

## Original Article

# TLR9 is dispensable for intestinal ischemia/reperfusion-induced tissue damage

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**Abstract:** The mortality rate due to intestinal ischemia/reperfusion (IR) remains at 60-80%. As toll-like receptor (TLR) 4 has been shown to be critical for IR injury in several organs, including the intestine, and TLR9 is necessary for IR-induced damage of the liver, we investigated the hypothesis that TLR9 is involved in intestinal IR-induced damage. Wildtype (C57Bl/6) and *TLR9*<sup>-/-</sup> mice were subjected to intestinal IR or Sham treatment. Several markers of damage and inflammation were assessed, including mucosal injury, eicosanoid production, cytokine secretion and complement deposition. Although IR-induced injury was not altered, PGE<sub>2</sub> production was decreased in *TLR9*<sup>-/-</sup> mice. Attenuated PGE<sub>2</sub> production was not due to differences in percentage of lipids or COX-2 transcription. The data indicate that TLR9 is not required for IR-induced injury or inflammation of the intestine.

**Keywords:** Mouse, intestine, complement, TLRs, ischemia

## Introduction

Intestinal ischemia/reperfusion (IR), while not as common as myocardial or cerebral IR, is associated with a 60-80% mortality rate [1-3]. Mesenteric ischemia induces cellular damage which is exacerbated upon reperfusion resulting in tissue injury and often systemic inflammation and death. Reperfusion initiates an excessive inflammatory response involving complement activation and cytokine and eicosanoid production which recruit inflammatory neutrophils and macrophages (reviewed in [4]).

In response to hypoxia, cells express cryptic antigens similar to those expressed on the plasma membrane of apoptotic cells [5, 6]. During reperfusion, naturally occurring antibodies (Ab) recognize newly expressed antigens, triggering complement activation and the innate immune response. Identification of naturally occurring monoclonal Ab which result in damage when administered to IR-resistant, Ab-deficient *Rag-1*<sup>-/-</sup> mice have suggested the neoantigens include DNA, non-muscle myosin, and cardiolipin [6-11]. Additionally, oxygen deprivation is known to cause membrane lipid

alterations and results in the liberation of arachidonic acid and subsequent production of eicosanoids. We have previously shown that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is necessary but not alone sufficient for tissue damage [12, 13]. Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) is chemotactic for neutrophils, which are also involved in IR-induced damage [14].

Recent studies indicate a significant role for toll-like receptors (TLRs) in IR-induced tissue damage and inflammation [12, 15]. As pathogen-associated molecular pattern receptors, TLRs recognize distinct microbial components. Although TLRs recognize commensal microflora to maintain intestinal homeostasis [16], activation of these pathogen recognition receptors also induces inflammation following tissue damage [17]. As a regulator of complement activation, TLR4 is required for IR-induced tissue injury and inflammation in the intestine, kidney, brain, lung and heart [12, 18-23]. TLR9 has been shown to be critical in liver IR [24, 25]. Upon activation, most TLRs, including TLR4 and TLR9, signal through the common MyD88 pathway. Recently, we demonstrated that MyD88 is necessary for intestinal IR-induced tissue dam-

age [12] and that both TLR4 and MyD88 are critical for PGE<sub>2</sub> production and the inflammatory response.

TLR9 localizes to endosomal and lysosomal compartments, where it can recognize internalized ligand. In addition to bacterial CpG DNA, TLR9 recognizes self DNA, particularly histones and mitochondrial DNA [25, 26]. As IR-induced injury involves both cellular damage and death, self DNA is released into the extracellular environment for uptake by macrophages and other cells. Furthermore, anti-DNA and anti-histone monoclonal Ab restored intestinal IR-induced injury in *Rag-1*<sup>-/-</sup> mice [9]. Although TLR9 is a key component for IR-induced liver damage, its role in intestinal IR is not clear. It is possible that TLR9 regulates complement activation, PGE<sub>2</sub> production or other critical components in IR-induced injury. We hypothesized that TLR9 is critical to IR-mediated intestinal damage. We tested the hypothesis by subjecting C57Bl/6 and *TLR9*<sup>-/-</sup> mice to intestinal IR and examined several markers of intestinal tissue damage, including complement deposition, eicosanoid production and cytokine secretions, in both *TLR9*<sup>-/-</sup> and wildtype mice. Contrary to expectations, TLR9 appears to be dispensable in intestinal IR-induced tissue injury.

## Methods

### Mice

*TLR9*<sup>-/-</sup> mice were obtained from S. Akira (Osaka University, Osaka, Japan) and bred as homozygote deficient mice along with C57Bl/6 mice (wildtype control) (Jackson Laboratory, Bar Harbor, ME) in the Division of Biology at Kansas State University with free access to food and water. All mice were backcrossed to the C57Bl/6 background for at least 9 generations and maintained as specific pathogen free (*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). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and was approved by the Institutional Animal Care and Use Committee at Kansas State University.

