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Helicobacter infection alters MyD88 and Trif signaling in response to intestinal ischemia/reperfusion

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Non-technical summary:

When the lack of blood flow (ischemia) to an organ is followed by reperfusion or return of the blood flow, components of the innate immune response induce tissue damage. Previous studies showed that inhibitors of complement prevent injury. Helicobacter infections, which are frequently undiagnosed, induce expression of a specific complement inhibitor. We show that an undiagnosed Helicobacter infection alters the mechanism of tissue damage such that therapeutic complement inhibitors would not be effective.

ABSTRACT

Ischemia/reperfusion-induced intestinal injury requires both toll-like receptor 4 (TLR4) signaling through myeloid differentiation primary response gene (88) (MyD88) and complement activation. As a common Gram negative intestinal pathogen, *Helicobacter hepaticus* signals through TLR4 and up-regulates the complement inhibitor, decay accelerating factor (DAF; CD55). Since ischemia/reperfusion (IR) is complement dependent, we hypothesized that Helicobacter infection may alter IR-induced intestinal damage. Infection increased DAF transcription and subsequently decreased complement activation in response to IR without altering intestinal damage in wildtype mice. IR induced similar levels of DAF mRNA expression in uninfected wildtype, MyD88$^{-/-}$ or Trif deficient mice. However, during infection, IR-induced DAF transcription was significantly attenuated in Trif deficient mice. Similarly, IR-induced intestinal damage, complement component 3 (C3) deposition and prostaglandin E_2 (PGE_2) production were attenuated in Helicobacter-infected, Trif deficient but not MyD88$^{-/-}$ mice. While infection attenuated IR-induced cytokine production in wildtype and MyD88$^{-/-}$ mice, there was no further decrease in Trif deficient mice. These data indicate distinct roles for MyD88 and Trif in IR-induced inflammation and chronic, undetected infections such as Helicobacter alter the use of the adaptor proteins to induce damage.

INTRODUCTION

Intestinal ischemia/reperfusion (IR) induces tissue damage, leading to systemic inflammation and death in 60-80% of affected individuals (Deitch, 2001; Clark & Coopersmith, 2007; Leaphart & Tepas, 2007). Damage occurring during ischemia is significantly amplified upon restoration of blood flow. During reperfusion, multiple inflammatory events occur including antibody recognition of neo-antigens expressed on the surface of damaged cells and subsequent complement activation (reviewed in (Fleming, 2006)). An influx of neutrophils and monocytes also results in the production of reactive oxygen and nitrogen species and cytokines (Sisley *et al.*, 1994; Cicalese *et al.*, 1996; Cerqueira *et al.*, 2005). Together, the inflammatory response induces significant local and systemic damage.

As part of the inflammatory response, toll-like receptors (TLRs) play a key role in maintaining intestinal homeostasis through recognition of commensal microflora (Rakoff-Nahoum *et al.*, 2004). These pathogen recognition receptors also induce inflammation after tissue damage (Mollen *et al.*, 2006). TLR4 activation plays a role in IR-induced tissue injury and inflammation in the intestine, kidney, brain, lung and heart (Li *et al.*, 2004; Wu *et al.*, 2007; Yang *et al.*, 2008; Gao *et al.*, 2009; Moses *et al.*, 2009; Takeishi & Kubota, 2009; Victoni *et al.*, 2010). Upon activation, most TLRs signal through the common MyD88 pathway. However, TLR4 can signal through either the MyD88 or Trif pathway resulting in cytokine and eicosanoid production (Moses *et al.*, 2009). In many studies, the TLR4 mediated inflammatory response was MyD88 dependent (Wu *et al.*, 2007; Cao *et al.*, 2009; Gao *et al.*, 2009). For example, MyD88 is required for intestinal, lung and cardiac polymorphonuclear leukocyte (PMN) migration and facilitates bacterial translocation (Feng *et al.*, 2008; Feng *et al.*, 2010; Victoni *et al.*, 2010). In response to

intestinal IR, MyD88, not Trif, is required for injury and intestinal PGE_2 production (Moses *et al.*, 2009). However, TLR4 but not MyD88 is necessary for edema after lung IR (Zanotti 2009). In addition, IR-induced inflammation in the liver requires TLR4 and interferon regulatory factor 3 (IRF3) activation (Zhai *et al.*, 2004), suggesting a role for Trif. Thus, despite the requirement for TLR4, the specific signaling pathways differ for each inflammatory response studied.

Helicobacter is a gram negative bacterium which induces chronic infections of the gastrointestinal tract in roughly half the world's population (Malaty, 2007). Recent evidence associates *Helicobacter pylori* with Crohn's disease (Huang *et al.*, 2004; Oliveira *et al.*, 2006). Similarly, *H. hepaticus*, *H. bilis* and *H. rodentium* have been implicated in rodent models of IBD and colon cancer (Foltz *et al.*, 1998; Solnick *et al.*, 2006). In addition, Helicobacter infection attenuates complement-mediated, shock-induced intestinal damage (Hylton *et al.*, 2010b). As intestinal IR-induced tissue damage is also complement mediated, it was possible that infection may change the mechanism of tissue injury in response to IR. Similar to IR, the pathogenesis of persistent Helicobacter infection includes unregulated cytokine production and oxidative stress via TLR4 (Mandell *et al.*, 2004). Interestingly, *H. pylori* infection increased gastric expression of a complement inhibitor, DAF (decay accelerating factor; CD55) (O'Brien *et al.*, 2006). Importantly, a recent study indicated that a natural chronic H. hepaticus infection up-regulated DAF expression and prevented complement activation during hemorrhage (Hylton *et al.*, 2010b). Therefore, we hypothesized that *H. hepaticus* infection may attenuate IR-induced, complement-mediated tissue damage by altering the TLR signaling pathway.

