (Moses et al., 2009; Rehrig et al., 2001). IR-induced damage in wild-type mice is mediated by complement activation; however, the role of complement in the attenuated damage of TLR4 deficient mice is unclear. To determine if the limited damage and PGE_2 production were complement mediated, we subjected wild-type (C57Bl/10) and TLR4 deficient (B10/ScN) mice to intestinal IR and compared C3 deposition on the intestinal tissue and serum C5a production. Similar to previous studies, TLR4 deficiency attenuated IR-induced intestinal damage (Fig.1A). After IR, intestinal tissues from wild-type mice (C57Bl/10) contained significant levels of C3 deposition when compared to Sham treated mice (Fig.1C-D). In contrast, mid-jejunal tissue sections from IR treated B10/ScN mice contained C3 deposits similar to the deposits observed in Sham treated mice and significantly fewer deposits compared to wild-type IR treated mice (Fig.1D). In addition, compared to sham treatment, IR significantly elevated the serum C5a concentrations in wild-type mice but not TLR4 deficient mice (Fig.1B).

Extrahepatic IR-induced Complement C3 and Factor B production requires TLR4 expression

It was possible that IR increased production of complement proteins, either in the liver or locally in the intestine (Andoh et al., 1998; Laufer et al., 2000; Wang et al., 1998). Using real time PCR, we found that hepatic transcription of complement components C3 and FB did not change after IR in either wild-type or TLR4 deficient mice (Fig.2A and data not shown). In contrast, IR induced a significant increase in the intestinal production of C3 (Fig.2B) and FB (Fig.2C) in

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wild-type, but not TLR4 deficient mice. In addition, a significant increase in intestinal TLR4

mRNA was observed in wild-type mice in response to IR (Fig. 2D). These results suggest that IR

induces TLR4 expression which is required for increased extrahepatic production of C3 and FB.

Attenuation of IR-induced intestinal inflammation in TLR4 deficient mice is further reduced by

CR2-Crry treatment.

Since mesenteric IR-induced damage and complement activation was attenuated, but not

eliminated in TLR4 deficient mice, we hypothesized that complement inhibition would synergize

with TLR4 deficiency. To test this hypothesis, we treated wild-type and TLR4 deficient mice

with CR2-Crry. Treatment with CR2-Crry eliminated IR-induced C3 mRNA production by both

strains of mice (Fig. 2B). In response to IR treatment, FB expression in wild-type but not TLR4

deficient mice decreased with the inhibitor as well (Fig. 2C). Surprisingly, CR2-Crry treatment

significantly reduced TLR4 mRNA expression suggesting that complement also regulates TLR4

expression.

Examination of IR-induced mucosal injury indicated that in wild-type mice, administration of

CR2-Crry during reperfusion attenuated mid-jejunal intestinal damage (Fig.3A-G). Importantly,

treatment with CR2-Crry did not further reduce IR-induced intestinal damage in B10/ScN mice

(Fig. 3A, D, F, G). Neither strain sustained intestinal damage after Sham treatment in the

presence or absence of complement inhibitor. Similar to previous studies, administration of

CR2-Crry significantly decreased C3d deposition in all mice (Fig.4) indicating that CR2-Crry

inhibited complement activation.

Since TLR4 mediates PGE_2 production during IR-induced intestinal damage, we examined the intestinal PGE_2 concentrations after administration of CR2-Crry to wild-type and B10/ScN mice. Administration of CR2-Crry significantly reduced the IR-induced PGE_2 production in wild-type mice despite the presence of TLR4 (Fig.5A). However, CR2-Crry treatment did not alter PGE_2 production in the absence of TLR4 (Fig.5A).