### Ischemia/Reperfusion

Animals were subjected to IR using a protocol similar to that of previous studies [12]. Briefly, a laparotomy was performed on ketamine (16 mg/kg) and xylazine (80 mg/kg) anesthetized mice with buprenorphine (0.06 mg/kg) administered for pain. After a 30 min equilibration period, ischemia was induced by applying a small vascular clamp (Roboz Surgical Instruments, Gaithersburg, MD) to the isolated superior mesenteric artery. Blanching of the intestine and absence of pulsations distal to the clamp confirmed ischemia. Covering the bowel with warm normal saline moistened surgical gauze prevented desiccation. After 30 min of ischemia, the clamp was removed allowing 2 h mesenteric reperfusion, confirmed by color change of the bowel and the return of pulsatile flow to the superior mesenteric artery. Some experiments reconstituted *Rag-1*<sup>-/-</sup> mice by i.v. injection of 200 µg of Protein L purified Ab from *TLR9*<sup>-/-</sup> or wildtype (C57Bl/6) mice at the time of laparotomy. Sham treated animals underwent the same surgical intervention except for vessel occlusion. All procedures were performed with the animals breathing spontaneously and body temperature maintained at 37°C using a water-circulating heating pad. Additional ketamine and xylazine was administered as needed and immediately prior to sacrifice. After sacrifice, 2 cm sections of the small intestine 10 cm distal to the gastroduodenal junction were harvested for histologic evaluation, and eicosanoid determination.

### Histology and immunohistochemistry

Mid-jejunal specimens were promptly fixed in 10% buffered formalin phosphate prior to being embedded in paraffin, sectioned transversely (8 µm), and H & E stained. The mucosal injury score was graded on a six-tiered scale defined by Chiu et. al. [27]. 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. Photomicrographs were obtained from H & E stained slides using a 20X, 0.5 Plan Fluor objective on Nikon 80i microscope and images acquired at room temperature using a Nikon DS-5M camera with DS-L2 software (Nikon, Melville, NY).

An additional 2 cm intestinal section was immediately snap-frozen in O.C.T. freezing medium and 8  $\mu$ m sections were transversely cut and placed on slides for immunohistochemistry. Nonspecific antigen binding sites were blocked via treatment with a solution of 10% donkey sera in phosphate buffered saline (PBS) for 30 min. After washing in PBS, the tissues were incubated with primary Ab overnight at 4°C. The C3 deposition, IgM and F4/80 expression on the tissue sections was detected by staining with a purified rat-anti-mouse C3 (Hycult Biotechnologies, Plymouth Meeting, PA) or anti-IgM Ab (eBioscience, San Diego, CA) or anti-F4/80 Ab (eBioscience) followed by a Texas-red conjugated donkey-anti-rat IgG secondary Ab (Jackson ImmunoResearch, West Grove, PA). Each experiment contained serial sections stained with the appropriate isotype control Abs. All slides were mounted with ProLong Gold (Invitrogen, Grand Island, NY). A blinded observer obtained images at room temperature using a Nikon Eclipse 80i microscope equipped with a CoolSnap CF camera (Photometrics, Tucson, AZ) and analyzed using Metavue software (Molecular Devices, Sunnyvale, CA).

### *Eicosanoid and cytokine determination*

The *ex vivo* generation of eicosanoids in small intestine tissue was determined as described previously [28]. Briefly, fresh mid-jejunum sections were minced, washed and resuspended in 37°C oxygenated Tyrode's buffer (Sigma, St. Louis, MO). After incubating for 20 min at 37°C, supernatants were collected and supernatants and tissue were stored at -80°C until assayed. The concentration of LTB<sub>4</sub> and PGE<sub>2</sub> were determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). Cytokine analysis of the same intestinal supernatants was determined using a Milliplex MAP immunoassay kit (Millipore, Billerica, MA) and read on a Milliplex Analyzer (Millipore, Billerica, MA). The tissue protein content was determined using the bicinchoninic acid assay (Pierce, Rockford,

IL) adapted for use with microtiter plates. Eicosanoid and cytokine production was expressed per mg protein per 20 min.

### *Lipid extraction*

Lipids were extracted as described previously [13]. Briefly, after Sham or IR treatment, intestinal sections (jejunum) were snap frozen in liquid nitrogen. Samples were ground into a fine powder using a liquid nitrogen-cooled stainless steel mortar and pestle. Samples were transferred to glass tubes that had been washed with a cation/phosphate-free liquid detergent (Contrex, Decon Labs, King of Prussia, PA). One ml chloroform and 2 ml methanol were added and tubes shaken vigorously. An additional 1 ml chloroform and 1 ml distilled water were added and tubes shaken vigorously again. Tubes were centrifuged at 4000 rpm for 5 min at 4°C. The organic layer was transferred to a clean glass tube and 1 ml chloroform added to the aqueous phase. Twice more, the tubes were shaken, centrifuged, and organic layer removed. Distilled water (0.5 ml) was added to the combined organic layers for each sample; the tubes were shaken and centrifuged once more. The organic layer was submitted to the Kansas Lipidomics Research Center for mass spectrometry analysis.

