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## Domain V peptides inhibit $\beta$ 2-glycoprotein I mediated mesenteric ischemia/reperfusion-induced tissue damage and inflammation<sup>1</sup>

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### Abstract

Reperfusion of ischemic tissue induces significant tissue damage in multiple conditions, including myocardial infarctions, stroke and transplantation. Although not as common, the mortality rate of mesenteric ischemia/reperfusion (IR) remains over 70%. Although complement and naturally occurring antibodies are known to mediate significant damage during IR, the target antigens are intracellular molecules. We investigated the role of the serum protein,  $\beta$ 2-glycoprotein I as an initiating antigen for antibody recognition and  $\beta$ 2-GPI peptides as a therapeutic for mesenteric IR. The time course of  $\beta$ 2-GPI binding to the tissue indicated binding and complement activation within 15 min post-reperfusion. Treatment of wildtype mice with peptides corresponding to the lipid binding domain V of  $\beta$ 2-GPI blocked intestinal injury and inflammation, including cellular influx and cytokine and eicosanoid production. The optimal therapeutic peptide (peptide 296) contained the lysine rich region of domain V. In addition, damage and most inflammation was also blocked by peptide 305 which overlaps with peptide 296 but does not contain the lysine rich, phospholipid binding region. Importantly, peptide 296 retained efficacy after replacement of Cys residues with Ser. In addition, infusion of wildtype serum containing reduced levels of anti- $\beta$ 2-GPI antibodies into Rag-1<sup>-/-</sup> mice prevented IR-induced intestinal damage and inflammation. Together these data suggest that the serum protein,  $\beta$ 2-GPI initiates the IR-induced intestinal damage and inflammatory response and as such is a critical therapeutic target for IR-induced damage and inflammation. This is an author-produced version of a manuscript accepted for publication in *The Journal of Immunology (The JI)*. The American Association of Immunologists, Inc. (AAI), publisher of *The JI*, holds the copyright to this manuscript. This version of the manuscript has not yet been copyedited or subjected to editorial proofreading by *The JI*; hence, it may differ from the final version published in *The JI* (online and in print). AAI (*The JI*) is not liable for errors or omissions in this author-produced version of the manuscript or in any version derived from it by the U.S. National Institutes of Health or any other third party. The final, citable version of record can be found at [www.jimmunol.org](http://www.jimmunol.org)

### Keywords

mouse; intestine; complement; antibodies

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## Introduction

During an ischemic event, the lack of blood flow to an organ induces tissue damage. However, return of blood flow during reperfusion enhances pathology significantly. The inflammatory response to ischemia/reperfusion (IR)-induced organ damage may subsequently lead to a systemic inflammatory response with multiple organ failure. Intestinal IR results in severe inflammatory-induced mucosal damage, barrier dysfunction, and subsequent bacterial translocation leading to sepsis (1) and frequently results in liver and lung damage (2).

Mesenteric IR-induced tissue injury is mediated by at least two components of the innate immune response, neutrophil infiltration and complement activation (3–5). Initial studies demonstrated that neutrophil depletion attenuated intestinal IR-induced injury (4–5). However, the presence of neutrophils was not sufficient for tissue damage when complement activation was inhibited (6). Complement activation increased adhesion molecule expression after IR and released a cascade of inflammatory mediators including leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) which also contributed to tissue damage (7–9). Additionally, depletion or inhibition of complement activation products prevented both local and remote organ injury in response to intestinal IR (10–13).

Cells subjected to hypoxic conditions express cryptic antigens on the plasma membrane (14–15). Cryptic antigens expressed on apoptotic cells are recognized by natural antibodies which frequently exhibit low affinity binding (16). Previous studies indicated that administering naturally occurring monoclonal antibodies (mAb) reconstituted IR-induced intestinal damage in antibody-deficient Rag-1<sup>-/-</sup> mice (15,17–19). Multiple natural antibodies which recognized intracellular antigens, DNA, non-muscle myosin and ribonucleoprotein, and cardiolipin induced damage in the IR-resistant Rag-1<sup>-/-</sup> mouse suggesting that the antibodies and antigens are critical to IR-induced damage (18–21). In conjunction with anti-phospholipid mAb, antibodies to the serum protein, β<sub>2</sub>-glycoprotein I (β<sub>2</sub>-GPI) also restored tissue damage in Rag-1<sup>-/-</sup>, IR resistant mice (19). These data suggest that ischemia induces a cellular response resulting in expression of multiple cryptic antigens targeted by low-affinity, naturally occurring antibodies also found in autoimmune diseases.

The serum protein, β<sub>2</sub>-GPI, also known as apolipoprotein H, is a member of the complement control protein family (22–23) but has no known complement regulating function (24). However, β<sub>2</sub>-GPI is a cofactor for plasminogen activation (25) and an opsonin for the clearance of apoptotic cells by phagocytes (26). By binding to anionic phospholipids, DNA or other negatively charged molecules (22), β<sub>2</sub>-GPI is the major antigenic target for anti-phospholipid antibodies found in the serum of anti-phospholipid antibody syndrome (APLS) patients (27). Increased anti-β<sub>2</sub>-GPI antibody titer also correlated with increased risk of ischemic stroke or heart disease in APLS or systemic lupus erythematosus patients, respectively (28–29). Together, these data suggest anti-β<sub>2</sub>-GPI antibodies are involved in ischemic events.

Based on these data, we hypothesized that during reperfusion, serum protein β<sub>2</sub>-GPI binds ischemic cell membranes and is recognized by naturally occurring antibodies which leads to complement activation and inflammation. Using an in vitro model, our findings demonstrate that anti-β<sub>2</sub>-GPI antibodies recognized β<sub>2</sub>-GPI bound to the surface of hypoxic endothelial cells. In a mouse model of intestinal ischemia, β<sub>2</sub>-GPI binding to damaged ischemic intestinal tissue correlated with tissue injury and reduction of anti-β<sub>2</sub>-GPI antibodies mitigated intestinal damage and inflammation. As reduction of antibodies in vivo is difficult, we injected β<sub>2</sub>-GPI peptides to compete with β<sub>2</sub>-GPI binding to the tissue. Importantly, injection of peptides specific for the lipid binding domain of β<sub>2</sub>-GPI blocked intestinal

injury as well as eicosanoid and cytokine production. Administration of peptides containing the phospholipid binding, lysine rich region and adjacent regions were most effective. Together these data suggest that  $\beta$ 2-GPI initiates the IR-induced intestinal damage and inflammatory response and as such is a critical therapeutic target for IR-induced damage and inflammation.

## Materials and methods

### Mice

C57Bl/6 and Rag-1<sup>-/-</sup> (backcrossed to C57Bl/6 for 10 generations) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred and maintained under 12 h light/dark cycles at Kansas State University, Division of Biology (Manhattan, KS). All mice were allowed access to food and water ad libitum and maintained under specific pathogen free conditions. 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.

### Ischemia/reperfusion Procedure

Animals were subjected to IR similar to previously described studies (30). Briefly, ketamine (16mg/kg) and xylazine (80 mg/kg) anesthetized mice were administered buprenorphine (0.06mg/kg) for pain. After laparotomy and a 30 min equilibration period, a small vascular clamp (Roboz Surgical Instruments) was applied to the isolated superior mesenteric artery. After 30 min of ischemia, the clamp was removed and the intestines reperfused for up to 2 h. Sham animals sustained the same surgical intervention without superior mesenteric artery occlusion. Mice treated with the various  $\beta$ 2-GPI peptides underwent the same procedure with i.v. administration of the peptides (40  $\mu$ M) 5 min prior to artery occlusion. Peptides 296, 305, and 296Cys to Ser were soluble in N. Saline and injected i.v. in 100ul volumes. Peptides 100 and 181 were dissolved in DMSO prior to diluting 1/100 in N. Saline. Additional mice received peptides prior to Sham treatment.

