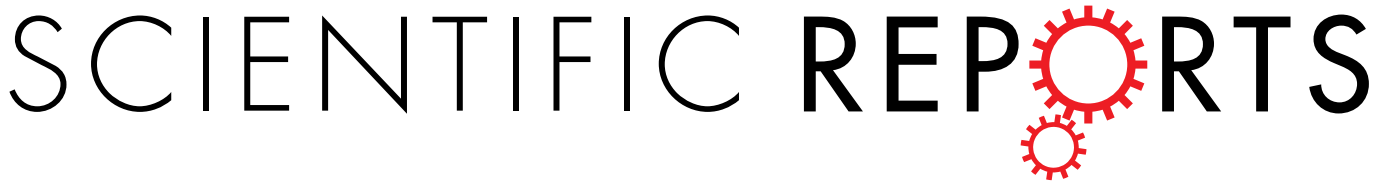

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Gram Negative Bacterial Inflammation Ameliorated by the Plasma Protein Beta 2-Glycoprotein I

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Lipopolysaccharide (LPS) is a major component of the outer wall of gram negative bacteria. In high doses LPS contributes to the inflammation in gram negative sepsis, and in low doses contributes to the low grade inflammation characteristic of the metabolic syndrome. We wanted to assess the role of beta2-glycoprotein I (β2GPI) a highly conserved plasma protein and its different biochemical forms in a mouse model of LPS systemic inflammation. Normal and β2GPI deficient mice were administered LPS through their veins and assessed for a range of inflammation markers in their blood and liver. Different biochemical forms of β2GPI were measured in normal mice given either saline or LPS. We show that β2GPI has a significant role in inhibiting LPS induced inflammation. In this study we provide some evidence that β2GPI serves a protective role in a mouse model of LPS inflammation. This resolves the controversy of previous studies which used LPS and β2GPI in test tube based models of LPS induced activation of white cells. We also highlight the potential relevance of a newly discovered biochemical form of β2GPI in LPS mediated inflammation and we speculate that this form has a protective role against LPS induced pathology.

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram negative organisms. In high concentrations in the plasma it has been implicated in the systemic inflammatory response associated with organ dysfunction in the setting of infection[1]. In low concentrations in the plasma it has been implicated in contributing to initiating obesity and insulin resistance in the metabolic syndrome by inducing a chronic inflammatory state[2,3]. LPS consists of three parts, lipid A, a core oligosaccharide, and an O side chain[4]. LPS activates both pro-inflammatory and anti-inflammatory mediators through toll-like receptor 4 (TLR4) signalling[5]. Dysregulation of both these processes can lead to hyperinflammation and immunosuppression[5]. LPS in plasma is bound by lipopolysaccharide binding protein which enables the interaction of LPS with its membrane receptor CD14[6]. LPS is subsequently transferred to the MD-2/TLR4 complex[6]. This leads to oligomerization of MD-2/TLR4 complexes resulting in the recruitment of the intracellular adaptor proteins MyD88 and TRIF[6]. MyD88 recruitment leads to sequential phosphorylation of IRAK-4 and IRAK-1, activation of TRAF-6, culminating in NF-κB activation and the generation of pro-inflammatory cytokines[6]. TRIF forms a complex with TRIF related adaptor molecule and TLR4 resulting in the activation of IRF3, and the transcription of IFN-β[6].

Beta 2-glycoprotein I (β2GPI/Apolipoprotein H) is an abundant plasma protein that is produced by the liver[7]. It is composed of five domains (DI-V), and has a molecular weight of approximately 50 kDa[7]. Domains I to IV each have two disulfide bridges, whereas domain V has three, including a disulfide bridge that incorporates the C-terminal cysteine[7]. Domain V also contains a positively charged lysine rich region, as well as a hydrophobic flexible loop segment, and both these regions are required for binding of β2GPI to negatively charged macromolecules[7].

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The domain V disulfide bond is susceptible to cleavage by the oxidoreductases thioredoxin I (TRX-1) and protein disulfide isomerase (PDI) leading to the generation of free thiols at Cysteine (Cys) 288 and Cys 326[8,9]. A large proportion of plasma β2GPI exists in the free thiol form[10]. The free thiol form has distinct properties to the oxidized form, the former protecting endothelial cells against hydrogen peroxide mediated cell death[11]. The implications being that *in-vitro* studies that look to delineate the biological function of β2GPI have limited *in-vivo* relevance if they only study the oxidized form and do not take the predominant *in-vivo* free thiol form into consideration.