### *Mass spectrometry*

Mass spectrometry analysis was carried out similar to that described previously [13]. An automated electrospray triple quadrupole mass spectrometry (ESI-MS/MS) approach was used and data acquisition and analysis carried out at the Kansas Lipidomics Research Center as described previously with modifications [29, 30]. Solvent was evaporated from the extracts and each was dissolved in 1 ml of chloroform. Precise amounts of internal standards, obtained and quantified as previously described [31], were added to each sample to be analyzed. The sample and internal standard mixture were combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.2 ml. These unfractionated lipid extracts were introduced by continuous infusion into the ESI source of a triple quadrupole MS (API 4000, Applied Biosystems, Foster City, CA) using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland)

fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30  $\mu$ l/min.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: phosphatidylcholine (PC), sphingomyelin (SM), and lysoPC,  $[M + H]^+$  ions in positive ion mode with Precursor of 184.1 (Pre 184.1); Phosphatidylethanolamine (PE) and lysoPE,  $[M + H]^+$  ions in positive ion mode with Neutral Loss of 141.0 (NL 141.0); phosphatidylinositol (PI),  $[M + NH_4]^+$  in positive ion mode with NL 277.0; phosphatidylserine (PS),  $[M + H]^+$  in positive ion mode with NL 185.0; phosphatidic acid (PA),  $[M + NH_4]^+$  in positive ion mode with NL 115.0; phosphatidylglycerol (PG),  $[M + NH_4]^+$  in positive ion mode with NL 189.0; lysoPG,  $[M - H]^-$  in negative mode with Pre 152.9; and free fatty acids (i.e., free arachidonic acid, (AA)),  $[M - H]^-$  in negative mode with single stage MS analysis. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode.

The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software (Applied Biosystems, Carlsbad, CA). The lipids in each class were quantified in comparison to the two internal standards of that class. The first and typically every 11<sup>th</sup> set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the "internal standards only" spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. An "internal standards only" sample was included for each set of 10 samples. Finally, the data were expressed as percent of the signal (intensity) of total lipid analyzed after the described normalization to internal standards. Each class of lipid was also normalized to intestinal proteins and expressed as nmol lipid class per mg protein.

#### Real-time PCR

Total RNA was isolated from the jejunum, liver and spleen using TRIzol reagent (Invitrogen, Grand Island, NY) according to manufacturer's instructions. RNA integrity and genomic DNA contamination was assessed using a BioAnalyzer (Agilent, Santa Clara, CA) and the quantity was determined by Nanodrop (Nanodrop 1000 spectrophotometer) evaluation. Only samples with no measurable DNA contamination and RIN values greater than 7.0 were used for cDNA synthesis. Total RNA (2  $\mu$ g) was reverse transcribed using RevertAid first strand cDNA synthesis kit (Fermentas, Glen Burnie, MD) using random primers. Quantitative real-time PCR was performed in 25  $\mu$ l volumes using a Mini-Opticon real time thermal cycler (Bio-Rad, Hercules, CA) and B-R SYBR Green Supermix for iQ reagent (Quanta Biosciences, Gaithersburg, MD) using the following protocol: 3 min at 95°C; 50 cycles of 10 sec at 95°C, 20 sec at  $T_m$ , 10 sec at 72°C; melt curve starting at 65°C, increasing 0.5°C every 5 sec up to 95°C. Primer sequences for Cox-2 were forward: 5'ATCCTGCCAGCTCCACCG and reverse: 5'TGGTCAAATCCTGTGCTCATACAT with  $T_m$  54°C. After amplification, gene of interest Ct values were normalized to 18s rRNA (forward: 5'GGTTGATCCTGCCAGTAGC and reverse: 5'GCGACCAAAGGAACCATAAC with  $T_m$  58°C) and then  $\Delta\Delta C_t$  fold change relative to Sham-treated wildtype mice was determined as described previously [32]. Melt-curve analysis of the PCR products ensured amplification of a single product.

#### Statistical analysis

Data are presented as mean  $\pm$  SEM and were compared by one-way analysis of variance with *post hoc* analysis using Newman-Kuels test (Graph Pad/InStat Software Inc., Philadelphia, PA) unless otherwise specified. The difference between groups was considered significant when  $p < 0.05$ .

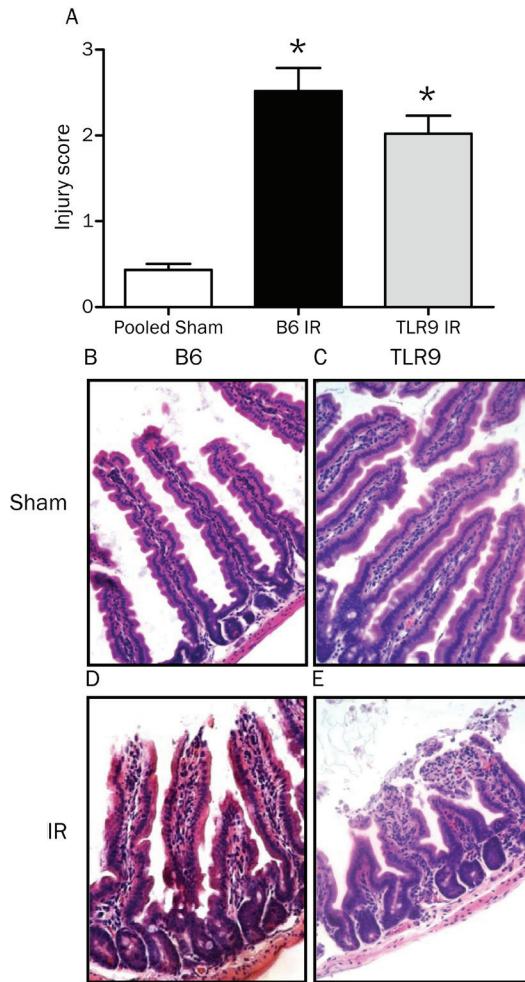
#### Results

##### *TLR9 deficiency does not protect from intestinal ischemia/reperfusion-induced injury*

Previous studies indicated that MRL/lpr autoimmune mice sustain greater tissue damage than wildtype mice following intestinal IR [33].