Using wildtype, MyD88$^{-/-}$ and Trif deficient mice in a model of intestinal IR, we demonstrate that MyD88 is critical to IR-induced injury, C3 deposition and eicosanoid production, while Trif is required for IL-12p40 and TNF-α production. However, during Helicobacter infection the absence of Trif significantly attenuated intestinal injury, complement activation and eicosanoid production after IR treatment. These data suggest that although both MyD88 and Trif contribute to IR-induced inflammation resulting in tissue damage, a chronic, subclinical Helicobacter infection alters the Trif-mediated response to IR.

METHODS

Ethical approval: All procedures were approved by the Kansas State University Institutional Animal Care and Use Committee and were in compliance with the Animal Welfare Act.

Mice: C57Bl/6 (wildtype) and Trif deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred at Kansas State University (Manhattan, KS). MyD88$^{-/-}$ mice were obtained from Dr. Tammy Killian (University of Nebraska Medical School, Omaha, NE). All mice were housed in the Kansas State University Division of Biology rodent facility and were maintained in 12 hr light/dark cycles with access to rodent chow and water ad libitum. All uninfected 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).

Helicobacter Infection: Mice were naturally colonized with *H. hepaticus* either by being reared by an infected female or by contacting infected feces during normal grooming. The presence of *H. hepaticus* was verified by PCR analysis of the feces from each infected mouse (data not shown). Fecal DNA was purified using the Qiagen DNA Stool mini kit according to the manufacturer's protocol and PCR amplified for 35 cycles at 54°C using Helicobacter-specific 16s rRNA primers. The PCR products were imaged using AlphaImager (Alpha Innotech) and semi-quantitative analysis performed using Image J (National Institutes of Health). Each mouse was infected for a minimum of 4 to 8 weeks before treatment. Feces from uninfected mice were also analyzed by PCR with 100% negative results. Liver, cecum, and colon DNA was purified by

TRIzol according to the manufacturer's protocol, and a similar PCR analysis was performed. Preliminary data indicated a constant level of shed bacteria at 1 to 2 months post-infection (data not shown) with *H. hepaticus* DNA also detectable in the liver, cecum, and colon of all infected mice. In contrast, *H. hepaticus* DNA was found in the jejunum of only 10% of the infected mice (data not shown).

Intestinal Ischemia/Reperfusion (IR): Animals were subjected to IR as previously described (Fleming *et al.*, 2002). Briefly, mice were anesthetized with ketamine/xylazine (16 mg/kg and 80 mg/kg respectively) administered i.p. and a midline laparotomy preformed. Pain was also controlled by the i.p. administration of buprenorphine (0.06 mg/kg). The superior mesenteric artery was isolated and a small vascular clamp applied (Roboz Surgical Instruments) for 30 min. Sham animals were subjected to the same surgical intervention without superior mesenteric artery occlusion. After clamp removal, the intestine was allowed to reperfuse for 2 hr with an additional dose of anesthetic administered prior to euthanization by exsanguination and tissue collection. Segments of small intestine, 10–20 cm distal to the gastroduodenal junction, were removed for subsequent analysis. There was no significant difference in survival between treatment and control groups.

Histological Analyses: Immediately after euthanasia, 2 cm segments of small intestine were fixed in 10% buffered formalin, embedded in paraffin, and 8 μm sections cut transversely and H&E stained. A blinded observer graded mucosal injury on a six-tiered scale adapted from Chiu et al. (Chiu *et al.*, 1970) as described previously (Fleming *et al.*, 2002). Briefly, the average injury score of each segment (75-150 villi) was determined by grading each villus from 0-6. A

score of 0 was assigned to normal villi; villi that had distortion of the tip were assigned a score of 1; villi with Guggenheims' spaces were assigned a score of 2; villi with patchy disruption of the epithelial cells were assigned a score of 3; a score of 4 was assigned to villi with epithelial sloughing and exposed but intact lamina propria; villi with exuding lamina propria were assigned a score of 5; hemorrhaged or denuded villi were assigned a score of 6.

Immunohistochemistry: Immediately after euthanasia, 2 cm intestinal sections were snap frozen in O.C.T. freezing medium and stored at -80^{o}C until 8 μm cryosections were cut and placed on slides. Slides were fixed in cold acetone and non-specific binding was blocked using 10% donkey serum in PBS. Tissues were stained for C3 deposition with a rat-anti-mouse C3 antibody (Hycult Biotechnologies) followed by a Texas-red conjugated secondary antibody (Jackson Immunoresearch). Serial sections stained with isotype control antibodies were used as background. A blinded observer examined the slides by fluorescent microscopy using a Nikon 80i fluorescent microscope and images acquired using a CoolSnapCf camera (Photometrics) and MetaVue Imaging software (Molecular Devices).