In some experiments, 200 $\mu$ l of C57Bl/6 sera with or without the reduction of anti- $\beta$ 2-GPI Ab was administered i.v. to Rag-1<sup>-/-</sup> mice 20 min prior to clamp application. After euthanization, mid-jejunum 10–20cm distal to the gastroduodenal junction was removed for analysis. Survival was not significantly different between treatment groups.

### $\beta$ 2-GPI peptides

To decrease  $\beta$ 2-GPI binding to the cells and tissue, peptides from mouse  $\beta$ 2-GPI were designed. As Domain V is proposed to contain the lipid binding site (31), we designed three overlapping 24–25 aa peptides from domain V (peptides 296, 305 and 322) using the NCBI sequence, AAB30789 (32). Peptide 296 contains the lysine rich region previously identified as the critical lipid binding region (31,33). The overlapping peptide 305 contains the final 3 residues of the lysine rich region and continues into the tail region which is proposed to insert into the lipid membrane. Peptide 322 is contained within the tail region. Additional control peptides 100 and 181 are contained within domains II and III respectively. Most peptides used in this study were purchased from Invitrogen with purity (>90%) and sequence determined by the manufacturer. Production of  $\beta$ 2-GPI peptide 296Cys to Ser was generated by solid-phase synthesis with 9-fluorenylmethoxycarbonyl chemistry, as described in detail previously (34). The peptides were purified by reversed-phase HPLC and characterized by matrix-assisted laser desorption time-of-flight mass spectroscopy. All lyophilized peptides were stored at  $-20^{\circ}$ C until time of use.

## Histology and Injury Scoring

Immediately after euthanasia, a 2 cm mid-jejunum tissue section was immediately fixed in 10% buffered formalin, embedded in paraffin, and 8 $\mu$ m sections were cut transversely and H&E stained. Mucosal injury was graded on a six-tiered scale adapted from Chiu et al. (35) as described previously (36). 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.

## Ex vivo eicosanoid and cytokine generation

*Ex vivo* generation of eicosanoids by mid-jejunal tissue was determined as previously described (30). 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<sub>2</sub> and LTB<sub>4</sub> concentrations were determined using enzyme immunoassay kits (Cayman Chemical). IL-6 and IL-12 concentrations were determined using a Milliplex MAP immunoassay kit (Millipore) and read on a Milliplex Analyzer (Millipore). All eicosanoid and cytokine concentrations were standardized to the total tissue protein content.

## C3 deposition and immunohistochemistry

After euthanasia, a 2 cm intestinal section was immediately snap frozen in O.C.T. freezing medium and 8  $\mu$ m sections were placed on slides for immunohistochemistry. The C3 deposition and F4/80 expression on the tissue sections was detected by staining with a purified rat-anti-mouse C3 (Hycult Biotechnologies) or F4/80 (eBioscience) antibody followed by a Texas-red conjugated donkey-anti-rat IgG secondary antibody (Jackson ImmunoResearch). CD31 (PECAM-1), CD106 (VCAM-1) were detected by FITC conjugated rat anti-mouse CD31 or CD106 (Biolegend) antibodies. Each experiment contained serial sections stained with the appropriate isotype control antibodies. All slides were mounted with ProLong Gold (Invitrogen). Images were obtained at room temperature using a Nikon eclipse 80i microscope equipped with a CoolSnap CF camera (Photometrics) and analyzed using Metavue software (Molecular Devices).

## Immunoprecipitation of $\beta$ 2-GPI complexes from tissue

Mid-jejunum (25–30 mm) was longitudinally opened, adhered to a 6-well plate and incubated at 4°C for 2 h in freshly oxygenated Tyrodes buffer containing 15 $\mu$ g/ml FC1 mAb (mouseIgG1, anti- $\beta$ 2-GPI)(37). The crosslinker, DTSSP (Pierce), was added to the Ab solution at a final concentration of 1.5mM and incubated at 4°C for an additional 2 h. The reaction was stopped with Tris, pH 7.5 and the washed mucosa was lysed in 1 ml of MES/Brij58 (145 mM NaCl, 0.2 mM EDTA, 0.5% w/v Brij58 (Sigma), 25 mM MES (Sigma), pH 6.5). The lysate was incubated for 30 min on ice, with periodic vortexing and clarified by centrifuging at 5,000 $\times$ g for 10 min at 4°C. Antibody was immunoprecipitated overnight at 4°C with Protein G beads (Pierce) and the samples were boiled in non-reducing Laemmli sample buffer prior to SDS-PAGE (10%) and Western blot analysis. Human  $\beta$ 2-GPI (Fitzgerald) was used as a positive control. The blots were probed with anti- $\beta$ 2-GPI antibody, MAB1066 (Chemicon) followed by goat anti-mouse IgG HRP conjugate (Pierce).

Protein was visualized using SuperSignal Detection Kit (Pierce) according to the manufacturer's protocol.

### Hypoxia and immunocytochemistry

Hypoxia was conducted similar to previous studies with the following modifications (38). Hypoxic MS-1 endothelial cells (ATCC) received degassed, serum-free DMEM and were placed in a hypoxia chamber containing 94% nitrogen and 5% CO<sub>2</sub>. Normoxic cells received DMEM supplemented with 10% heat-inactivated sera from Rag-1<sup>-/-</sup> mice in 8% CO<sub>2</sub>. After 4 h at 37°C, all cells received fresh medium containing 10% heat-inactivated Rag-1<sup>-/-</sup> sera and were incubated in normoxic conditions for 1 hr at 37°C. Additional peptide studies were performed by addition of peptides (40 μM final concentration) during the hypoxic period. Cells were methanol fixed and stained with the anti-β2-GPI mAb (Millipore) followed by an anti-mouse IgG antibody to determine β2-GPI binding. Anti-β2-GPI binding was determined by allowing anti-β2-GPI mAb (Millipore) to bind during the 1 h normoxic period. The cells were then stained with anti-mouse IgG antibodies (Jackson ImmunoResearch) as previously described (19). The fluorescence was determined in a blinded manner using a Nikon 80i fluorescent microscope with a 40x Plan Fluor objective and images acquired using a CoolSnap Cf camera (Photometrics) and MetaVue Imaging software (Molecular Devices).

### Anti-β2-GPI concentrations and isotyping

Anti-β2-GPI concentrations were determined based on optimal conditions previously described (39–40). The specific isotypes of anti-β2-GPI antibodies were determined after binding serum in duplicate to coated and blocked wells and incubating for 1 h. After washing, the appropriate biotinylated anti-mouse Ig isotype antibodies were added to each well for 1 h at RT while gently shaking. After incubation with avidin peroxidase (Sigma) the plate was developed using TMB (Kirkegaard).

### Reduction of anti-β2-GPI activity from C57Bl/6 serum

An ELISA plate was coated for 2 h at room temperature with 2 μg of β2-GPI (Fitzgerald) in PBS. After blocking for 2 h with 100 μl of 3% bovine serum albumin in PBS, 50 μl of heat-inactivated C57Bl/6 sera was added to half of the coated wells for 2 h at room temperature. The sera was then transferred to the remaining coated set of wells and incubated for an additional 2 h at room temperature. The reduced serum was removed, pooled and then administered as described above. The reduction procedure removed approximately 50% of the anti-β2-GPI antibodies as determined by ELISA.

### Statistical analysis

Data are presented as mean ± SEM and significance ( $p < 0.05$ ) determined by one-way ANOVA with Newman-Keuls post hoc analysis (GraphPad/Instat Software).