## TLR9 is not required for gut IR injury



**Figure 1.** *TLR9*<sup>-/-</sup> mice sustain intestinal injury following ischemia/reperfusion. Wildtype and *TLR9*<sup>-/-</sup> mice were subjected to Sham or intestinal IR treatment. Intestinal sections were collected and formalin fixed following 30 min ischemia and 120 min reperfusion. A: intestinal injury scores as determined by H&E stained intestinal sections and described in *Methods*; B-E: representative images of H&E stained intestinal sections; each bar represents 10-11 animals; \* indicates  $p < 0.05$  compared to Sham treatment.

In addition, administration of anti-DNA or anti-histone Ab (also found in systemic lupus erythematosus) resulted in intestinal injury in Ab-deficient *Rag-1*<sup>-/-</sup> mice after IR treatment [33]. As TLR9 recognizes CpG DNA, we hypothesized that intestinal IR-induced damage would be attenuated in *TLR9*<sup>-/-</sup> mice. We tested the hypothesis by subjecting *TLR9*<sup>-/-</sup> mice to intestinal Sham or IR treatment. As indicated in **Figure 1**, Sham treatment did not result in injury in either strain of mice with injury scores of 0.35

$\pm 0.07$  in wildtype and  $0.64 \pm 0.10$  in *TLR9*<sup>-/-</sup> mice (**Figure 1A-C**). As expected, after IR, wildtype C57Bl/6 mice sustained significant tissue injury (**Figure 1A and 1D**). To our surprise, the injury sustained by the *TLR9*<sup>-/-</sup> mice ( $2.02 \pm 0.21$ ) was not significantly different from wildtype ( $2.52 \pm 0.27$ ) (**Figure 1A and 1E**), suggesting that TLR9 is not required for intestinal IR-induced injury.

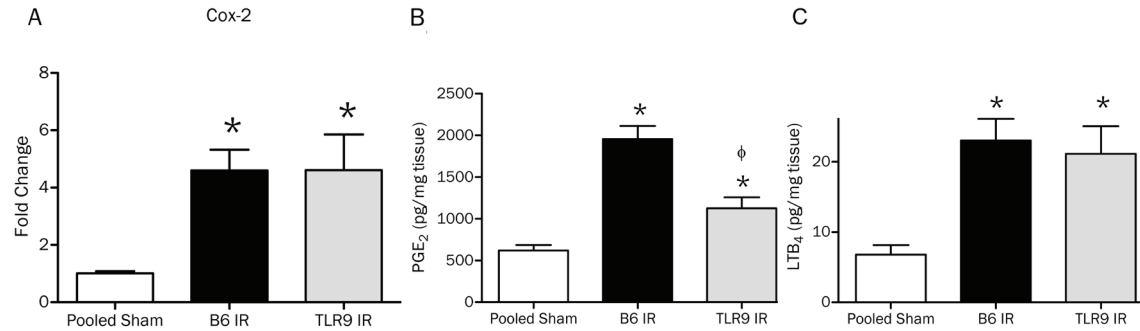
### *TLR9 deficiency attenuates prostaglandin production*

Previous studies indicated that Cox-2-mediated PGE<sub>2</sub> production is required for IR-induced tissue damage and that the signal is transduced via MyD88 [12, 15]. As TLR9 also signals through MyD88, we examined the intestinal eicosanoid production in response to IR in the *TLR9*<sup>-/-</sup> mice. Similar to intestinal damage, Cox-2 transcript was elevated in response to IR in wildtype and *TLR9*<sup>-/-</sup> mice (**Figure 2A**). Although IR resulted in an increase of PGE<sub>2</sub> production in the *TLR9*<sup>-/-</sup> mice, it was attenuated in comparison to wildtype PGE<sub>2</sub> levels (**Figure 2B**). To determine if the decreased PGE<sub>2</sub> levels were specific for PGE<sub>2</sub> or common to all eicosanoids, we examined the secretion of LTB<sub>4</sub> which recruits neutrophils. Neither the production of the chemotactic eicosanoid LTB<sub>4</sub> nor peroxidase secretion from the intestine was significantly different between the strains (**Figure 2C** and data not shown). Thus, it appears that neutrophils may be recruited normally but PGE<sub>2</sub> production is decreased.

### *TLR9 deficiency does not alter cellular lipid composition or metabolism following IR*

As eicosanoids are derived from the fatty acid arachidonic acid, we examined the lipid profiles of both wildtype and *TLR9*<sup>-/-</sup> mice by mass spectrometry. The relative intestinal lipid composition between Sham treated wildtype and *TLR9*<sup>-/-</sup> mice was not different (**Table 1**). However, the relative amounts of several classes of phospholipids did change with IR treatment (**Table 1**). The relative amount of PC decreased from  $25.07 \pm 1.88$  to  $13.41 \pm 1.66$  after IR treatment in the wildtype mice and from  $25.98 \pm 4.16$  to  $14.05 \pm 1.83$  in the *TLR9*<sup>-/-</sup> mice. PE similarly decreased in both strains of mice following IR treatment (wildtype:  $14.15 \pm 1.35$  to  $7.81 \pm 1.56$ ; *TLR9*<sup>-/-</sup>:  $15.31 \pm 3.44$  to  $7.47 \pm 1.28$ ). In contrast, IR treatment resulted in an