Ex vivo Secretions: Intestinal ex vivo eicosanoid generation was determined as described previously (Fleming *et al.*, 2002). Briefly, 2 cm mid-jejunal sections were collected and immediately minced, washed, and resuspended in 37°C oxygenated Tyrode's buffer (Sigma-Aldrich). The intestines were incubated for 20 min at 37°C, then the supernatants collected and stored at -80°C until assayed. Enzyme immunoassay kits (Cayman Chemical) were used to determine leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) concentrations. Cytokine concentrations in the intestinal supernatants were determined with a Milliplex MAP kit

(Millipore) following the manufacturer's instructions and analyzed on a Luminex 200 using xPONENT 3.1 and Analyst software (Millipore). All concentrations were standardized to the total tissue protein content as determined by BCA assay (Pierce) adapted to microtiter plates.

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 RNA integrity number values greater than 7.0 were used for cDNA synthesis. Using random primers, total RNA (2 ug) was reverse transcribed using a RevertAid first strand cDNA synthesis kit (Fermentas). Real-time PCR primer sequences were designed using Primer 3. Sequences used include: DAF sense: 5'TAAGCAGAATCGCCACAGAG 3' and anti-sense: 5'TCTTGCCTTCATCTCCCAAA 3'; Factor H sense: 5'ACCACATGTGCCAAATGCTA3' and anti-sense: 5'TGTTGAGTCTCGGCACTTTG3' and 18s rRNA sense: 5'GGTTGATCCTGCCAGTAGC 3' and anti-sense: 5'GCGACCAAAGGAACCATAAC 3'. Quantitative real time PCR was performed in 25 ul volumes using a Mini-Opticon real time thermal cycler (Bio-Rad) and Maxima SYBR Green/Flourescein qPCR Supermix (Fermentas) using the following protocol: 3m at 95°C; 50 cycles of 10s at 95°C, 20s at 58°C, 10s at 72°C; melt curve starting at 65°C, increasing 0.5°C every 5s up to 95°C. After amplification, DAF Ct values were normalized to 18s rRNA and then ΔΔCt fold change relative to Sham-treated wildtype 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 average ± SEM and were compared by two-way ANOVA unless a significant interaction occurred between the infection status and the mouse strain. In this situation, unpaired T test or one-way ANOVA with post hoc analysis using Newman-Keuls test (GraphPad/Instat Software) established significance between specific strains and infection status. The difference between groups was considered significant when $p < 0.05$.

RESULTS:

Helicobacter infection changes IR-induced inflammation without changing damage

Helicobacter, a common Gram negative intestinal pathogen, alters expression of the complement inhibitor, DAF, in human infection and a mouse model of hemorrhage (O'Brien *et al.*, 2006; O'Brien *et al.*, 2008; Hylton *et al.*, 2010b). Since IR is complement dependent, we hypothesized that infection may protect from IR-induced intestinal damage. Therefore, we examined IR-induced damage and complement activation in wildtype mice with a naturally occurring Helicobacter infection. As indicated in Fig. 1A, DAF mRNA expression increased significantly in both Sham and IR-treated infected mice when compared to uninfected mice. Other complement inhibitors, Factor H and Crry were not upregulated in response to IR or infection in wildtype mice (Fig. 2C and data not shown). Correlating with increased DAF expression, Helicobacter infection significantly decreased intestinal C3 deposition (Fig. 1C and D). However, infection did not alter intestinal mucosal injury after Sham or IR treatment (Fig.1B).

As a gram negative bacterium, Helicobacter signals through toll-like receptors (TLRs) and MyD88 which are also required for IR-induced tissue injury and inflammation (Moses *et al.*, 2009). We sought to determine the role of the TLR signaling adaptors, MyD88 and Trif, in intestinal IR after Helicobacter infection. Wildtype, MyD88$^{-/-}$, and Trif deficient mice were subjected to 30 min of intestinal ischemia followed by 2 h of reperfusion and tissue damage evaluated. Similar to previous studies (Moses *et al.*, 2009), IR-induced intestinal injury was attenuated in uninfected MyD88$^{-/-}$ mice compared to wildtype mice. As two-way ANOVA determined the infection status differed significantly between mouse strains (significant interaction between the strain and infection status), p values were determined by one-way

ANOVA. As expected, uninfected Trif$^{-/-}$ mice sustained significant IR-induced intestinal damage, with injury scores comparable to wildtype control mice (Fig. 2A, open bars and Fig. 2D). Surprisingly, infection did not alter damage in MyD88$^{-/-}$ or wildtype mice (Fig. 2A solid bars and Fig. 2D). However, infection significantly decreased tissue damage in Trif deficient mice (Fig. 2A solid bars and Fig. 2D). In addition, IR-induced DAF expression was low but significantly increased in all uninfected mice (Fig. 2B). However, infection significantly increased DAF transcription in all strains except Trif deficient mice (Fig. 2B). Despite elevated Factor H transcripts in the MyD88$^{-/-}$ and Trif deficient mice, the infection status did not further change the Factor H transcripts (Fig. 2C). These data indicate that Helicobacter-induced DAF expression requires the adaptor protein, Trif.