A recent study has proposed that β2GPI may be able to attenuate the pro-inflammatory effects of LPS[12]. Using *in-vitro* techniques β2GPI bound to LPS, through domain V. The Kd range was between 62 nM and 23 nM depending on the LPS source[12]. Upon binding LPS, β2GPI undergoes a conformational change from a circular to an open form[12]. *In-vitro* β2GPI was able to attenuate LPS induced tissue factor activation and IL-6 release by human monocyte-like THP-1 cells and human umbilical vein endothelial cells[12]. This effect was negated with the use of receptor-associated protein (RAP), a potent inhibitor of endocytic receptors that belong to the low density lipoprotein (LDL) receptor gene family[12]. β2GPI has previously been shown to bind several members of the LDL receptor family, the most well studied being LDL receptor-related protein 8 (LRP-8), also known as apolipoprotein E receptor 2 (ApoER2)[13]. This receptor is expressed during differentiation of monocytes to macrophages[14]. These findings suggests that β2GPI may function as a scavenger protein for LPS, promoting its endocytosis by monocytes/macrophages through ApoER2[12]. Furthermore the magnitude of fever and plasma inflammatory cytokine rise post LPS injection demonstrated an inverse relationship with serum levels of total β2GPI prior to LPS administration in male healthy human volunteers[12].

In a different study β2GPI was shown to specifically bind to LPS[15]. However monocytes stimulated with LPS in the presence of β2GPI demonstrated comparable levels of pro-inflammatory cytokine production compared to cells stimulated with LPS in the absence of β2GPI[15]. A third report studying the *in-vitro* interaction of β2GPI with LPS failed to demonstrate specific binding of β2GPI to LPS[16].

In view of these contradictory *in-vitro* studies we have used C57BL/6 β2GPI−/− (deficient) mice to delineate the *in-vivo* relevance of β2GPI in LPS pathophysiology. Hence bypassing the limitations of *in-vitro* studies such as LPS contamination of β2GPI and other components of the *in-vitro* system. In has been proposed that β2GPI *in-vivo* has different conformations (open and closed)[17], as well as different post-translational redox forms (e.g. free thiol and non-free thiol)[10,11], the function of β2GPI can only be accurately delineated in an *in-vivo* setting.

Results

C57BL/6 β2GPI deficient mice have significantly higher levels of inflammatory cytokines compared to C57BL/6 wild type (WT) mice at 2 and 6 h post LPS challenge. Significantly higher levels of TNFα were noted at both 2 and 6 h post LPS injection in the β2GPI−/− mice compared to the WT mice (Fig. 1A,B). Significantly higher levels of IL-6, IFN-γ, MIP-1α,β and Eotaxin-1 (Fig. 1C–G) were detected in the β2GPI−/− compared to WT mice 6 h post intravenous LPS administration.

The levels of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17A, GM-CSF, KC, MCP-1, IL-12p40, IL-12p70, G-CSF and RANTES were either the same between WT and β2GPI−/− mice or below the detection limit of the assay (data not shown). Levels of cytokines noted in Fig. 1A–G were the same in WT and β2GPI−/− mice given pyrogen free saline.

Histological changes in the liver after LPS administration. Liver tissue was obtained at 6 h following intravenous LPS administration. β2GPI−/− compared to WT mice demonstrated increased staining with anti-Gr-1 antibody (representative images of Gr-1 staining Fig. 2A–F) consistent with increased neutrophil infiltration into the liver (211.2 vs. 90.2 (positive cells/per 10 high power fields (HPF)), $n = 5$, $p = 0.03$) (Fig. 2G). No significant difference was detected in the number of apoptotic cells in the livers of the β2GPI−/− mice 6 h post LPS compared to WT at the identical time point. There was a significant increase in neutrophil infiltration in the β2GPI−/− compared to WT livers when mice were administered pyrogen free saline $n = 5$, $p = 0.03$ (Fig. 2G).

β2GPI deficient mice have a significant increase in serum alanine aminotransferase (ALT) following LPS administration. Serum ALT, a specific marker of liver damage, was significantly increased in β2GPI−/− mice following LPS injection compared to injection of β2GPI−/− mice with pyrogen free saline $n = 8$, $p = 0.02$ (Fig. 3). There was no significant difference seen in WT mice (Fig. 3).