## TLR9 is not required for gut IR injury



**Figure 2.** The absence of TLR9 attenuates intestinal PGE<sub>2</sub> production but not LTB<sub>4</sub> production. Wildtype and *TLR9*<sup>-/-</sup> mice were subjected to Sham or intestinal IR treatment. Intestinal sections were collected following 30 min ischemia and 120 min reperfusion. A: RNA was extracted from snap frozen intestinal sections and fold change in Cox-2 transcript determined by real time PCR using  $\Delta\Delta Ct$  values; B-C: Intestinal sections were minced in oxygenated Tyrode's buffer and incubated at 37 °C for 20 min. Supernatants were collected for PGE<sub>2</sub> (B) and LTB<sub>4</sub> (C) quantitation by EIA; values are expressed as pg eicosanoid secreted per mg protein per 20 min; each bar represents 5-6 animals (A) or 8-11 animals (B-C); \* indicates  $p < 0.05$  compared to Sham treatment and Φ indicates  $p < 0.05$  compared to wildtype IR.

**Table 1.** Lipid composition of intestinal sections from mice subjected to Sham and IR treatment

Lipid class	C57Bl/6 Sham% of total	C57Bl/6 IR% of total	<i>TLR9</i> <sup>-/-</sup> Sham% of total	<i>TLR9</i> <sup>-/-</sup> IR% of total
LysoPC	1.10 ± 0.29	1.03 ± 0.21	0.58 ± 0.09	1.43 ± 0.22*
PC	25.07 ± 1.88	13.41 ± 1.66*	25.98 ± 4.16	14.05 ± 1.83*
SM + DSM	4.52 ± 0.51	2.67 ± 0.37	3.53 ± 0.23	2.30 ± 0.26*
ePC	1.42 ± 0.12	0.92 ± 0.13	1.36 ± 0.20	0.87 ± 0.12
LysoPE	0.19 ± 0.03	0.14 ± 0.01*	0.16 ± 0.04	0.17 ± 0.03
PE	14.15 ± 1.35	7.81 ± 1.56*	15.31 ± 3.44	7.47 ± 1.28*
PE-cer	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
ePE	3.24 ± 0.43	2.03 ± 0.45	2.94 ± 0.65	1.60 ± 0.19*
PI	2.09 ± 0.17	1.55 ± 0.33*	2.23 ± 0.16	1.19 ± 0.18*
PS	1.63 ± 0.34	1.25 ± 0.36	2.53 ± 1.08	0.99 ± 0.31
ePS	0.04 ± 0.00	0.02 ± 0.01	0.05 ± 0.02	0.02 ± 0.01
PA	0.28 ± 0.06	0.11 ± 0.02*	0.12 ± 0.02	0.05 ± 0.02*
PG	0.45 ± 0.09	0.32 ± 0.04	0.24 ± 0.07	0.39 ± 0.07
Free AA	2.67 ± 0.34	7.62 ± 0.89*	2.59 ± 0.53	7.49 ± 0.88*

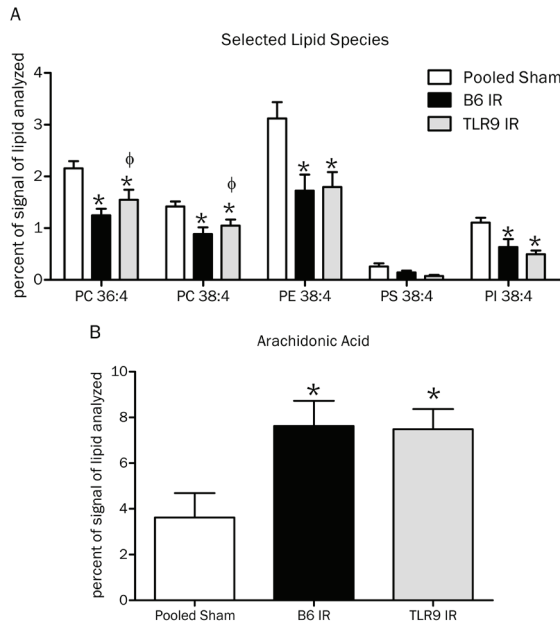
Data are presented as percent of mass spectral signal after normalization of signal to internal standards of each lipid class. Mean and SEM are reported. Lipids were extracted from intestinal sections of Sham and IR treated wildtype and *TLR9*<sup>-/-</sup> mice and subjected to analysis by mass spectrometry. Each treatment group includes 3-6 animals. \* indicates a significant difference ( $p < 0.05$ ) from respective Sham by Student's t-test. SM + DSM = sphingomyelin + dihydrosphingomyelin; ePC = ether-linked phosphatidylcholine; PE-cer = ceramide phosphatidylethanolamine; ePE = ether-linked phosphatidylethanolamine; ePS = ether-linked phosphatidylserine.

increase in the relative amount of free arachidonic acid in the intestine. Free arachidonic acid increased from  $2.67 \pm 0.34$  to  $7.62 \pm 0.89$  in the wildtype mice and from  $2.59 \pm 0.53$  to  $7.49 \pm 0.88$  in the *TLR9*<sup>-/-</sup> mice. We previously identified specific lipid species, that upon degradation, would likely result in release of free arachidonic acid [13]. The changes in these specific lipid species as a result of IR were similar between wildtype and *TLR9*<sup>-/-</sup> mice, except for the PC species where a greater decrease from Sham levels occurred in the wildtype mice

subjected to IR than the *TLR9*<sup>-/-</sup> mice subjected to IR (**Figure 3A**). The relative amount of free arachidonic acid released as a result of IR-induced injury was comparable between strains (**Figure 3B**).