## Results

### Mucosal injury and β2-GPI binding to ischemic or hypoxic tissue occurs early in reperfusion

C57Bl/6 mice were subjected to ischemic injury followed by 5, 10, or 15 min of reperfusion. Compared to pooled sham-treated animals, significant mid-jejunal mucosal injury was observed after 15 min of reperfusion and increased up to 2 h post-reperfusion (Fig. 1A). In contrast, Rag-1<sup>-/-</sup> mice did not sustain intestinal damage at any time point analyzed (Fig. 1A). When analyzed for β2-GPI, sera from both C57Bl/6 and Rag-1<sup>-/-</sup> mice contained similar concentrations of β2-GPI (data not shown). As previously shown, anti-β2-GPI binds



ischemic-damaged tissue within 2 h following reperfusion (19); however, we were interested in determining the early kinetics of  $\beta$ 2-GPI binding to tissue following ischemia. To examine the kinetics, tissue harvested after 5, 10, or 15 min of reperfusion was probed with the anti- $\beta$ 2-GPI mAb, FC1. The antibody/antigen complexes were cross-linked to the surface of the villi prior to immunoprecipitation and Western blotting. Immunoprecipitation indicated the presence of  $\beta$ 2-GPI bound to the cell surface at 15 min post-reperfusion but not at the earlier time points (Fig. 1B). The apparent molecular weight difference between human and mouse is likely due to differential glycosylation and different isoelectric points (41). Additionally the presence of detectable levels of tissue-bound  $\beta$ 2-GPI correlates positively with the earliest time point when significant damage was observed (Fig 1A).

MS-1 endothelial cells were subjected to hypoxia or normoxia to validate  $\beta$ 2-GPI binding in vitro. Addition of sera from Rag-1<sup>-/-</sup> mice during the subsequent normoxia (reperfusion) stage provided the  $\beta$ 2-GPI. After hypoxic but not normoxic treatment, cells were positive for  $\beta$ 2-GPI (Fig. 2A–C). The addition of anti- $\beta$ 2-GPI mAb to the cells during reperfusion, again showed that only hypoxic but not normoxic-treated cells stained positively for anti- $\beta$ 2-GPI antibodies (Fig. 2D–F). Similar to the in vivo results, in vitro studies showed that hypoxia-induced cellular changes facilitated the binding of both  $\beta$ 2-GPI and anti- $\beta$ 2-GPI antibodies to the surface of ischemic cells.

### Characterization of the anti- $\beta$ 2-GPI activity in C57Bl/6 serum

To further understand the role of anti- $\beta$ 2-GPI antibodies, we examined the presence of these antibodies in wildtype and Rag-1<sup>-/-</sup> mice. As shown in Figure 1C, we determined that approximately 60 ng/ml anti- $\beta$ 2-GPI antibody (total Ig) is present in C57Bl/6 serum but as expected Rag-1<sup>-/-</sup> serum contained no detectable antibodies (Fig. 1C). Interestingly, serum from IR-resistant, Cr2<sup>-/-</sup> mice contains significantly less anti- $\beta$ 2-GPI antibody (Fig. 1C). These results indicate that naturally occurring antibodies against  $\beta$ 2-GPI exist in wildtype mice. The anti- $\beta$ 2-GPI antibody concentration in wildtype sera was determined to be primarily of the IgM and IgG2b isotypes with minor amounts of IgG3 and IgG1 isotypes (Fig. 1C). The presence of IgG2b, IgG3 and IgM isotypes is consistent with complement activation. Therefore,  $\beta$ 2-GPI represents a significant target for forming antibody/antigen complexes capable of facilitating complement-mediated tissue damage.

### Reduction of serum anti- $\beta$ 2-GPI activity attenuated intestinal damage and inflammation

The effects of anti- $\beta$ 2-GPI antibody reduction on IR-mediated damage were assessed by subjecting Rag-1<sup>-/-</sup> mice to IR after reconstitution with wildtype serum after 2 rounds of adsorption to bound  $\beta$ 2-GPI. When Rag-1<sup>-/-</sup> mice were reconstituted with non-adsorbed C57Bl/6 serum, significant damage was observed after 2 h reperfusion (Fig. 3A) similar to previous results for C57Bl/6 mice (Fig. 1A). However, when mice were administered anti- $\beta$ 2-GPI reduced serum, no damage was observed similar to that seen in Rag-1<sup>-/-</sup> IR control mice (Fig. 3A, D–F). Moreover, the effects of anti- $\beta$ 2-GPI reduction extended to dramatically decreasing the intestinal inflammatory response. The IR-induced increase in PGE<sub>2</sub> and LTB<sub>4</sub> production was abrogated with the antibody-reduced serum to concentrations similar to Rag-1<sup>-/-</sup> IR controls (Fig. 3B, C). These data suggest that inhibition of anti- $\beta$ 2-GPI antibodies may provide a therapeutic target for IR-induced tissue damage.

### Domain V $\beta$ 2-GPI peptides block IR-induced intestinal damage

We hypothesized that if inhibition of antibody binding to  $\beta$ 2-GPI on the tissue attenuated injury, then peptides which block the lipid-binding domain of  $\beta$ 2-GPI may inhibit  $\beta$ 2-GPI binding and attenuate IR-induced intestinal damage and inflammation as well. Peptides were designed to match sequences from multiple domains of mouse  $\beta$ 2-GPI, including domains

II, III and lipid-binding domain V as indicated in Table 1 and Fig 4. Within domain V, three overlapping peptides were created, 296, 305 and 322 (Fig. 4 and Table 1) to cover the lysine rich domain (296) and the tail which is inserted into the lipid membrane (322) with peptide 305 spanning the intervening region. Additional peptides from Domains II and III were used as controls. Initial in vitro studies tested the ability of the peptides to block  $\beta$ 2-GPI binding to hypoxic endothelial cells. As indicated in Fig. 5, after 4 h hypoxia, anti- $\beta$ 2-GPI mAb bound to untreated MS-1 cells significantly more than isotype control mAb.  $\beta$ 2-GPI peptides 100 or 322 did not inhibit antibody binding to the hypoxic endothelial cell line. In contrast, anti- $\beta$ 2-GPI mAb did not bind to hypoxic MS-1 cells, which were pre-treated with peptides 296 or 305. Together these data indicated that the overlapping peptides 296 and 305 were capable of preventing  $\beta$ 2-GPI from binding to hypoxic endothelial cells. As these two peptides contain 3 Cys and may bind non-specifically, the Cys of peptide 296 were changed to Ser and used in the in vitro hypoxia assay. Similar to peptide 296, the Cys-Ser substituted peptide also attenuated  $\beta$ 2-GPI binding to the hypoxic cells.

The in vitro hypoxia studies suggested that peptides 296 and 305 may attenuate IR-induced tissue damage. To test this hypothesis, peptides were infused into C57Bl/6 mice 5 min prior to intestinal IR and mucosal damage and inflammation evaluated. Similar to in vitro results, mice which received peptides 296, 305 or 296 Cys to Ser sustained attenuated mucosal damage in response to IR (Fig. 6). In contrast, peptides 100, 181, and 322 sustained IR-induced intestinal damage similar to untreated mice (Fig. 6). Thus, peptide inhibition of  $\beta$ 2-GPI attenuates IR-induced intestinal damage.