Complement activation inhibits TLR4 induced IL-12 secretion (Hawlisch et al., 2005; Zhang et al., 2007) and increases IL-6 production (Zhang et al., 2007). Therefore, we hypothesized that complement inhibition would alter the expression profile of these cytokines. We examined IL-6, IL-12p40 and IL-12p70 intestinal secretion in response to IR in both strains of mice, with and without CR2-Crry treatment. As expected, IR resulted in elevated intestinal production of all three cytokines in wild-type mice (Fig.5B-D). In contrast, levels of the pro-inflammatory cytokines, IL-6 and IL-12p70, remained the same in sham and IR treated TLR4 deficient B10/ScN mice (Fig.5B,C). IL-12p40 increased moderately in TLR4 deficient mice subjected to IR (Fig.5D). Inhibition of complement activation by CR2-Crry resulted in decreased production of IL-6 and both IL-12 family members in wild-type mice (Fig.5B-D). In B10/ScN mice, CR2-Crry significantly decreased the intestinal production of IL-6 and IL-12p40 (Fig.5B, D). Together these data suggest that TLR4 activation enhances complement mediated, IR-induced inflammation and damage.

DISCUSSION

As innate immune components, both TLR4 and the complement cascade affect mesenteric IR-induced tissue damage (Arumugam et al., 2009; Moses et al., 2009; Williams et al., 1999). IR-induced tissue damage is largely dependent on complement activation, an event that generates C3a and C5a chemotactic peptides (Fleming et al., 2003; Williams et al., 1999). In addition, activation of TLR4 results in the production of inflammatory chemokines, cytokines and eicosanoids during intestinal IR (Moses et al., 2009). In other models of tissue damage, TLR4 interacts with complement (Hawlisch and Kohl, 2006; Zhang et al., 2007). The present study extends these findings and shows a complex bidirectional interaction between TLR4 and complement activation in intestinal IR-induced damage and inflammation.

There are a limited number of studies examining the role of TLR4 activation during complement-mediated tissue damage with conflicting results. A recent study indicated that alcohol-induced liver injury was complement-dependent, but TLR4 independent (Roychowdhury et al., 2009). In an LPS-induced mouse model of abortion, complement activation significantly enhanced TLR4- mediated tissue damage (Yu et al., 2008). In a recent study, the neutrophilic response to *E.coli* depended on complement components rather than CD14 (Lappegard et al., 2009). In contrast, monocytic cytokine response varied depending on the cytokine (Lappegard et al., 2009). Similarly, in response to intestinal IR, complement inhibition significantly decreased intestinal injury in wild-type mice. However, complement inhibition of TLR4 deficient mice resulted in no further decrease in injury and therefore was not synergistic. Thus, damage is likely the sum of multiple inflammatory insults suggesting that each inflammatory component must be examined individually. This is illustrated by the fact that TLR4 deficiency inhibited

PGE$_2$ and IL-6 production, but complement inhibition of the TLR4 deficient mice further reduced only IL-6 levels not PGE$_2$ concentrations. In addition, in the absence of the complement inhibitor, DAF, complement enhanced IL-6 production in response to LPS (Zhang et al., 2007) suggesting that complement inhibitors regulate TLR4 cytokine production. Together with the previous studies reported above, these data suggest that the relationship of TLR4 and complement activation differ based on the type of tissue damage and the mediator analyzed.

Mesenteric IR induced significant complement activation in the intestine which was attenuated in the absence of TLR4, possibly due to the dependence of local C3 and FB transcription on TLR4. These results are similar to recent studies showing that TLR4 activation increases C3 and FB transcription in chondrocytes (Haglund et al., 2008), and that blocking TLR4 activation in Lewis lung carcinoma cells results in a 50% decrease of C3, C5 and C9 production (Stott and Korbelik, 2007). Together, these data suggest a model (Fig. 6) in which IR activates both TLR4 and complement. In this model, complement activation enhances TLR4 stimulation and TLR4 activation up-regulates complement protein synthesis (Fig. 6A), which in turn enhances local inflammation and injury. CR2-Crry inhibited complement activation and TLR4 transcription, as well as decreased cytokine, C3 and FB production (Fig. 6B). Similar to our findings with CR2-Crry, DAF expression decreased TLR4 activation (Zhang et al., 2007) and dextran sulfate, an inhibitor of complement, decreased TLR4-mediated dendritic cell maturation (Spirig et al., 2008). These data suggest a feedback loop between TLR4 activation and the production of complement components.