*TLR9*<sup>-/-</sup> mice produce an antibody repertoire conducive to damage

Previous studies indicated that the appropriate Ab repertoire is required for recognition of IR-induced neo-antigens and complement acti-



**Figure 3.** Arachidonic acid release is TLR9 independent. Extracted intestinal lipids from Sham and IR treated wildtype and *TLR9*<sup>-/-</sup> mice were quantitated by ESI-MS/MS. A: percent of signal of lipids analyzed for selected phospholipids as determined by mass spectrometry; B: percent of signal of lipids analyzed for free arachidonic acid as determined by mass spectrometry; each bar represents 5-8 animals; \* indicates  $p < 0.05$  compared to Sham treatment.

vation [7, 34, 35]. In addition, Ab appeared to be required for IR-induced  $\text{PGE}_2$  production [13]. To verify that *TLR9*<sup>-/-</sup> mice have the proper Ab repertoire, we injected Ab deficient, IR-resistant, *Rag-1*<sup>-/-</sup> mice with purified Ab obtained from wildtype or *TLR9*<sup>-/-</sup> mice and subjected the mice to IR. As indicated in **Figure 4**, after IR, addition of Ab purified from wildtype mice induced significant intestinal damage and eicosanoid production in *Rag-1*<sup>-/-</sup> mice. Similarly, *Rag-1*<sup>-/-</sup> mice administered Ab from *TLR9*<sup>-/-</sup> mice sustained significant intestinal damage in response to IR (**Figure 4A**). In addition, eicosanoid production was at wildtype levels in *TLR9*<sup>-/-</sup> Ab reconstituted *Rag-1*<sup>-/-</sup> mice after IR treatment (**Figure 4B** and **4C**). Thus, the Ab repertoire of *TLR9*<sup>-/-</sup> mice is sufficient to induce intestinal damage,  $\text{PGE}_2$  production and inflammation in response to IR.

#### Complement deposition and inflammatory cytokine production are TLR9 independent

Similar to eicosanoid production and previous studies with *TLR4*<sup>-/-</sup> or *MyD88*<sup>-/-</sup> mice [15], intes-

tinal production of IL-6, IL-12p40 and TNF $\alpha$  increased in response to IR in both wildtype and *TLR9*<sup>-/-</sup> mice (**Figure 5**). Similar to myocardial ischemia, intestinal IR induced significant KC and MCP-1 (CCL2) production in wildtype mice that was significantly reduced in *TLR9*<sup>-/-</sup> mice (**Figure 5** and data not shown). These findings are in contrast with previous reports of IR in the liver, where IL-6 and TNF $\alpha$  levels were significantly lowered in *TLR9*<sup>-/-</sup> mice [24].

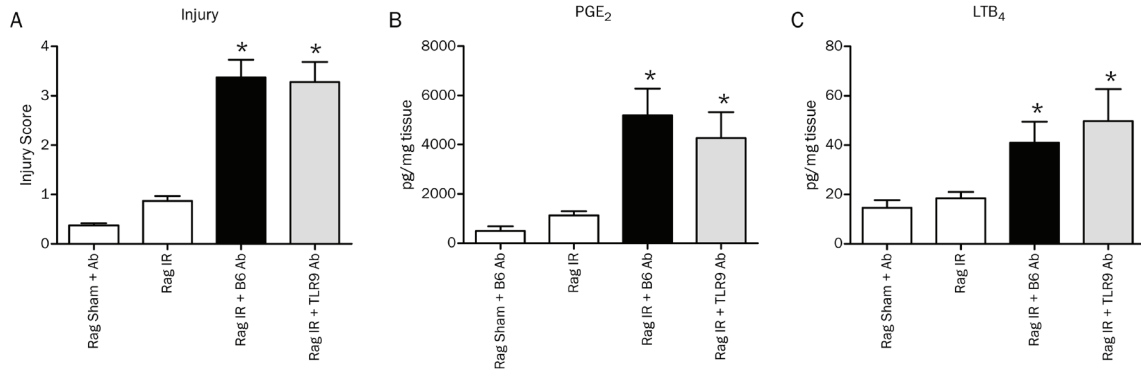
Because Ab activation of complement C3 is required for IR-induced intestinal damage, C3 and IgM deposition within the intestinal villi were determined by immunohistochemistry. As indicated in **Figure 6**, neither C3 nor IgM were deposited in response to Sham treatment. However, significant deposits of both IgM and C3 were present in wildtype and *TLR9*<sup>-/-</sup> mice after mesenteric IR. Furthermore, macrophages were identified in both wildtype and *TLR9*<sup>-/-</sup> IR-treated tissues, as detected by F4/80 staining (**Figure 6**). Both the immunohistochemistry staining and cytokine data support the conclusion that intestinal macrophages are not affected by the presence or absence of TLR9 in the context of IR.