Helicobacter infection changes mechanism of damage

IR-induced intestinal injury is complement dependent (Hill *et al.*, 1992). Therefore we examined C3 deposition on intestinal sections after Sham or IR treatment. Corresponding to intestinal damage, C3 deposition was observed in uninfected wildtype and Trif deficient mice (Fig. 3). Additionally, uninfected MyD88$^{-/-}$ mice showed little to no C3 deposition which did not change with infection (Fig 3). In contrast, Helicobacter infection significantly decreased C3 deposition on intestines from wildtype and Trif deficient mice (Fig. 3). These data suggest that Trif is required for complement activation in mice infected with *H. hepaticus*. It is likely that the excess Factor H production in the Trif deficient mice attenuated the complement deposition in response to IR.

Helicobacter mediated damage requires Trif mediated PGE_2 production

Since injury and complement activation were altered by the naturally occurring Helicobacter infection in Trif deficient mice, we also examined the production of inflammatory eicosanoid mediators, which are required for IR-induced tissue damage (Moses *et al.*, 2009). As expected, uninfected wildtype and Trif deficient mice secreted significant PGE_2 and LTB_4 concentrations while eicosanoid production by $MyD88^{-/-}$ mice was attenuated (Fig. 4A and B). As two-way ANOVA determined that infection status significantly interacted with the mouse strains, significance was examined by one-way ANOVA. Helicobacter infection did not alter production of PGE_2 in response to either Sham or IR treatment in wildtype mice (Fig. 4A). Compared to uninfected mice, mid-jejunal PGE_2 production significantly increased after IR in infected $MyD88^{-/-}$ mice (Fig. 4A). However, Helicobacter-infected Trif deficient mice produced significantly less PGE_2 than uninfected mice (Fig. 4A). The basal levels of LTB_4 production increased in all strains of infected mice tested after Sham treatment (Fig. 4B). Additionally, in response to IR, infection increased LTB_4 production by wildtype and $MyD88^{-/-}$ mice but not Trif deficient mice (Fig. 4B). These data suggest that Helicobacter-infected mice require Trif for IR-induced eicosanoid production.

Previous studies suggested that TNF-α and IL-12p40 secretions were also critical to IR-induced intestinal damage (Caty *et al.*, 1990; Pope *et al.*, 2010), and that Helicobacter infection alters intestinal cytokine production (Obonyo *et al.*, 2007; Hylton *et al.*, 2010b). Therefore, we examined IR-induced IL-12p40 and TNF-α intestinal secretion in mice infected with *H. hepaticus*. As expected, IR induced significant increases in both cytokines in wildtype mice (Fig. 4C and D). The strain significantly impacted the cytokine production as Trif but not MyD88 deficiency attenuated IL-12p40 and TNF-α production in uninfected mice (Fig. 4C and D).

Importantly, using two way ANOVA, Helicobacter infection significantly attenuated production of these cytokines. Specifically, infected wildtype and MyD88$^{-/-}$ mice produced significantly less cytokine while infection had no effect in Trif deficient mice (Fig 4C and D). Taken together, these data suggest distinctive roles for TLR4 signaling pathways and that these pathways may be modified in the presence of a chronic infection.

DISCUSSION

Previous studies demonstrated that complement and TLR4 activation are required for IR-induced tissue damage (Weiser *et al.*, 1996; Williams *et al.*, 1999; Moses *et al.*, 2009). As Helicobacter infection upregulates DAF expression, we hypothesized that infection may decrease IR-induced, complement-mediated tissue damage. Using mice with naturally occurring *H. hepaticus* infection, we demonstrated that IR induces tissue damage, despite diminished complement activation. As a gram negative bacterium, it was likely that toll-like receptor signaling pathways contributed to this unexpected finding. Similar to previous data, IR induced intestinal damage in uninfected Trif deficient but not MyD88$^{-/-}$ mice and the damage positively correlated with C3 deposition. However, Helicobacter infection induced increased transcription of complement inhibitors, DAF and/or Factor H resulting in decreased C3 deposition despite no change in IR-induced tissue damage. Examination of the inflammatory response indicated that IR-induced PGE_2 production required MyD88 expression in uninfected mice and Trif expression in infected mice. In addition, Trif was required for IL-12p40 and TNF-α production in the presence or absence of infection. Together these data suggest that although IR-induced damage and inflammation requires MyD88 expression, chronic subclinical infections may change the inflammatory response to a Trif-dependent mechanism and result in similar damage levels.

By administering complement inhibitors, previous studies indicated that intestinal IR is complement mediated (Eror *et al.*, 1999; Williams *et al.*, 1999; Harkin *et al.*, 2004; Souza *et al.*, 2005; Weeks *et al.*, 2007). Specifically, administration of soluble DAF limited deposition of the terminal complement complex, C5b-9, in response to skeletal muscle IR (Weeks *et al.*, 2007). Natural infections such as *H. pylori* increase DAF expression on the human gastric epithelium

(O'Brien *et al.*, 2008). In addition, *H. hepaticus* also up-regulates DAF in response to hemorrhage (Hylton *et al.*, 2010b). Similarly, we demonstrate that *H. hepaticus* infection increased DAF transcription with a subsequent decrease in complement activation in response to IR. In addition, the absence of either MyD88 or Trif increased Factor H production. However, during a subclinical Helicobacter infection, wildtype and MyD88$^{-/-}$ mice subjected to IR sustained significant intestinal injury which was similar to IR-treated, uninfected mice. In contrast, the injury was significantly reduced after infection of Trif deficient mice. Together these data suggest that in mice, *H. hepaticus* infection alters the mechanism of IR-induced damage in a Trif dependent manner.