### Domain V $\beta$ 2-GPI peptides block IR-induced intestinal inflammation

To examine the multiple pathways of inflammation involved in IR-induced damage, intestinal tissues from the peptide treated mice were examined for complement deposition, adhesion molecule expression and the macrophage marker, F4/80. As expected, IR induced C3 deposition on the intestines of C57Bl/6 mice in response to IR but not Sham treatment (Fig. 7A). Similar to injury results, peptides 100 and 322 did not significantly inhibit C3 deposition (Fig. 7A). In addition, infusion of peptides 296, 305 and 296Cys to Ser prior to IR significantly decreased C3 deposition (Fig. 7A). Similarly, the expression of adhesion molecules, CD31 and VCAM, was inhibited after treatment with peptides 296 and 296Cys to Ser but not after treatment with peptides 100 or 322 (Fig. 7B, data not shown). Expression of the mature macrophage marker increased in response to IR with or without peptide 100 or peptide 322 (Fig. 7C). Treatment with peptides 296 and 296 Cys to Ser reduced macrophage to Sham levels after treatment with peptide 296Cys to Ser (Fig. 7C). In contrast, peptide 305 which prevented injury and complement deposition did not attenuate CD31 or VCAM expression or macrophage infiltration.

The pro-inflammatory cytokines, IL-12 and IL-6, and eicosanoids, LTB<sub>4</sub> and PGE<sub>2</sub>, increase rapidly in response IR (42). Therefore, we examined the ability of peptides 296, 305 and 296 Cys to Ser to attenuate production of these inflammatory molecules. Similar to previous results, IR induced IL-12 and IL-6 production which was attenuated by protective peptides 296, 305 and 296Cys to Ser (Fig. 8A, B). Interestingly, peptide 100 also attenuated IL-6 production (Fig. 8B). However, peptide 322 did not inhibit IR-induced cytokine production (Fig. 8A,B). Thus,  $\beta$ 2-GPI binding occurs prior to IR-induced, pro-inflammatory cytokine production.

Previous studies demonstrated that IR also induces eicosanoid production within 2 hr post ischemia (36). To determine if  $\beta$ 2-GPI initiation of intestinal damage contributes to eicosanoid production, intestinal LTB<sub>4</sub> and PGE<sub>2</sub> production within the intestine was examined in mice subjected to Sham or IR in the presence or absence of the various  $\beta$ 2-GPI peptides. As demonstrated in injury, peptides 296 and 296Cys to Ser attenuated IR-induced

production of both eicosanoids while mice treated with peptides 100 and 322 sustained inflammation similar to untreated mice (Fig. 8C–D). Despite the ability to attenuate IR-induced intestinal damage, peptide 305 did not attenuate intestinal eicosanoid production (Fig. 8C–D). Together, these data indicate that  $\beta$ 2-GPI has a role in IR-induced tissue damage and initiation of inflammation. Further studies are required to determine if administration of  $\beta$ 2-GPI peptides at later time points attenuate injury and as such may provide clinically relevant therapeutics for a condition with a high mortality rate.

## Discussion

Antibody-dependent complement activation is required for initiation of IR-induced tissue damage (1,10). Although antibodies against multiple intracellular antigens have been implicated in initiating damage in response to IR, the identification of an extracellular antigen remained unclear (14,20). We hypothesized that a serum protein,  $\beta$ 2-GPI, binding to ischemic tissues is likely responsible for initiating the complement cascade. Both peptide inhibition of  $\beta$ 2-GPI activity in wildtype mice (Table II) and infusion of wildtype serum containing reduced levels of anti- $\beta$ 2-GPI antibodies into Rag-1<sup>-/-</sup> mice prevented IR-induced intestinal damage and inflammation. Thus, our results demonstrate that natural antibodies targeting  $\beta$ 2-GPI play a critical role in initiating antibody/antigen complexes required for subsequent complement activation in response to IR. In addition, these data suggest that binding of  $\beta$ 2-GPI to ischemic cells is critical for IR-induced damage and inflammation. Although many studies have associated anti-phospholipid antibodies with autoimmunity and acute graft rejection (reviewed in (43)),  $\beta$ 2-GPI and anti- $\beta$ 2-GPI antibodies also mediate reperfusion-induced organ damage.

We showed that  $\beta$ 2-GPI binding to the tissue within 15 min of reperfusion correlates with initiation of tissue damage. The binding to injured tissue also correlates with previously determined IR-induced lipid changes (44). Domain V of  $\beta$ 2-GPI is responsible for binding negatively charged substrates such as membranes containing phosphatidylserine and/or cardiolipin (45–47) which are significantly increased in response to IR (44). Thus, peptides 100 or 181 from domains II and III, respectively would not be expected to reduce damage in the IR model of tissue damage. In the analysis of domain V, mutagenesis studies suggested that the Lys286 in the CKNKEKCC sequence is critical for in vitro binding of  $\beta$ 2-GPI to cardiolipin (48). In addition, mutation of Lys284, Lys 286 and Lys287 abolished anti- $\beta$ 2-GPI binding as detected by ELISA (48). Peptide 296 contains this lysine rich sequence with three cysteine residues. Correlating with previous findings that the Cys residues were not as critical as lysine residues, peptide 296Cys to Ser also inhibited IR-induced damage and inflammation. A recent in vivo study found that a related lysine rich sequence from cytomegalovirus (KEKRKKK) inhibited antibody-induced thrombosis by competing with  $\beta$ 2-GPI (49). It is likely that a similar mechanism may be occurring in the peptide treated mice. However, protective peptide 305 contains only the last three amino acids in the CKNKEKCC sequence suggesting that additional residues are capable of binding to cells as well. Interestingly, peptide 305 did not prevent eicosanoid production indicating that distinct residues may be critical for the inflammatory response and intestinal damage.

Reperfusion is accompanied by the production of inflammatory mediators and immune cell infiltration (50) which are believed to be responsible for the subsequent systemic pathologies (1). IR-induced lipid changes result in increased arachidonic acid and subsequent production of the inflammatory mediators, LTB<sub>4</sub> and PGE<sub>2</sub> (44) which may contribute to cellular infiltration. Interestingly, anti- $\beta$ 2-GPI antibodies binding of  $\beta$ 2-GPI induced cellular infiltration and eicosanoid generation. Importantly, all these inflammatory mediators and the IR-induced pro-inflammatory cytokines were blocked by peptides 296 and 296Cys to Ser while peptide 305 inhibited IL-12 and IL-6 production but not eicosanoid



production (summarized in Table II). Together these data suggest that the inflammatory response is controlled by a larger sequence than the CKNKEKCC sequence of the lipid binding domain. Activation of complement also initiates immune cell infiltration and activation as treatment with C5a receptor antagonists attenuated neutrophil infiltration (4,6–7,50). Despite containing four complement regulatory domains,  $\beta$ 2-GPI exhibits no known complement regulating function (24). However, antibody reduction or treatment with peptides 296, 305 and 296Cys to Ser attenuated complement activation suggesting that antibody recognition of the serum protein,  $\beta$ 2-GPI initiates complement activation. As the inflammatory responses are also induced by LPS, TLR pathways involvement is also possible.