#### Discussion

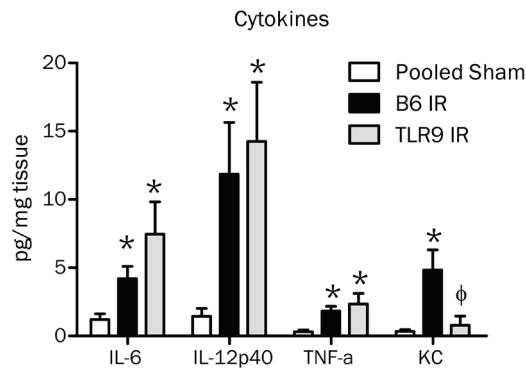
This is the first study, to our knowledge, to examine the role of TLR9 in intestinal IR-induced injury. As TLR9 is critical to IR-induced liver damage, we hypothesized that intestinal IR would also require TLR9. Surprisingly, *TLR9*<sup>-/-</sup> mice sustained significant tissue damage and inflammation following intestinal IR. Mucosal injury scores and the production of eicosanoids and cytokines were very similar between *TLR9*<sup>-/-</sup> and wildtype mice. In addition, it was verified that *TLR9*<sup>-/-</sup> mice produce an Ab repertoire conducive to injury. The intestinal lipids and release of arachidonic acid as well as complement deposition mimic that observed for wildtype mice. These data support the conclusion that TLR9 is not required for intestinal tissue damage or inflammation following IR.

TLRs, while originally identified as pathogen-associated molecular pattern receptors, recognize various self ligands. As IR-induced tissue injury results in cellular damage and apoptosis, several self ligands are released and available for TLR recognition. TLR4 has been shown to be involved in mediating IR-induced injury in several organs. We have previously shown TLR4 to

## TLR9 is not required for gut IR injury



**Figure 4.** Antibodies produced by *TLR9*<sup>-/-</sup> mice are sufficient to induce intestinal injury following IR. *Rag-1*<sup>-/-</sup> mice were administered antibodies from wildtype or *TLR9*<sup>-/-</sup> mice prior to Sham or IR treatment. A: Intestinal injury scores were determined from H&E stained intestinal sections as described in *Methods*; B-C: Intestinal sections were minced in oxygenated Tyrode's buffer and incubated at 37 °C for 20 min. Supernatants were collected and assayed for PGE<sub>2</sub> (B) and LTB<sub>4</sub> (C) by EIA. Values are expressed as pg eicosanoid secreted per mg protein per 20 min. Each bar represents 4-7 animals; \* indicates *p* < 0.05 compared to Sham treatment.



**Figure 5.** TLR9 deficiency does not affect cytokine secretion. Wildtype and *TLR9*<sup>-/-</sup> mice were subjected to Sham or IR treatment. Intestinal sections were minced in oxygenated Tyrode's buffer and incubated at 37 °C for 20 min. Supernatants were collected and assayed for cytokine secretion. Values are expressed as pg cytokine secreted per mg protein per 20 min. Each bar represents 4-8 animals. \* indicates *p* < 0.05 compared to Sham treatment.

be involved in intestinal IR, as TLR4 mutant mice had attenuated injury and eicosanoid and cytokine secretion [12, 36]. Furthermore, TLR4 mutant mice were protected from liver injury in a model of liver IR [37]. Additionally, TLR2 has been implicated in IR-induced damage to the kidney [38].

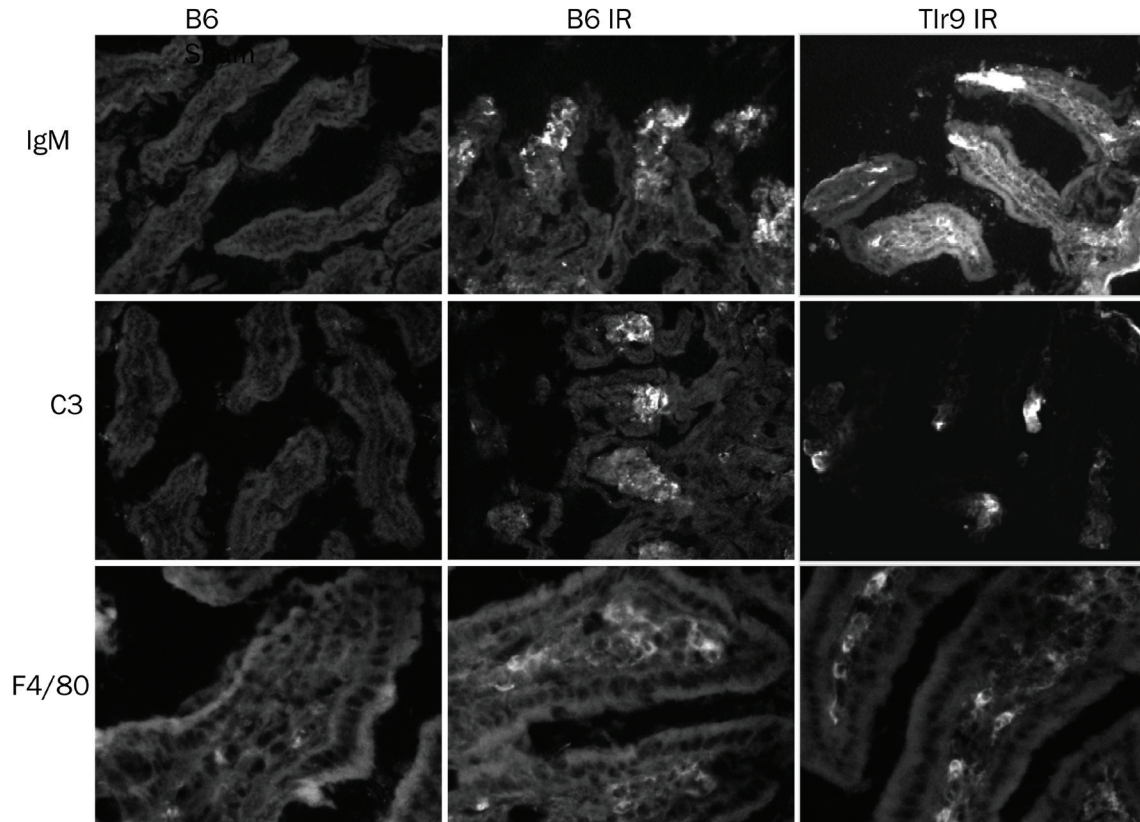
Limited data exists regarding the role of TLR9 in sterile inflammation and injury models. However, recent studies have demonstrated involvement of TLR9 in the pathogenesis of tissue injury and inflammation of the liver in both