The complexity of TLR4 and complement activation is further illustrated by the production of IL-12 family members. As a pro-inflammatory cytokine, IL-12p70 is composed of two subunits, IL-12p40 and IL-12p35. The IL-12p40 subunit also complexes with either p19 to form IL-23, EBV-induced gene 3 to form IL-27, or another IL-12p40 subunit to form a homodimer (reviewed in (Cooper and Khader, 2007; Hunter, 2005)). In wild-type mice, IR significantly increased intestinal concentrations of IL-12p70 and IL-12p40, while TLR4 deficiency attenuated, but did not eliminate, production of these cytokines after IR. In addition, complement inhibition during reperfusion of the TLR4 deficient mice significantly reduced IL-12p40 concentrations to levels below that found in Sham treated mice. Similarly, C5 deficient macrophages produce significantly less IL-12p70 than TLR4 deficient macrophages after in vitro stimulation (Karp et al., 2000). In contrast, previous in vitro studies demonstrated that C5a treatment decreased LPS-induced macrophage IL-12 production (Hawlisch and Kohl, 2006).   These data suggest that the mechanism of IR-induced IL-12 production may be different than that of LPS-induced IL-12 production. It is also possible that PGE_2 regulates IR-induced IL-12 production, as both complement inhibition and TLR4 deficiency resulted in decreased PGE_2 production. Despite a slight increase in PGE2 concentrations after CR2-Crry treatment, there is no similar increase in IL-12p40 production. This may be due to normal variation within the intestine. Previous in vitro studies indicated that PGE_2 mediates IL-12p40 production by dendritic cells in the absence of IL-12p35 (Kalinski et al., 2001), while in vivo studies indicated that PGE_2 is TLR4 dependent (Moses et al., 2009). Thus, complement may regulate IL-12p40 directly and indirectly by altering TLR4-regulated, PGE_2 induction of IL-12p40.

In summary, we have demonstrated a complex relationship between TLR4 and the complement system during IR, and that this possible feedback loop is important in the subsequent inflammation and tissue injury. CR2-Crry, a targeted inhibitor that locally inhibits complement activation, further reduced IR-induced inflammation in the presence of TLR4. Thus, targeted therapeutics attenuate tissue damage without compromising the innate immune response.

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Figure 1. TLR4 deficiency attenuates intestinal injury and complement activation. A) Injury scores were determined from H+E stained intestinal sections scored as described in Materials and Methods. Each symbol is representative of an individual animal within the treatment group, with the line representing the average. B) Serum C5a activation was determined as stated in Materials and Methods. C-D) Frozen sections were stained for C3 deposition and quantified by Image J as described in Materials and Methods. Each bar represents a separate treatment group average ± 95% CI, with 6-12 samples per group. Photomicrographs for each treatment group (200X) are representative of 3 experiments of 5-7 photos per slide. The white bar represents 50μm. Mann-Whitney analysis determined p-values; *p <0.05; ** $p < 0.01$; ***$p < 0.001$.

Figure 2. CR2-Crry significantly alters TLR4, C3 and Factor B transcription within the intestine. Real time PCR ΔΔCt values were calculated for A) liver C3, B) intestinal C3, C) intestinal FB and D) intestinal TLR4 mRNA as described in Materials and Methods. Each bar represents a separate treatment group average ± CI, with 3-10 samples per group. Mann-Whitney analysis determined p-values; *p <0.05; ** $p < 0.01$; ***$p < 0.001$ ND = not detectable

Figure 3. CR2-Crry attenuates IR-induced intestinal injury in wild-type mice. Injury scores (A) were determined from H+E stained intestinal sections scored as described in Materials and Methods.. Representative H&E stained intestinal sections are shown (B-G). Each symbol

represents an individual animal within a separate treatment group, with the line representing the average. Mann-Whitney analysis determined p-values; *p <0.05; ** $p < 0.01$; ***$p < 0.001$.