The mechanism of  $\beta$ 2-GPI binding to cells is not fully understood. Previous studies demonstrated that mice lacking specific immune regulatory proteins such as toll-like receptor 4 (TLR4) also render mice resistant to IR-induced damage (30). Despite having the proper antibody repertoire, TLR4<sup>lpsd</sup> mice remain resistant to intestinal IR-induced damage (30). One possible explanation is that anti- $\beta$ 2-GPI antibodies recognize  $\beta$ 2-GPI in conjunction with TLR4 (51–52) resulting in signaling through TLR4. This hypothesis is supported by the fact that anti- $\beta$ 2-GPI antibodies induce phosphorylation, NF- $\kappa$ B activation and TNF production by monocytes (53). Another possibility is presented by recent evidence illustrating that  $\beta$ 2-GPI binds TLR2 on endothelial cells (54). Correlating with these data, we demonstrated that peptides 296 and 296Cys to Ser inhibit IR-induced IL-12 and IL-6 as well as upregulation of adhesion molecules and subsequent increases in cellular infiltration. Thus, the lack of TLR expression may prevent intestinal damage by interfering with antibody recognition of  $\beta$ 2-GPI. In contrast, other studies indicate that  $\beta$ 2-GPI-antibody complexes interact with other proteins including Ro60 on apoptotic cells (55) or annexin II (Ma et al., 2000). Binding to either of these proteins would suggest the antigen/antibody complexes resulted in monocyte stimulation, as limited evidence exists for a transmembrane domain in either Ro60 or annexin II. Although the nature of the interaction remains unclear, binding of the serum protein,  $\beta$ 2-GPI, appears to initiate the subsequent inflammatory response during IR.

Similar to the TLR4 deficient mice, Cr2<sup>-/-</sup> mice are also resistant to IR-induced tissue damage. Despite having normal serum levels of antibodies, Cr2<sup>-/-</sup> mice do not produce the necessary antibody repertoire required for IR-mediated tissue damage (19,36,56). The lack of anti- $\beta$ 2-GPI antibodies in the Cr2<sup>-/-</sup> mice correlates with initial studies indicating that infusion of an anti- $\beta$ 2-GPI mAb was sufficient to restore injury (19). Although the exact role that CR2 plays in generating  $\beta$ 2-GPI reactive antibodies is unclear, CR2 is associated with the B cell Ig receptor and therefore may influence the selection of  $\beta$ 2-GPI reactive B cells (57). Thus, the interactions of TLR4 and CR2 with  $\beta$ 2-GPI and/or anti- $\beta$ 2-GPI antibodies remain unclear and require additional investigation.

It has been proposed that binding of  $\beta$ 2-GPI to the cell membrane exposes cryptic epitope(s) recognized by natural antibodies (58). Natural antibody recognition of  $\beta$ 2-GPI is a characteristic of APLS and results in tissue damage and fetal loss (59–61). When compared to IR in normal patients, APLS patients have significantly higher anti- $\beta$ 2-GPI antibody titers and the antibodies exhibit greater affinity for the target antigen which is suggested to result in damage (62). By recognizing stressed or damaged tissue,  $\beta$ 2-GPI recognition by anti- $\beta$ 2-GPI antibodies appears to lead to IR-induced pathology. Although CR2 may play a role in generating antibodies against  $\beta$ 2-GPI, it is unclear why, under normal immunological functioning,  $\beta$ 2-GPI elicits autoantibodies. The generation of anti- $\beta$ 2-GPI antibodies may be for removal of apoptotic cells by phagocytes. This hypothesis suggests that  $\beta$ 2-GPI binds to ischemic tissue because the membrane changes are similar to early apoptotic cells and that the process will facilitate clearance of the damaged cells (26,63). When significantly lower

concentrations of anti- $\beta$ 2-GPI antibodies exist, such as in Cr2<sup>-/-</sup> mice or in the reduced serum, the hypoxic cells are not targeted and complement activation is significantly reduced. Similarly, transfer of Cr2<sup>-/-</sup> serum or antibodies to Rag-1<sup>-/-</sup> mice did not restore IR-induced intestinal damage (36). Although the exact nature of the alterations occurring in IR or apoptotic tissues is not fully characterized, ischemia exposes changes in the lipid and/or protein composition of the membrane allowing  $\beta$ 2-GPI binding and subsequent natural antibody recognition during reperfusion.

Our previous studies indicated that IR-induced damage in Rag-1<sup>-/-</sup> mice required a combination of two IgG monoclonal antibodies recognizing  $\beta$ 2-GPI and negatively charged phospholipids (19). Importantly, neither mAb alone was sufficient to induce damage. These data suggest that IR-induced damage requires a complex of antibodies recognizing multiple antigens, including  $\beta$ 2-GPI bound to phospholipids. Based on these results, prevention of either the phospholipid changes or  $\beta$ 2-GPI binding would attenuate injury. Recently, we demonstrated that IR-induced lipid changes occur in both Rag-1<sup>-/-</sup> and C57Bl/6 wildtype mice within 15 min post-reperfusion (44). As lipid mobility is critical to cellular signaling, blocking the lipid changes may produce significant side effects. In contrast, peptide inhibition of either  $\beta$ 2-GPI binding to the lipids or antibody binding to  $\beta$ 2-GPI would prevent binding by one mAb and subsequently prevent intestinal damage. In addition, as a natural serum protein, the expected side effects may be significantly lower than lipid blockade.

Previous studies indicated that IR-induced damage is due to natural antibodies with reactivity to non-muscle myosin, glycogen phosphorylase or annexin IV. However, attenuated damage following peptide inhibition of  $\beta$ 2-GPI binding suggests that these additional target antigens may be exposed after  $\beta$ 2-GPI binding. It is possible that  $\beta$ 2-GPI binding induces a signal which leads to either apoptosis with annexin IV expression or necrosis and non-muscle myosin exposure. As specific  $\beta$ 2-GPI peptides reduced IR-induced tissue damage to Sham levels,  $\beta$ 2-GPI appears to be a critical therapeutic target for mesenteric IR. In addition, reperfusion-induced tissue damage in response to myocardial infarction, stroke, and transplantation appears to use similar mechanisms (42,64). Thus, understanding the exact role of  $\beta$ 2-GPI itself or the natural antibodies recognizing  $\beta$ 2-GPI in mediating tissue damage may lead to effective strategies of preventing reperfusion injury in multiple organs.

## Acknowledgments

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## Non-standard abbreviations include

<b>IR</b>	ischemia/reperfusion
<b>LTB<sub>4</sub></b>	leukotriene B <sub>4</sub>
<b><math>\beta</math>2-GPI</b>	$\beta$ 2-glycoprotein I (apolipoprotein H)
<b>APLS</b>	anti-phospholipid antibody syndrome