an IR model [25] and a hemorrhagic-shock and trauma model [39]. In addition, inhibitory CpG oligodeoxynucleotides (ODN) attenuated liver IR-induced injury [40]. Oka, *et al.* found *TLR9*<sup>-/-</sup> mice to experience attenuated injury and less severe cardiac failure following transverse aortic constriction [26] and inhibitory CpG ODN attenuated cardiac IR, suggesting a role for TLR9 [41]. In contrast, there are conflicting data on the role of TLR9 in brain IR. The absence of TLR9 in brain IR was not protective [42] but CpG ODN was protective when administered prior to IR [43]. These conflicting data may be resolved by a current study which indicates that complement receptor 2 may function as a receptor for DNA in addition to TLR9 [44]. Similar to brain IR, our data indicate that *TLR9*<sup>-/-</sup> mice are not protected from injury following intestinal IR. It is currently unknown if CpG ODN attenuates intestinal IR; however, complement receptor 2 deficient mice are protected from IR-induced tissue damage [34]. These data provide another mechanism by which IR-induced injury differs between organs. This is an important finding as the field continues to uncover contributing factors to IR-induced damage and the differences between organs.

We previously demonstrated that tissue damage requires PGE<sub>2</sub> production and the PGE<sub>2</sub> production requires the appropriate Ab repertoire [12, 13]. It is interesting to note that PGE<sub>2</sub> was secreted but levels were attenuated in the *TLR9*<sup>-/-</sup> mice. However, the attenuated PGE<sub>2</sub>



## TLR9 is not required for gut IR injury



**Figure 6.** IR-induced complement and IgM deposition occurs in both C57Bl/6 and *TLR9*<sup>-/-</sup> mice. Wildtype and *TLR9*<sup>-/-</sup> mice were subjected to Sham or IR treatment. Intestinal sections were frozen in OCT and serial sections cut for immunohistochemistry. Antibody staining for IgM deposition, C3 deposition and F4/80+ macrophages was performed as described in *Methods*. Original magnification was 200x. Each photomicrograph is representative of 5-6 photos per treatment in each of 3-4 experiments.

production was not due to an improper Ab repertoire, as intestinal sections from *Rag-1*<sup>-/-</sup> mice administered Ab from *TLR9*<sup>-/-</sup> mice secreted similar concentrations of PGE<sub>2</sub> as *Rag-1*<sup>-/-</sup> mice receiving Ab from wildtype mice. Thus, *TLR9*<sup>-/-</sup> mice contain the proper Ab repertoire to induce normal PGE<sub>2</sub> production.

PGE<sub>2</sub> is produced from free arachidonic acid after conversion by Cox-2. It is possible that TLR9 influences the production or release of arachidonic acid to promote eicosanoid production. Our data indicate that Cox-2 transcription is normal despite the fact that previous studies indicated that CpG DNA stimulates Cox-2 production [45]. Other studies indicate that other TLR agonists also stimulate Cox-2 [46]. Thus, IR-induced PGE<sub>2</sub> production does not require TLR9 stimulation of Cox-2. It is possible that arachidonic acid is not released in the absence of TLR9 stimulation. Our data indicate that the relative amount of free arachidon-

ic acid is the same between wildtype and *TLR9*<sup>-/-</sup> mice (and *Rag-1*<sup>-/-</sup> mice, data not shown). However, *TLR9*<sup>-/-</sup> mice do have significantly lower levels of arachidonic acid per mg protein (data not shown). Conflicting reports exist in the literature regarding arachidonic acid release following TLR9 stimulation. Lee, *et. al.* showed an increase in arachidonic acid release from RAW264.7 cells after stimulation with CpG ODN and that TLR9 knockdown reduced the amount of arachidonic acid released from the stimulated cells [47]. However, Ruiperez, *et. al.* found no release of arachidonic acid from RAW264.7 or P388D1 cells when stimulated with synthetic ODN 1826 [48]. Thus, further investigation on the effects of TLR9 stimulation-induced release of arachidonic acid is needed.

Previous studies indicated that anti-DNA and anti-histone mAb restored injury in the *Rag-1*<sup>-/-</sup> mice, suggesting that DNA acted as a neo-anti-

gen. Our data indicate that while TLR9 is involved in IR-induced injury of some organs, it is not vital for injury of the intestine. A recent study suggests that in addition to TLR9, complement receptor 2 functions as a receptor for DNA [44]. Thus, complement receptor 2 or other DNA receptors may be recognized by naturally occurring Ab to promote tissue damage and inflammation in the absence of TLR9.

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## Conflict of interest statement

The authors declare no conflicts of interest.

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