LPS administration to C57BL/6 WT mice decreased total, but increased free thiol β2GPI serum levels. Total β2GPI levels were significantly decreased in WT mice at 6 h after LPS injection compared to WT mice injected with pyrogen free saline, (86.1 ± 8.5 vs. 101 ± 10.28 mean (μg/ml) ± SD, $n = 9$, $p = 0.004$) (Fig. 4A). In contrast. the percentage of serum β2GPI in the free thiol form significantly increased after LPS injection compared to mice injected with pyrogen free saline, 108.4 ± 13.18 vs. 83.5 ± 16.94, mean (% of pooled normal standard) ± SD, $n = 9$, $p = 0.003$) (Fig. 4B). As expected there was no detectable β2GPI or free thiol β2GPI in the serum of β2GPI deficient mice (data not shown).

Treatment of oxidized β2GPI with reduced thioredoxin (TRX)-1 in the presence of LPS increases free thiol generation in β2GPI. In view of the increase in the percentage of free thiol β2GPI demonstrated in the serum of LPS treated mice compared to the decrease in total β2GPI levels, we assessed the *in-vitro* effect of LPS on TRX-1 induced free thiols in β2GPI. LPS but not lipid A at equivalent concentrations significantly

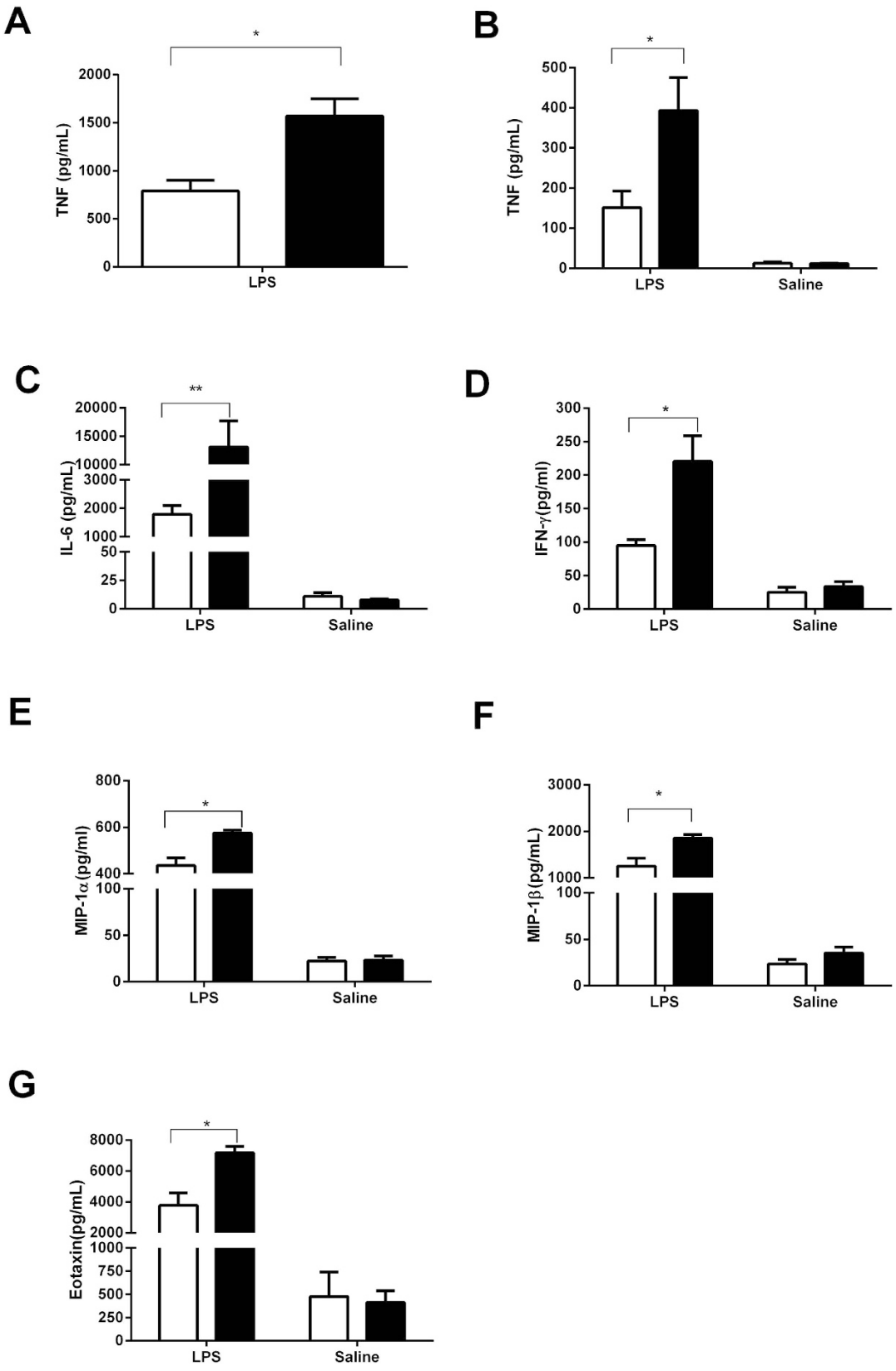

Figure 1. (**A**–**G**) β2GPI deficient mice had a significant increase in plasma inflammatory cytokines at 2 h and 6 h time points following LPS challenge. (**A**) TNFα at 2 h (**B**) TNFα at 6 h. For IL-6, IFNγ, MIP-1α, MIP-1β and Eotaxin (**C**–**G**) at 6 h. (□) = WT = Wild Type mice, (■) = β2GPI−/− = β_2-glycoprotein I deficient mice. Mann-Whitney test n = 5 *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

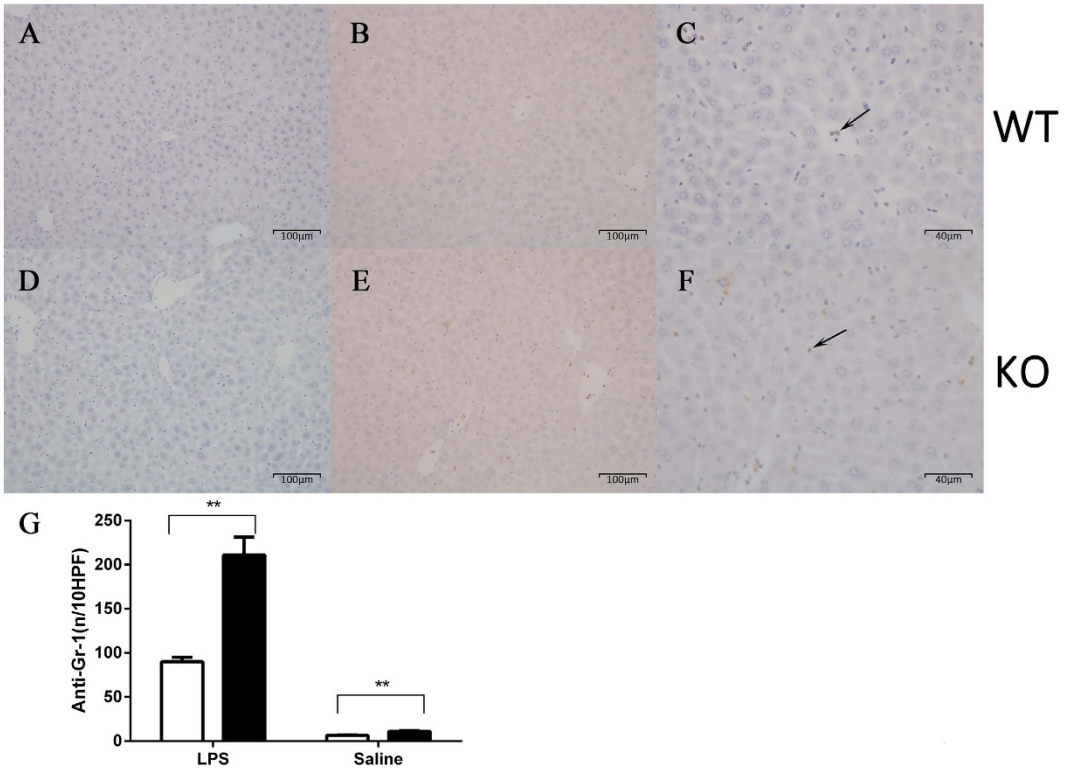

Figure 2. Representative images of livers immunostained for Gr-1. WT (**A**–**C**); (**A**) Normal Saline (**B**) LPS at 200× magnification (**C**) LPS at 400× magnification. β2GPI−/− (**D**–**F**); (**D**) Normal Saline (**E**) LPS at 200× magnification (**F**) LPS at 400× magnification. Arrows indicate representative positive immunostaining for Gr-1 (brown staining). Quantitation of inflammatory cells staining for Gr-1 (granulocyte marker). (**G**) The number of anti-Gr-1 positive cells per 10 high power fields (HPF) at 400× magnification. Data represent mean ± SEM, n = 5 ***$p < 0.001$. Mann-Whitney test.