Recent studies indicated that DAF interacts with TLR4 (Zhang *et al.*, 2007) and TLR4 is critical for IR-induced damage and PGE_2 production (Moses *et al.*, 2009). In addition, these studies indicated a role for MyD88 but not Trif in intestinal IR (Moses *et al.*, 2009). Confirming the previous results, the current data also correlate with a recent study indicating that MyD88$^{-/-}$ mice demonstrated a decreased PMN migration, myeloperoxidase production and attenuated intestinal injury after IR (Victoni *et al.*, 2010). The requirement of MyD88 but not Trif for IR-induced injury was demonstrated in cardiac IR as well (Feng *et al.*, 2010). However, cold heart IR associated with transplantation involves both MyD88 and Trif signaling pathways with deficient mice expressing decreased serum cytokines and inflammatory transcripts in the graft (Kaczorowski *et al.*, 2009). This phenomenon may be associated with the difference between warm and cold IR. Ischemia/reperfusion-induced injury in other organs may differ. For example, MyD88$^{-/-}$, Trif deficient and wildtype mice showed similar responses to renal IR (Pulskens *et al.*, 2008). In contrast, LPS preconditioning protects from subsequent cerebral ischemia by IFNβ

production through Trif/IRF3 pathways (Marsh *et al.*, 2009). Similarly, liver IR required TLR4 signaling through IRF3 for damage and inflammation, suggesting use of the Trif pathway (Zhai *et al.*, 2004). Together, these data suggest that TLR4 may be able to use either pathway to reach an inflammatory threshold and induce tissue damage.

The current study demonstrates that significant damage occurs in infected wildtype mice despite diminished complement activation and cytokine production. However, eicosanoid production remains elevated. This enhanced damage may be explained by two possibilities. First, it is possible that damage occurs when a threshold of inflammatory insults has been reached. Additional evidence for requiring an inflammatory threshold is present in the literature. Multiple inflammatory processes contribute to intestinal IR-induced injury including complement activation (Eror *et al.*, 1999; Williams *et al.*, 1999; Harkin *et al.*, 2004; Souza *et al.*, 2005; Weeks *et al.*, 2007), cytokine production (Chen *et al.*, 2008), eicosanoid production (Moses *et al.*, 2009) and PMN infiltration (Hill *et al.*, 1992). Importantly, the inhibition of any one of these inflammatory processes attenuated IR-induced intestinal damage. Thus, multiple components of the inflammatory response may contribute to this threshold. As such, an undetected infection contributes to these insults, allowing increased damage despite decreased complement activation and cytokine production by wildtype mice. As a second possibility, macrophages may mediate the increased damage in response to infection. As LTB_4 is a macrophage chemotactic factor and remains elevated during infection, it is likely that IR-induces a macrophage infiltration even after infection. In addition, Helicobacter infection changes hemorrhage-induced intestinal damage from complement mediated to macrophage and IL-12p70 mediated (Hylton *et al.*, 2010a; Hylton *et al.*, 2010b). Further investigation of cytokines and macrophage infiltration may provide a

specific mechanism for IR-induced tissue damage during infection.

Thus, chronic but undiagnosed infections may critically alter the mechanisms of IR-induced tissue damage. With a high mortality rate, new therapeutics which focus on complement activation are currently being tested for intestinal IR. However, the present data suggests that complement focused therapeutics may be ineffective for patients with Helicobacter infection. As over half of the world is infected with *H. pylori* and the majority of these infections are asymptomatic, additional therapeutics must also be identified.

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FIGURE LEGENDS:

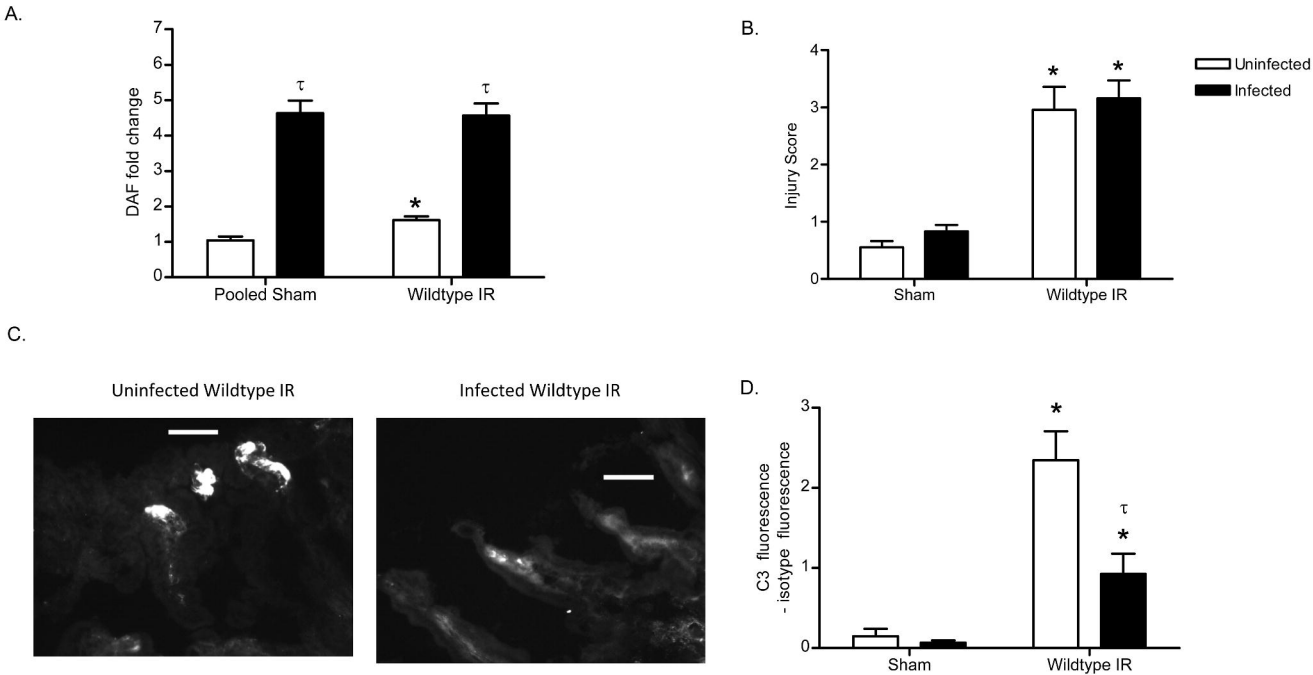
FIGURE 1. Helicobacter infection increases DAF transcription and decreases C3 deposition. A. DAF expression was determined by real-time RT-PCR. B. Mid-jejunal injury was scored using H&E stained intestinal sections taken from C57Bl/6 (B6) mice subjected to Sham or ischemia/reperfusion (IR) treatment. C. Intestinal sections were stained for C3 deposition. Photomicrographs are representative of 3 independent experiments. D. Immunofluorescence was quantitated using Image J. n = 5-10 animals per group, * = $p \leq 0.05$ compared to respective Sham, ϕ = $p \leq 0.05$ compared to respective wildtype IR, τ = $p \leq 0.05$ compared to respective uninfected treatment group.

FIGURE 2. Trif is required for IR-induced injury and DAF expression in Helicobacter-infected mice. A. Mid-jejunal injury was scored using H&E stained intestinal sections taken from C57Bl/6 (wildtype), MyD88$^{-/-}$ or Trif$^{-/-}$ mice subjected to Sham or ischemia/reperfusion (IR) treatment. As no difference was observed between strains, sham treated animals were pooled. B. DAF or C. Factor H mRNA expression was determined by real-time RT-PCR. D. Representative H&E stained intestinal sections. Bar = 50μm. n = 5-10 animals per group, * = $p \leq 0.05$ compared to respective Sham, ϕ = $p \leq 0.05$ compared to respective wildtype IR, τ = $p \leq 0.05$ compared to respective uninfected treatment group.

FIGURE 3. MyD88 deficiency or Helicobacter infection attenuates IR-induced C3 deposition. Intestinal sections from C57Bl/6 (wildtype), MyD88$^{-/-}$ or Trif$^{-/-}$ mice subjected to Sham or ischemia/reperfusion (IR) treatment were stained for C3 deposition. Photomicrographs are representative of 3 independent experiments. Immunofluorescence was quantitated using

Image J. * = $p \leq 0.05$ compared to respective Sham, $\phi = p \leq 0.05$ compared to respective wildtype IR, $\tau = p \leq 0.05$ compared to respective uninfected treatment group.

FIGURE 4. Helicobacter infection requires Trif for IR-induced intestinal injury and inflammation. Ex vivo intestinal supernatants from C57Bl/6 (wildtype), $MyD88^{-/-}$ or $Trif^{-/-}$ mice were used to determine A. PGE_2, B. LTB_4, C. IL-12p40, or D. TNF-α production in response to Sham or ischemia/reperfusion (IR) treatment. As no difference was observed between strains, sham treated animals were pooled. All concentrations were normalized to tissue protein content and expressed as pg per mg of intestinal tissue. n = 5-10 animals per group, * = $p \leq 0.05$ compared to respective Sham, $\phi = p \leq 0.05$ compared to respective wildtype IR, $\tau = p \leq 0.05$ compared to respective uninfected treatment group.