Figure. 4. CR2-Crry decreases C3d deposits within the intestine. Mid-jejunal sections from Sham or IR treated wild-type (C57Bl/10) and TLR4 deficient (B10/ScN) mice were stained for C3d deposition. Photomicrographs represent 3 experiments of 5-7 photos per treatment group. Each bar represents 50μm.

Figure 5. Eicosanoid production in response to CR2-Crry. A) PGE_2, B) IL-6, C) IL-12p70, and D) IL-12p40 concentrations were determined as described in Materials and Methods. Each bar represents a separate treatment group average ± 95% CI, with 4-8 samples per group. Mann-Whitney analysis determined p-values; *p <0.05; ** $p < 0.01$; ***$p < 0.001$.

Figure 6. Proposed interactions of TLR4 and complement in response to intestinal IR. IR induces complement activation which enhances TLR4 activation (A). IR also activates TLR4 resulting in cytokine production and increased C3 and FB production (A). CR2-Crry inhibits both C3 and TLR4 activation (B).

Table 1

Table I: Primer Sequences

Gene	Sense (5'→ 3')	Antisense (5'→ 3')	Tm (°C)	Cycle #
18s rRNA	5'GGTTGATCCTGCCAGTAGC 3'	5'GCGACCAAAGGAACCATAAC 3'	58	50
C3	5'AAGCATCAACACACCCAACA 3'	5'CTTGAGCTCCATTCGTGACA 3'	58	50
Factor B	5'CCAGCATTTGGGTTTCAGTT 3'	5'CACACCTCCAGAGGAGAAGC 3'	58	50
TLR4	5'ATGGAAAAGCCTCGAATCCT 3'	5'CTCTCGGTCCATAGCAGAGC 3'	58	50

A. Injury Score — Sham, B10 IR, B10/ScN IR
B. C5a (ng/ml) — Sham, B10 IR, B10/ScN IR
C. C3 area/isotype — Sham, B10 IR, B10/ScN IR
D. Sham, B10 IR, B10/ScN IR

Figure 2

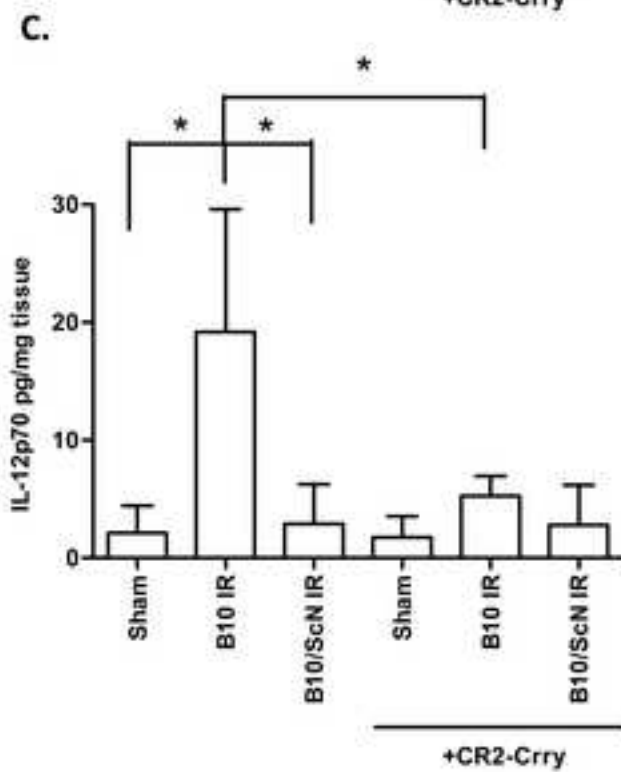**Figure 3**
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A.