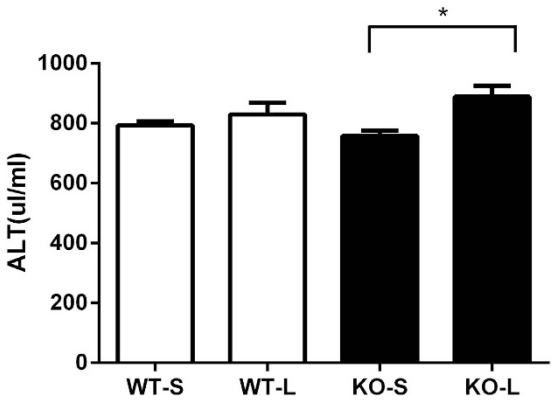

Figure 3. β2GPI deficient mice have an increase in serum ALT following LPS administration. (□) = WT = Wild Type mice, (■) = β2GPI−/− = β_2-glycoprotein I deficient mice. n = 8 *$p < 0.05$, S = Pyrogen Free Saline, L = LPS. Mann-Whitney test.

increased free thiols in β2GPI (Fig. 5). There was no free thiol β2GPI generation when β2GPI was incubated alone or with LPS in the absence of TRX-1.

Discussion

The liver plays a major role in the clearance of LPS from blood, removing from the plasma up to 85% of LPS within 15 min of intravenous administration[18]. LPS is scavenged by hepatic sinusoidal macrophages (Kupffer cells) and hepatocytes[19,20]. Both cell types express the TLR4/CD14/MD-2 complex and respond to LPS with activation of NF-κB and MAPK, leading to rapid secretion of pro-inflammatory cytokines[21]. Tumour necrosis factor alpha (TNFα) concentrations have been reported to peak within 2 h of LPS challenge[22]. In C57BL/6 WT mice

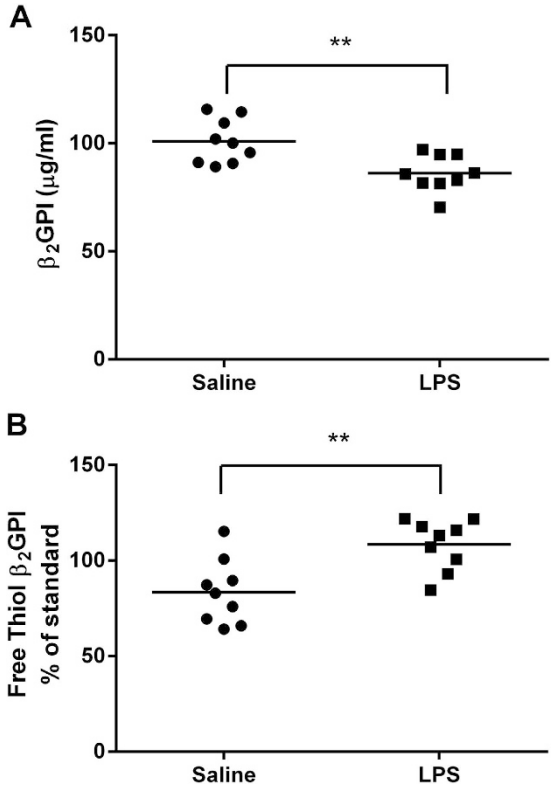

Figure 4. Total and free thiol β2GPI levels in mice following LPS injection. LPS decreased the (**A**) total but increased (**B**) free thiol β2GPI levels at 6 hrs after injection of LPS 1 μg/g body weight compared to injection of pyrogen free saline. n = 9 **p < 0.01. Unpaired two tailed Students t-test.

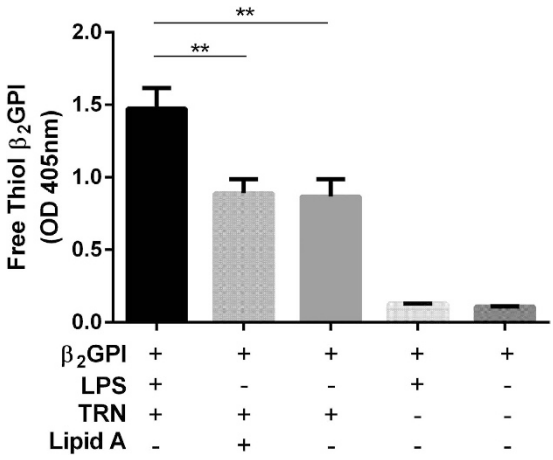

Figure 5. LPS but not Lipid A increases free thiol generation in β2GPI in the presence of TRX-1. Free thiol β2GPI generation increased only when β2GPI was incubated with LPS, but not Lipid A or PBS in the presence of reduced thioredoxin-1 (TRX-1). There was no free thiol β2GPI generation when β2GPI was incubated with LPS or PBS in the absence of TRX-1. Data are mean ± SEM, n = 3 **p = <0.01. One Way ANOVA with Tukey's multiple comparison test.