A.
DAF fold change
0
1
2
3
4
5
6
7
τ
τ
*
Pooled Sham
Wildtype IR
B.
Injury Score
0
1
2
3
4
*
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Sham
Wildtype IR
Uninfected
Infected
C.
Uninfected Wildtype IR
Infected Wildtype IR
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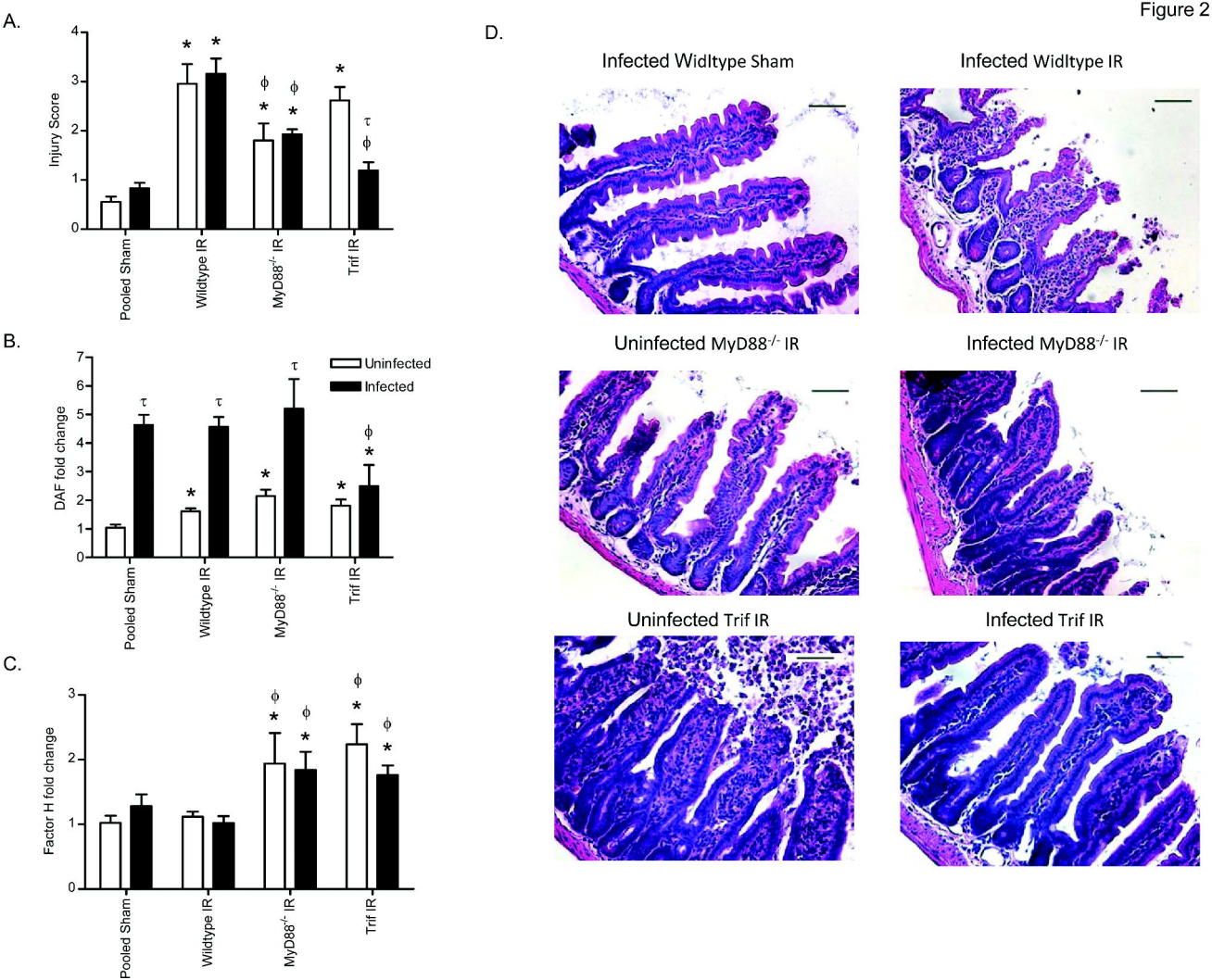
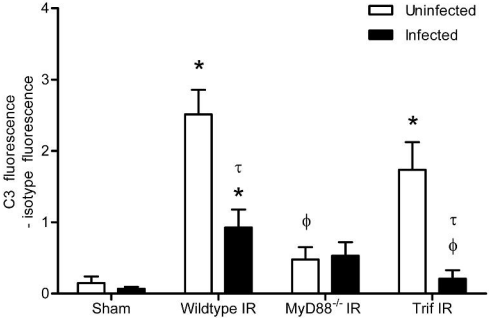
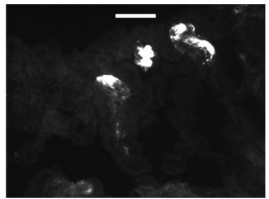

A.
Injury Score
Pooled Sham
Wildtype IR
MyD88-/- IR
Trif IR
B.
DAF fold change
Uninfected
Infected
C.
Factor H fold change
D.
Infected Widltype Sham
Infected Widltype IR
Uninfected MyD88-/- IR
Infected MyD88-/- IR
Uninfected Trif IR
Infected Trif IR

Figure 3


Uninfected
Infected
C3 fluorescence - isotype fluorescence
4
3
2
1
0
*
*
τ
ϕ
*
τ
ϕ
Sham
Wildtype IR
MyD88-/- IR
Trif IR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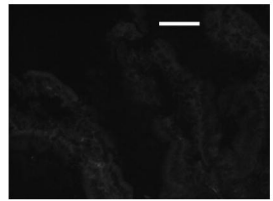
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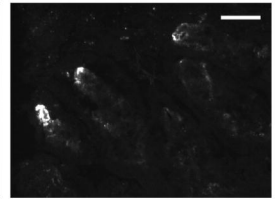
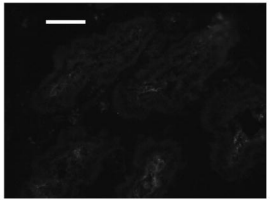
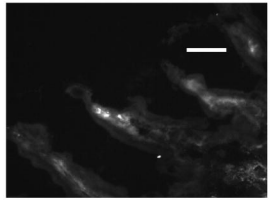
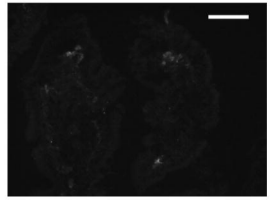