E. Sham + CR2-Crry

B. Sham
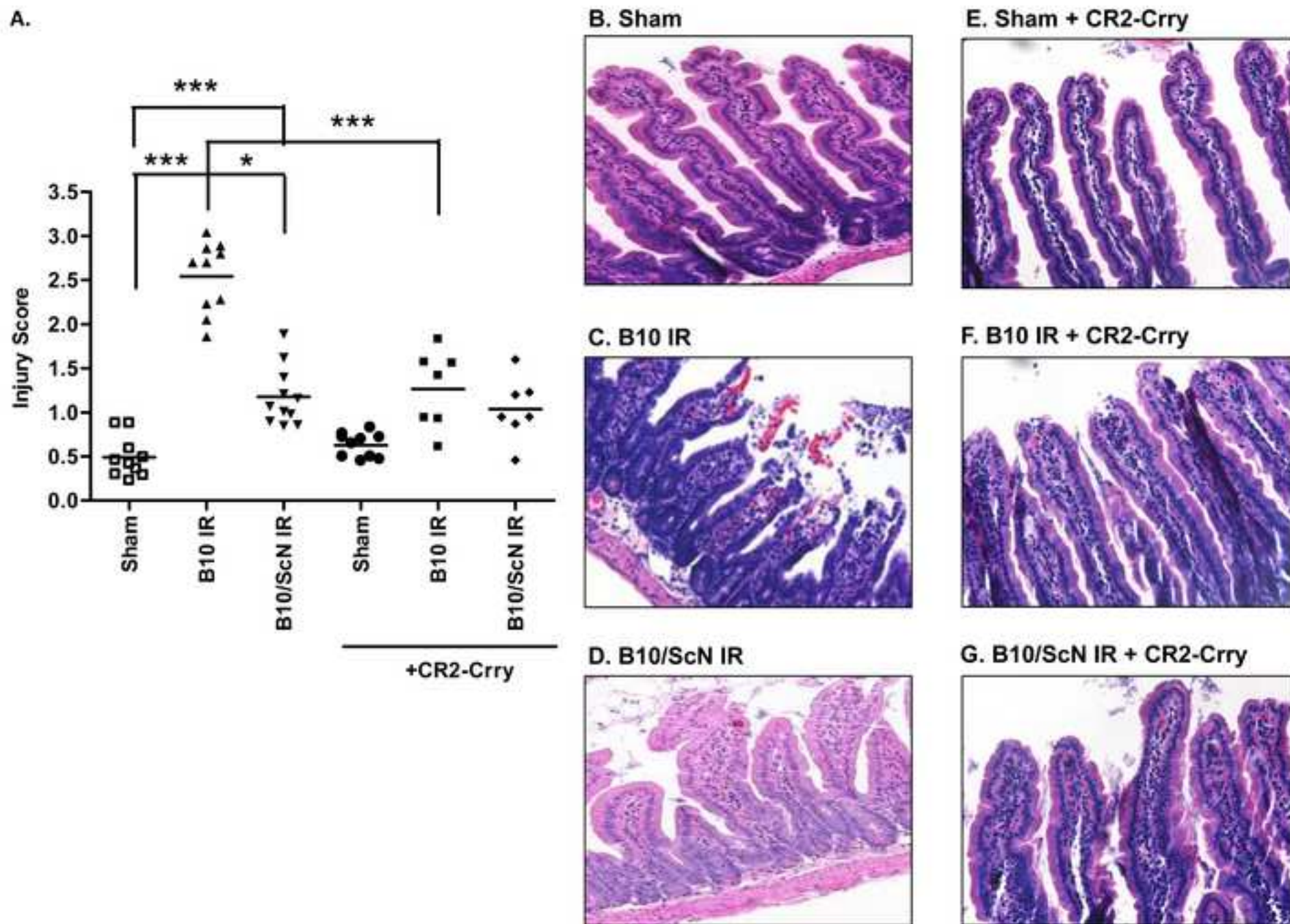
C. B10 IR

F. B10 IR + CR2-Crry

D. B10/ScN IR

G. B10/ScN IR + CR2-Crry

A. Sham
B. B10 IR
C. B10/ScN IR