given a sublethal dose of LPS pro-inflammatory cytokines and chemokines peaked at 6 h post LPS injection (except TNFα)[23]. Both TNF-α and IL-1 can act in an autocrine and paracrine manner to regulate their own biosynthesis and that of IL-6 in the liver[22]. In our current study β2GPI deficient, compared to WT mice, have significantly higher TNFα levels at 2 and 6 h following LPS challenge and, as would be expected, TNFα levels peaked at 2 h. Serum protein levels of the pro-inflammatory cytokines IL-6, IFN-γ and the chemokines MIP-1α and MIP-1β were also significantly increased in β2GPI−/− compared to WT at the 6 h time point after the intravenous administration of LPS. On histological analysis the liver tissue of β2GPI−/− mice administered LPS displayed elevated levels of infiltrating anti-Gr-1 positive inflammatory cells within the liver parenchyma compared to WT mice. The β2GPI−/− mice administered LPS had

an increase in serum levels of ALT which was not seen in the WT suggesting that there is a degree of liver injury. Collectively these findings support the concept that β2GPI in plasma binds to LPS and diverts the LPS molecules from activating TLR4 on cell surfaces including hepatocytes and Kupffer cells, thus ameliorating the subsequent activation of downstream transcription factors such as NF-κB that induce cascades of pro-inflammatory cytokine and chemokine production and release. Interestingly we noted that there was a significant increase of neutrophils in the liver of β2GPI deficient compared to WT mice given pyrogen free saline. This raises the possibility of low grade LPS translocation to the liver from the gut microbiome inducing increased neutrophil infiltration in the absence of β2GPI. Metabolic endotoxemia is the phenomenon of increased translocation of LPS from the gut microbiome into the systemic circulation in the setting of a high fat diet[3].

It is pertinent to note that β2GPI has been associated with a protective effect against obesity in humans and other animal species[24,25]. Though the exact mechanism(s) has not been determined we speculate that β2GPI may protect against the inflammatory effects of metabolic endotoxemia by neutralizing LPS.

In this study we also demonstrate that the administration of approximately 1 μg/g of body weight LPS to WT mice, when compared to WT mice administered pyrogen free saline, resulted in a drop of approximately 15% of total serum β2GPI levels in the LPS treated group, from 101 μg/ml to 86 μg/ml. Total human β2GPI responses are more sensitive to LPS administration as it was noted there was a reduction of 25% of baseline total plasma β2GPI levels within 30 min of administration of 4 ng/kg body weight of LPS to healthy adult male volunteers[12]. In the study involving human volunteers it was proposed that the drop in total β2GPI levels is due to the uptake of the β2GPI-LPS complex by monocytes/macrophages involving the ApoER2 receptor[12]. It is apparent however that the magnitude of the drop in total β2GPI levels is out of proportion to the relatively small amounts of LPS administered when taking into account a 1:1 stoichiometry of the β2GPI and LPS interaction. This suggests that it is not only β2GPI bound to LPS that is removed from the circulation, but also β2GPI not attached to LPS. We propose that it is the drop in the non free thiol form of β2GPI (oxidized β2GPI) that is likely to account for the fall in total β2GPI levels. Total β2GPI plasma levels are the sum of free thiol plus non-free thiol β2GPI levels. In the study by Agostinis *et al.* oxidized (perchloric acid treated) human β2GPI when administered to mice intravenously promptly localized in an intracellular location with a granular pattern in the liver[26]. These findings suggest that the liver may be the major organ responsible for the drop in total plasma β2GPI levels post LPS administration by taking up β2GPI that is oxidized plus also the β2GPI fraction that is bound to LPS. The finding of a higher percentage of free thiol serum β2GPI post LPS injection, despite the concomitant reduction in total β2GPI levels, suggests that free thiol β2GPI may mediate an important role in modulating LPS pathophysiology.