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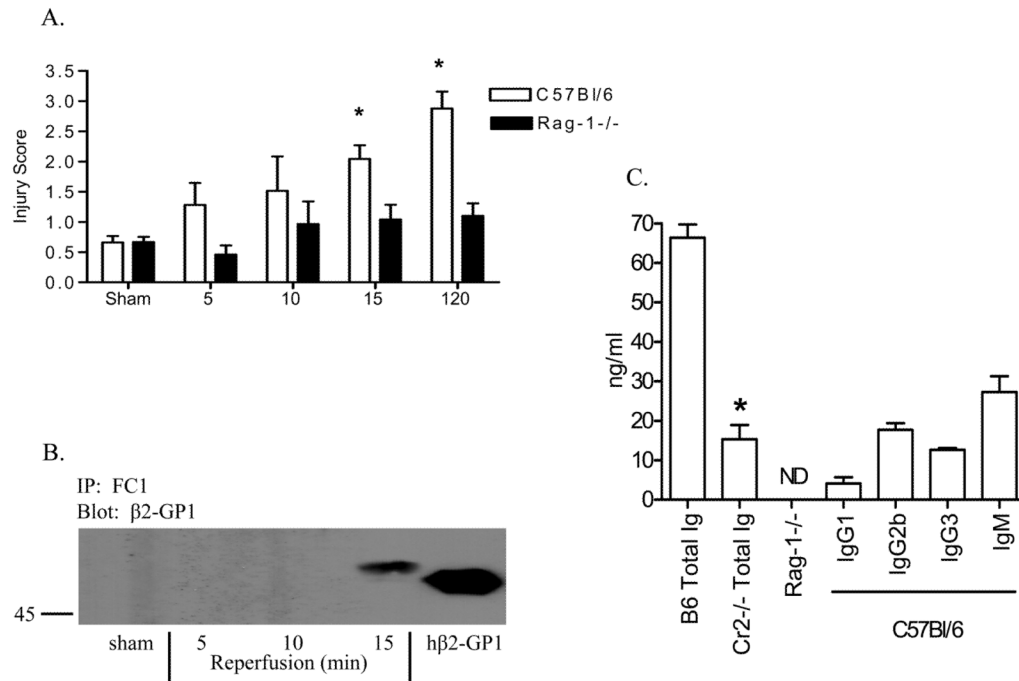
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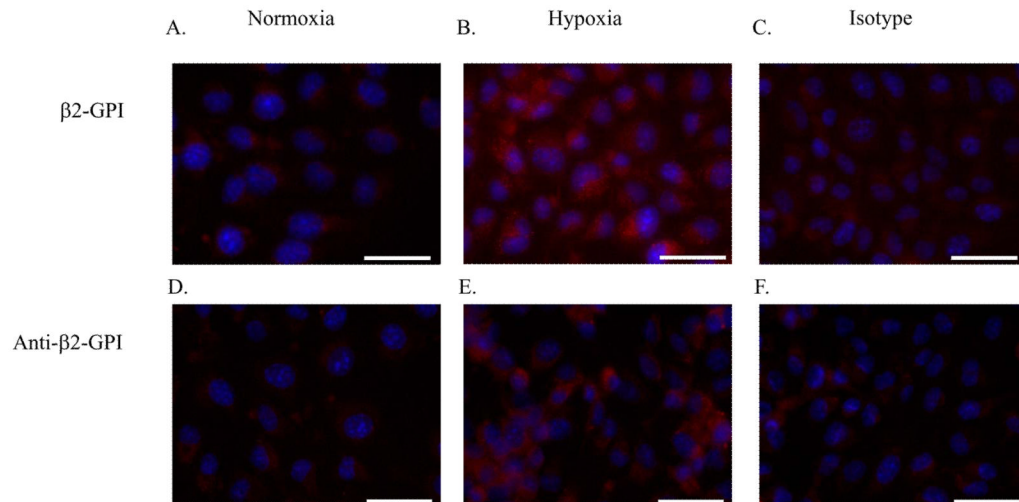


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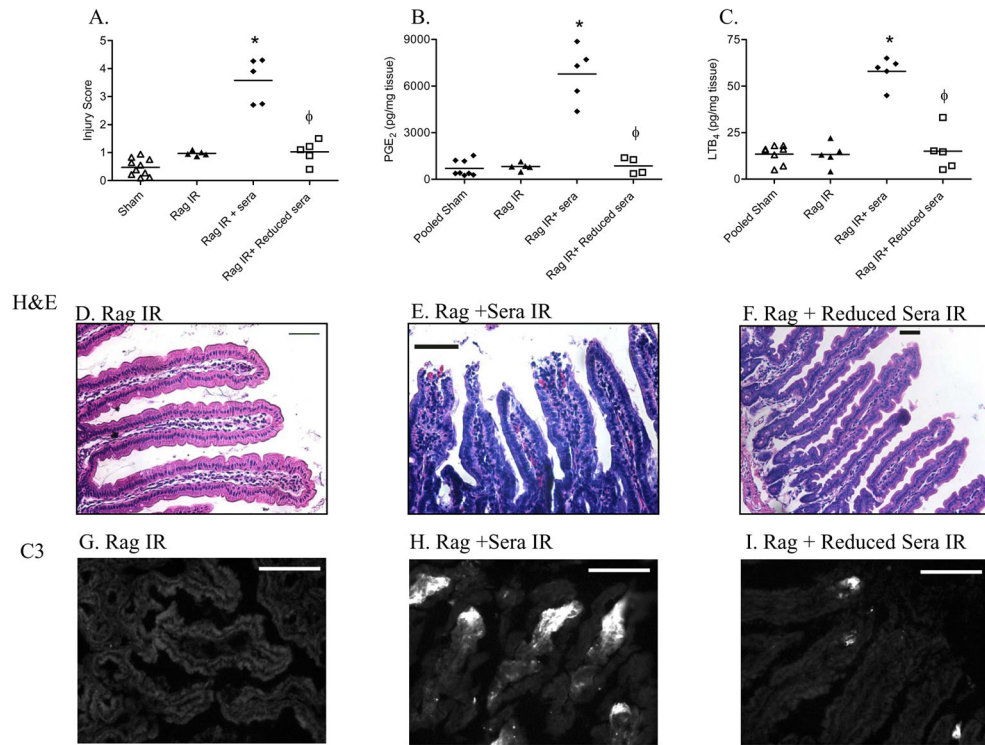


**Figure 1. The presence of  $\beta$ 2-GPI and anti- $\beta$ 2-GPI correlates with IR-induced intestinal damage**

**A.** Mid-jejunal sections collected from C57Bl/6 or Rag-1<sup>-/-</sup> mice at 5, 10 and 15 min after reperfusion or from Sham-treated mice were scored for intestinal injury (75–150 villi per animal with 3–10 animals/treatment and each treatment was performed on at least 2 separate days). **B.**  $\beta$ 2-GPI was immunoprecipitated with FC1 from tissue sections collected at 5, 10 and 15 min post-reperfusion or from Sham-treated mice and subjected to Western blot analysis. Human  $\beta$ 2-GPI (50 kDa) was run as a control for mouse  $\beta$ 2-GPI (54 kDa). Blot is representative of 4 experiments. **C.** Serum concentrations of anti- $\beta$ 2-GPI antibodies in C57Bl/6, C3<sup>-/-</sup>, CR2<sup>-/-</sup> or Rag-1<sup>-/-</sup> mice as determined by ELISA. Isotypes of the specific antibodies bound to  $\beta$ 2-GPI were determined using specific rat anti-mouse isotyping antibodies. Each bar represents the mean  $\pm$ SEM of 3 independent experiments. \* =  $p \leq 0.05$  compared to Sham.