D. Sham + CR2-Crry
E. B10 IR + CR2-Crry
F. B10/ScN IR + CR2-Crry
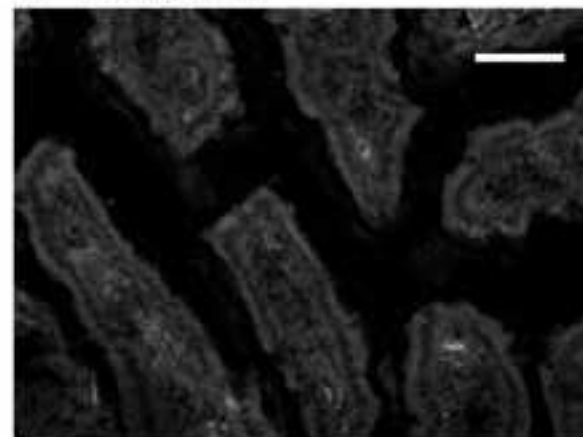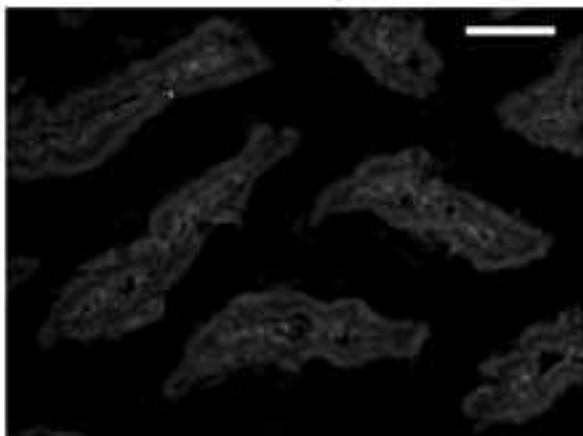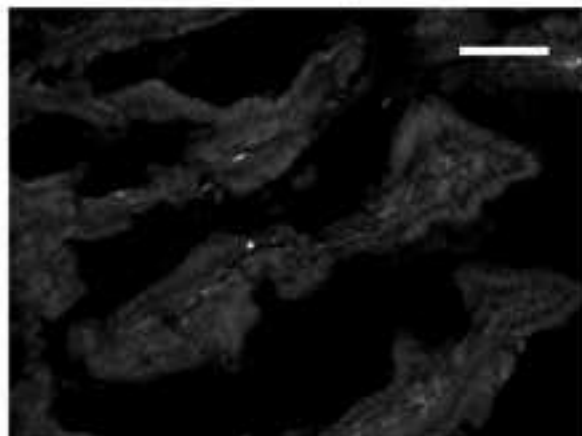