We have previously demonstrated that free thiol β2GPI can be generated when oxidized β2GPI is treated with TRX-1[8]. Furthermore, it was demonstrated that free thiol β2GPI, once generated was able to protect endothelial cells *in-vitro* from hydrogen peroxide induced cell injury[11]. In the current study, conversion of free thiol β2GPI by TRX-1 was upregulated by the non-lipid A component of LPS. There are likely to be additional mechanisms that allow oxidized β2GPI to be converted to free thiol β2GPI in the setting of LPS exposure. It has been noted in a previous study that the disulfide reducing enzyme PDI is upregulated and expressed on the surface of splenocytes when mice are exposed to LPS[27]. We have previously shown that like TRX-1, PDI is able to reduce the Cys288-Cys326 disulfide bond in β2GPI[9]. Whether other thiol reducing enzymes secreted during LPS stimulation are able to act on β2GPI remains an open question. One possible candidate is gamma interferon-inducible lysosomal thiol reductase (pro-GILT) which is secreted by monocytes treated with LPS[28].

The administration of LPS to mice in the study by Metcalfe *et al.* was noted to lead to the generation of free thiols in a number of diverse proteins on the surface of cells involved in B and T cell regulation and activation, suggesting the existence of a generic, extracellular, cell surface 'redox-regulator' mechanism in the setting of immune activation[27]. It is pertinent to note that both CD14 and MD-2 which form an important part of the LPS-TLR4 recognition/activation complex have been noted to have free thiols, as does LRP-8/ApoER2[27,29]. We speculate that the dampening of the LPS pro-inflammatory response seen in the WT compared to the β2GPI−/− mice may be due to the ability of free thiol β2GPI to potentially interact *in vivo* with the free thiols present in molecules such as CD-14 or MD-2 or ApoER2, perhaps leading to the formation of covalent disulfide bonds between β2GPI and the respective molecules. For example, if our hypothesis is correct, the interaction between free thiol β2GPI and free thiol ApoER2, may allow for more efficient scavenging of β2GPI-LPS complexes. The interaction between free thiol β2GPI and free thiol MD-2 we speculate may inhibit LPS induced cell activation. In the study by Mancek-Keber *et al.* it was noted that compounds that form a covalent bond with MD-2 through the free thiol at Cys133 were able to inhibit LPS signaling[29]. Cys133 is located in the hydrophobic pocket of MD-2[29], raising the possibility that the hydrophobic flexible loop within domain V of β2GPI may play a role in allowing access of the nearby β2GPI domain V free thiols Cys288 and Cys326. Such speculations warrant further investigation in order to precisely delineate how β2GPI dampens LPS pro-inflammatory responses. This has relevance not only in understanding mechanisms by which hyperinflammation in the setting of severe gram negative sepsis and septic shock can be abated, but also in terms of understanding how low grade endotoxemia encountered in the setting of metabolic syndrome, and which contributes to low grade systemic inflammation[2], may be attenuated by understanding the role of redox β2GPI interacting with LPS.

Methods

Materials. *E. coli* LPS, (serotype 0111:B4), haematoxylin and eosin (H&E), bovine serum albumin (BSA), Tween 20 were purchased from Sigma (Sigma-Aldrich Inc, St Louis, MO). Lipid A was from Hycult biotech (Uden, The Netherlands). Bio-plex Pro™ mouse cytokine 23-plex kit was purchased from Bio-Rad (Hercules, CA). Affinity purified murine IgG2 anti-β2GPI monoclonal antibody (moAb) (4B2E7) and affinity purified rabbit polyclonal anti-β2GPI antibody were produced as previously described[30,31]. Isotype control murine IgG2 and rabbit polyclonal IgG and 70 μm nylon mesh were purchased from BD PharMingen (San Diego, CA). Streptavidin-HRP, anti-rabbit HRP, anti-mouse HRP and anti-goat HRP were purchased from Dako (Carpinteria, CA).

N-(3-maleimidylpropionyl) biocytin (MPB) was from Life Technologies (Grand Island, NY). PolyScreen polyvinyldiethylene fluoride (PVDF) transfer membrane, Western blot chemiluminescence reagents were purchased from GE Healthcare (Bucks, UK). An alanine aminotransferase activity assay kit was from Sigma. Streptavidin coated plates were from Thermo Fischer Scientific (Waltham, MA). Recombinant human TRX-1 and sheep polyclonal anti-mouse β2GPI was from R&D Systems (Minneapolis, MN), recombinant thioredoxin reductase (TRX-R) was from Vital Diagnostic (Lincoln, RI), and NADPH was from Merck (Billerica, MA).