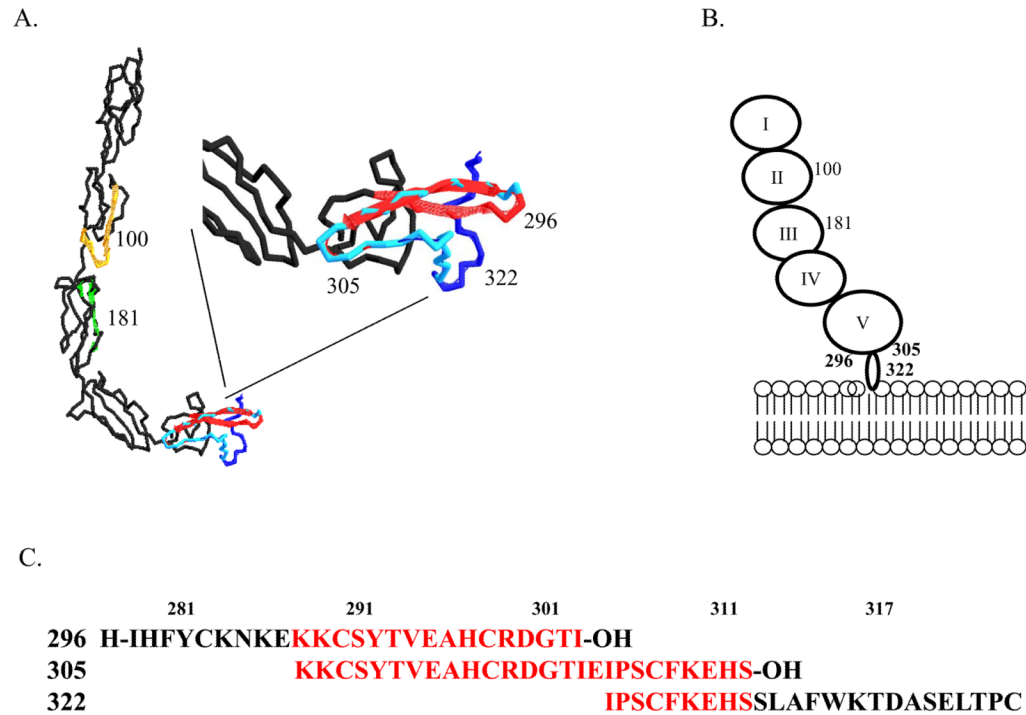


**Figure 2.  $\beta$ 2-GPI and anti- $\beta$ 2-GPI antibodies bind to MS-1 cells following hypoxia**  
 Cells were subjected to 4 hours of normoxia in media containing 10% heat-inactivated Rag-1<sup>-/-</sup> sera (A, D) or hypoxia under serum-free conditions (B–C, E–F), followed by 1 hour of normoxia in media containing 10% Rag-1<sup>-/-</sup> serum in the absence (A–C) or presence of anti- $\beta$ 2-GPI (D–E) or isotype control (F) antibody. The cells were fixed with methanol, probed with a primary anti- $\beta$ 2-GPI antibody (A–B) or isotype control antibody (C) then stained with an anti-mouse secondary or stained with secondary antibody only (Red; D–F). Slides were mounted with DAPI (Blue) to identify the nuclei. Each photomicrograph is representative of 3 experiments with 4–6 photomicrographs per treatment group in each experiment. Bar = 40 $\mu$ m.



**Figure 3. Reduction of anti-β2-GPI antibody attenuates tissue injury and inflammation**

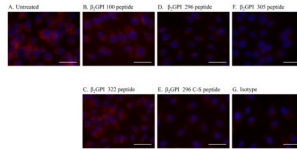
A. Mid-jejunal sections were scored (75–150 villi per animal) from Rag-1<sup>-/-</sup> mice with or without injection of C57Bl/6 sera or anti-β2-GPI antibody reduced C57Bl/6 serum prior to Sham or IR treatment. PGE<sub>2</sub> (B) or LTB<sub>4</sub> (C) production was measured in Rag-1<sup>-/-</sup> mice injected with C57Bl/6 sera or anti-β2-GPI antibody reduced C57Bl/6 serum prior to Sham or IR treatment. Values are represented as pg/mg of intestinal protein. \* =  $p \leq 0.05$  compared to Sham,  $\phi$  =  $p \leq 0.05$  compared to animals receiving non-reduced sera. Each animal is represented by an individual point with the bar representing the average. Each treatment was performed on at least 2 separate days. D–I. Representative intestinal sections H&E stained (D–F) or stained for C3 deposition (G–I) from IR-treated Rag-1<sup>-/-</sup> mice (D,G), IR-treated Rag-1<sup>-/-</sup> mice receiving C57Bl/6 serum (E, H), or IR-treated Rag-1<sup>-/-</sup> mice receiving anti-β2-GPI antibody reduced C57Bl/6 serum are shown (F, I). Microphotographs are representative of 3–4 animals stained in at least 3 independent experiments. H&E bar = 50 μm and immunohistochemistry bar = 40 μm.



**Figure 4. Location of overlapping  $\beta$ 2-GPI peptides**

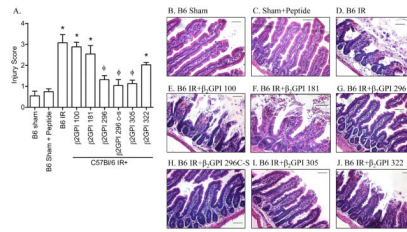
A. Ribbon diagram of human  $\beta$ 2-GPI with peptide locations identified by color, peptide 100 (gold), peptide 181 (green), peptide 296 (red), peptide 322 (dark blue) and overlapping peptide 305 (light blue). Inset is magnification of Domain V. B. Cartoon of  $\beta$ 2-GPI binding to lipid membrane with peptide locations indicated. C. Sequence identification of overlapping regions of peptides 296, 305 and 322. Red indicates regions of overlap. Peptides were designed based on the published sequences (32) to mimic the lipid binding domain and tail inserted into the lipid bilayer.





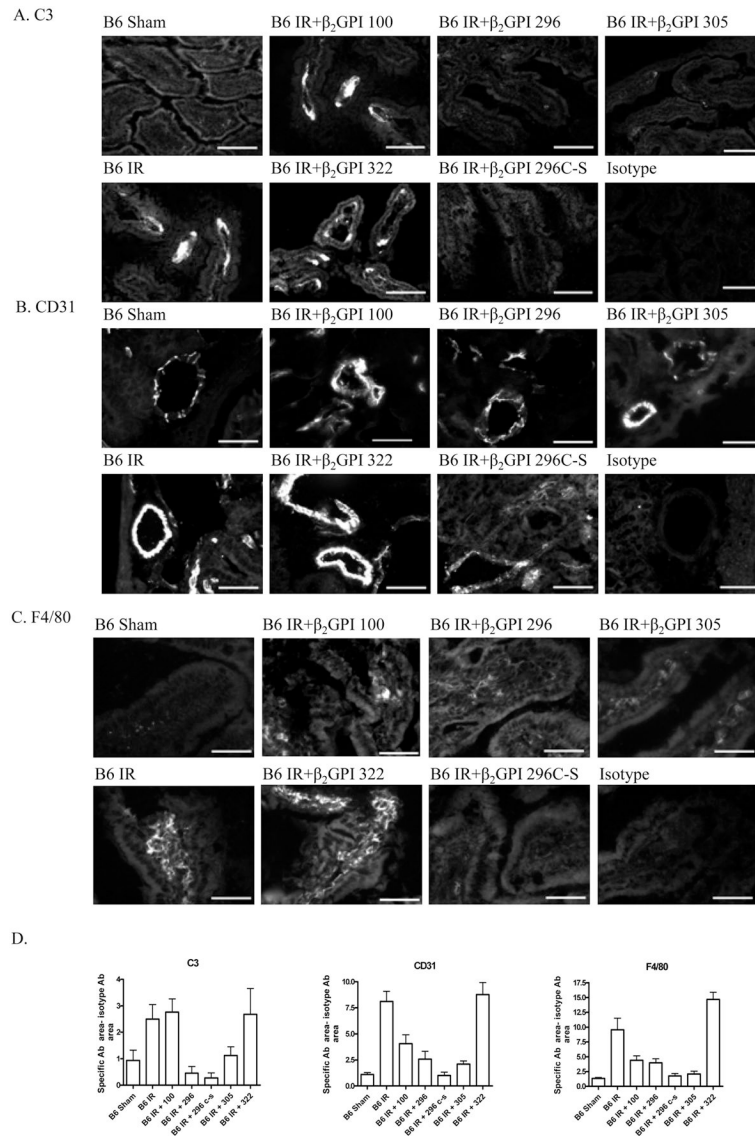
**Figure 5.  $\beta$ 2-GPI peptides inhibit anti- $\beta$ 2-GPI staining of hypoxic MS-1 cells**

Cells were subjected to 4 hours of hypoxia under serum-free conditions with (A) or without (B–G)  $\beta$ 2-GPI peptides prior to 1 hour normoxia in media containing 10% heat-inactivated Rag-1<sup>-/-</sup> sera. The cells were fixed with methanol, probed with a primary anti- $\beta$ 2-GPI antibody (A–F) or isotype control antibody (G) followed by a Texas red labeled, anti-mouse secondary antibody. Slides were mounted with DAPI (Blue) to identify the nuclei. Each photomicrograph is representative of 3 experiments with 4–6 photomicrographs per treatment in each experiment. Bar = 40 $\mu$ m.