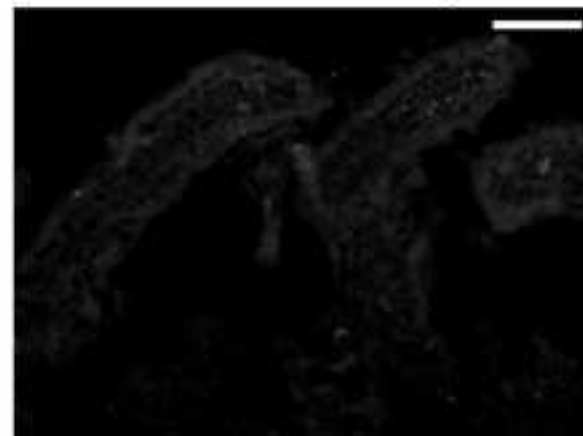
Figure 5
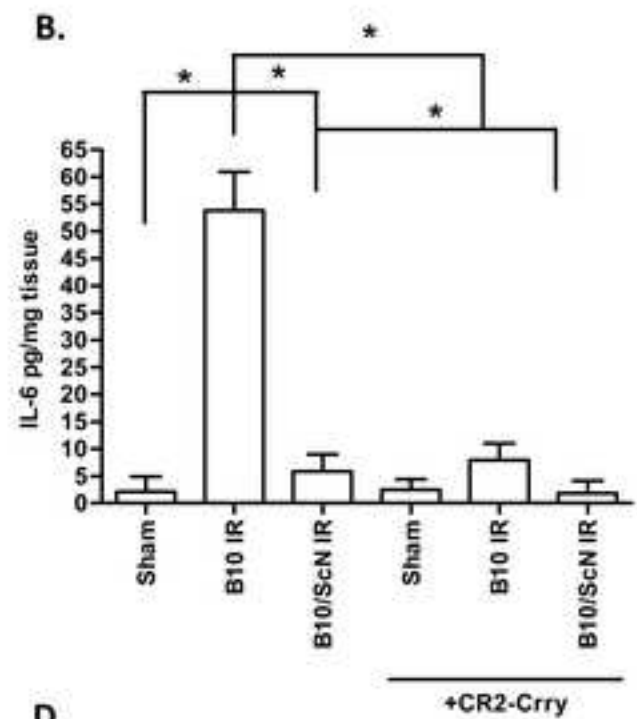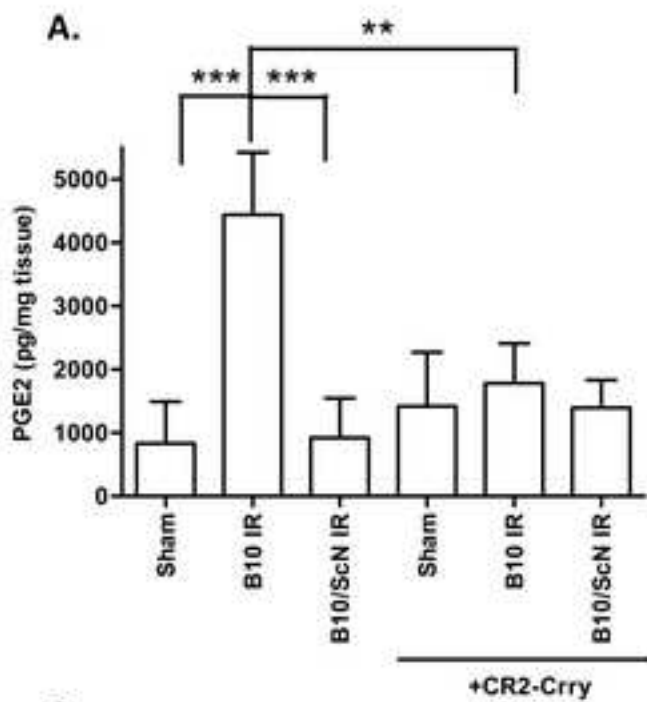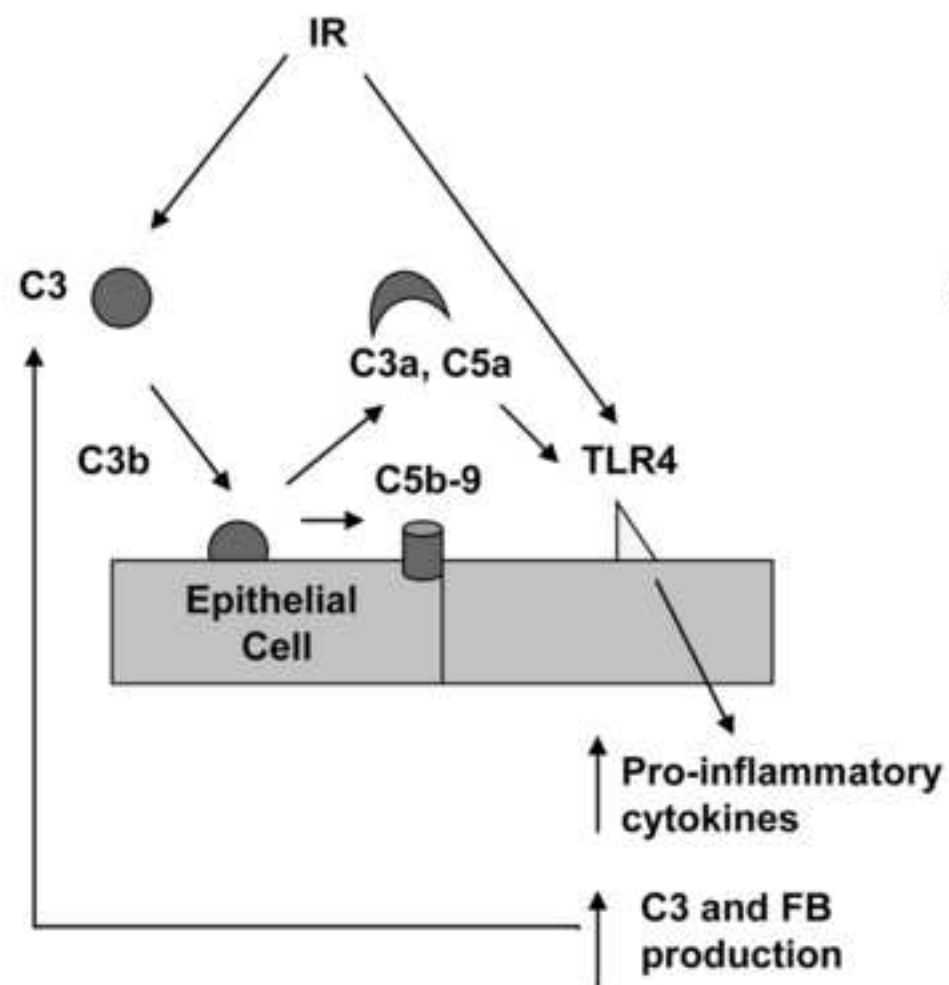**Click here to