Uninfected Trif IR


Infected Wildtype Sham


Infected Wildtype IR


Infected MyD88-/- IR

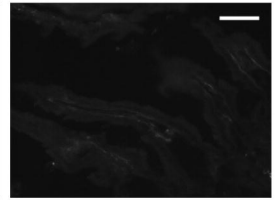
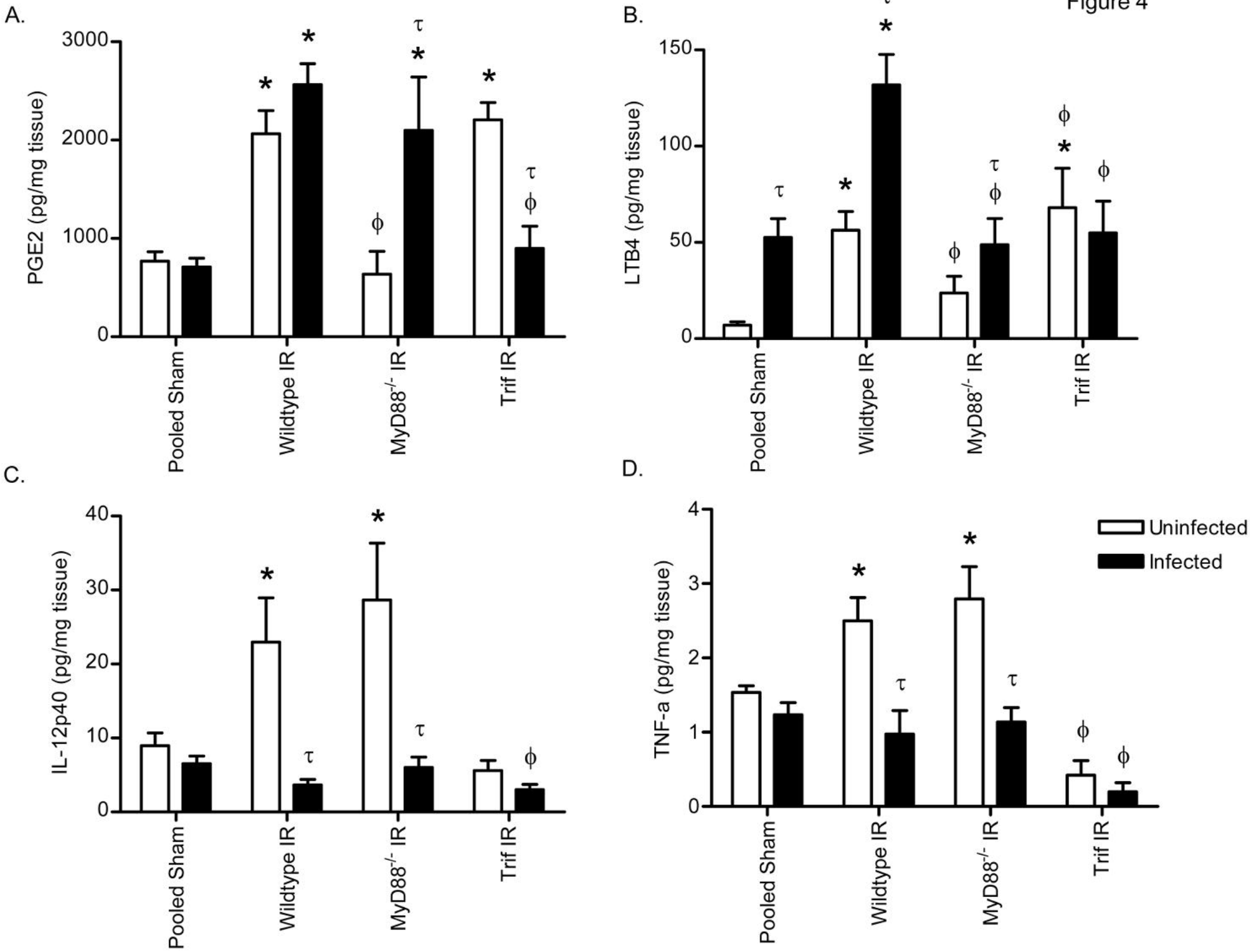

Infected Trif IR

Figure 4


A.
PGE2 (pg/mg tissue)
3000
2000
1000
0
Pooled Sham
Wildtype IR
MyD88-/- IR
Trif IR
B.
LTB4 (pg/mg tissue)
150
100
50
0
C.
IL-12p40 (pg/mg tissue)
40
30
20
10
0
D.
TNF-a (pg/mg tissue)
4
3
2
1
0
Uninfected
Infected