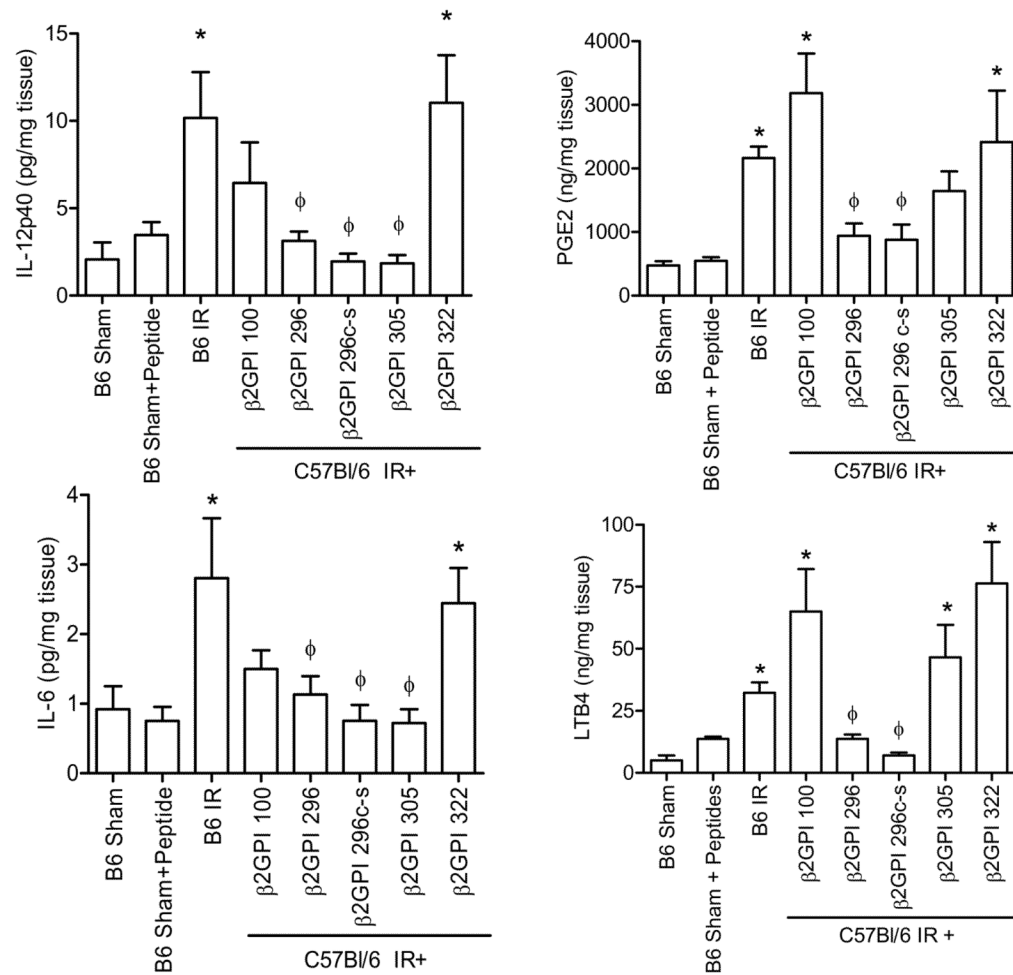


**Figure 6.  $\beta$ 2-GPI peptides attenuate IR-induced mucosal damage in wildtype mice**

A. Mid-jejunal sections were scored (75–150 villi per animal) from C57Bl/6 mice with or without injection of  $\beta$ 2-GPI peptides prior to Sham or IR treatment. B-G. Representative intestinal sections H&E stained from C57Bl/6 Sham-treated mice (B), IR-treated C57Bl/6 mice in the absence of peptide (C) or receiving  $\beta$ 2-GPI peptide 100 (D), peptide 296 (E), peptide 206Cys to Ser (F), peptide 305 (G), and peptide 322 (H). Microphotographs are representative of 3–4 animals stained in at least 3 independent experiments. Bar = 50 $\mu$ m. \* =  $p \leq 0.05$  compared to Sham + peptide,  $\phi = p \leq 0.05$  compared to IR treatment animals not receiving peptides. Each bar is representative of 3–4 animals and each treatment was performed on at least 2 separate days.



**Figure 7.  $\beta_2$ -GPI peptides attenuate IR-induced complement deposition, adhesion molecule expression and macrophage infiltration**  
 Representative intestinal sections stained for C3 (A), CD31 (B), or F4/80 (C) from Sham-treated C57Bl/6 mice, IR-treated C57Bl/6 in the absence or presence of  $\beta_2$ -GPI peptides as indicated. Microphotographs are representative of 3–4 animals stained in at least 3 independent experiments. Bar = 40 $\mu$ m.



**Figure 8.  $\beta$ 2-GPI peptides attenuate IR-induced pro-inflammatory cytokine and eicosanoid production**

IL-12 (A), IL-6 (B), PGE<sub>2</sub> (C) or LTB<sub>4</sub> (D) production was measured in C57Bl/6 mice with or without injection of  $\beta$ 2-GPI peptides prior to Sham or IR treatment. Values are represented as pg/mg of intestinal protein. \* =  $p \leq 0.05$  compared to Sham,  $\phi = p \leq 0.05$  compared to animals not receiving peptide. Each bar is representative of 3–4 animals and each treatment was performed on at least 2 separate days.

**Table I** $\beta$ 2-GPI peptide sequences

Peptide name	Sequence <sup>a</sup>	Residue numbers	MW <sup>b</sup> (Da)
100	H-KNISFACNPGFFLNG-NH2	105–118	1627
181	H-GNDTVMCTEQQN-NH2	182–193	1338
296	H-IHFYCKNKEKKCSYTVEAHC RDGTI-OH	296–320	2974
296 Cys-Ser	H-IHFYSKNKEKKSSYTVEAHS RDGTI-OH	296–320	2925
305	H-KKCSYTVEAHC RDGTIEIPSCFKEHS-OH	305–330	2969
322	H-IPSCFKEHSSLAFWKTDASELTPC-NH2	322–345	2629

<sup>a</sup> Amino acid sequence based on NCBI sequence, AAB30789, as described in the Materials and Methods.

<sup>b</sup> MW is Molecular weight



**Table II**

Summary of IR-induced injury and inflammation C57Bl/6 mice with or without peptide treatment

	B6 IR <sup>a</sup>	B6 + $\beta_2$ -100	B6 + $\beta_2$ -296	B6 + $\beta_2$ -296c-s	B6 + $\beta_2$ -305	B6 + $\beta_2$ -322
Injury <sup>b</sup>	+	+	-	-	-	+
C3 Deposition	+	+	-	-	-	+
CD31 Deposition	+	+	-	-	+	+
F4/80 Deposition	+	+/-	+/-	-	-	+
IL-12p40 Induction	+	+/-	-	-	-	+
IL-6 Induction	+	+/-	-	-	-	+
PGE <sub>2</sub> Production	+	+	-	-	+/-	+
LTB <sub>4</sub> Production	+	+	-	-	+	+

<sup>a</sup>C57Bl/6 mice subjected to IR with or without peptide treatment.

<sup>b</sup>Measure of injury or inflammation.

<sup>c</sup>+ indicates significant difference from Sham treated mice; - indicates not significantly different from C57Bl/6 sham treated mice; +/- indicates no significant difference from either C57Bl/6 mice subjected to either Sham or IR