download high resolution image**
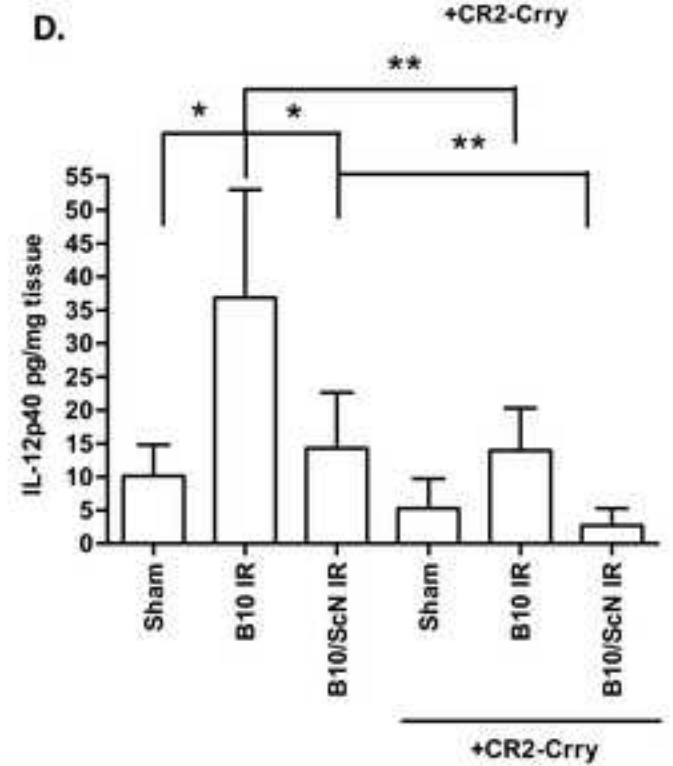
Figure 6
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A. Untreated cells

B. CR2-Crry treated cells