Anti-mouse Gr-1 antibody, Anti-Ig HRP detection kit were purchased from BD Bioscience. DeadEnd™ Fluorometric TUNEL System was purchased from Promega (Madison, WI).

Mice. Male C57BL/6 (WT) mice, 8–9 weeks old, were purchased from the animal resources centre (ARC) (Perth, WA, Australia). C57BL/6 β2GPI−/− mice were generated in our laboratory and have been previously reported[32]. All mice were housed in a specific pathogen free environment and health status regularly monitored. Male mice were injected intravenously (IV) with 1 μg/gram body weight (gbw) with *E. coli* LPS (Sigma-Aldrich) in sterile saline or the same volume of saline. 6 h post injection, mice were euthanized and approximately 1 ml of blood was collected by cardiac puncture. Serum was collected by centrifugation of clotted blood at 1500 *g* for 10 min. The livers were removed, weighed and fixed in 10% formaldehyde. All animal studies were approved by the Animal Ethics Committee of the University of New South Wales. All methods were performed in accordance with the University of New South Wales Animal Care Ethics Committee guidelines.

Histopathological examination. To evaluate the histological changes in each group, the right lobe of the liver from C57BL/6 and β2GPI−/− mice were fixed in 10% neutral phosphate-buffered formalin solution for 24 h immediately after euthanasia, embedded in paraffin, and cut into 5 μm thick sections. Tissue sections were then stained with either H&E or with anti-Gr-1 antibody to detect and quantitate neutrophils and monocytes or with DeadEnd Fluorometric TUNEL System to assess and quantitate apoptotic cells according to the manufacturers' instructions. Anti-Gr-1 and apoptotic cells were quantified by counting in ten random high power fields at 400× magnification by an observer blinded to the β2GPI status of the mice and treatments.

ELISA for total and free thiol β2GPI. A sandwich ELISA for quantifying total β2GPI levels in mouse serum samples was performed based on a previously published method[10]. Briefly, a high binding 96 well plate was coated overnight at 4 °C with sheep polyclonal anti-mouse β2GPI (10 nM/well). Plates were washed 4 times. Unoccupied sites were blocked with 2% BSA in PBS-T. Following washing, 100 μl of rabbit polyclonal anti-mouse β2GPI was added (10 nM/well, diluted in 0.25% BSA/PBS–0.1% Tween 20 (PBST)) and then 100 μl of mouse serum diluted 4,000-fold in PBST was coincubated for 1 h at RT. After washing 4 times, AP-conjugated goat anti-rabbit IgG was added (1:1,500 dilution) and incubated for 1 h at RT, and samples read at OD 405 nm after addition of chromogenic substrate.

The relative amount of β2GPI with free thiols in mouse serum was performed as previously described[10,11].

Thioredoxin-1 reduction of oxidized β2GPI. Human recombinant TRX-1 (3.5 μM) was incubated for 1 h at 37 °C with TRX-R (500 nM) plus NADPH (200 μM) diluted in Hanks Balanced Salt Solution. TRX-R activated TRX-1 was diluted with an equal volume of β2GPI (4 μM) with LPS (10–1000 pg/ml) or Lipid A and PBS as control and incubated for another 1 h at 37 °C. β2GPI incubated with LPS or PBS in the absence of TRX-1, TRX-R and NADPH were further controls.

These reactions were performed under argon gas at all times to maintain proteins in their free thiol state. Free thiol β2GPI was labeled by MPB (200 μM) for 30 min at RT in the dark with agitation. After MPB labeling, mixtures were added to β2GPI deficient human plasma at a final concentration of 200 μg/ml. Unbound MPB was then removed by acetone precipitation. The protein pellet was resuspended in PBS-0.05% Tween 20 (final dilution 100 fold). The samples were then diluted a further 40 fold, added in duplicate to a streptavidin coated 96 well plate (100 μl/well), and incubated for 90 min at RT. Prior to addition of MPB labeled serum samples, streptavidin coated plates were washed 3 times with PBST and blocked with 2% BSA/PBST. After washing 3 times with PBST, a rabbit polyclonal anti-β2GPI was added (25 nM) and incubated for 1 h at RT. After 3 further washings with PBST, AP conjugated goat anti-rabbit IgG (1:1,500) was added for 1 h at RT and samples read at 405 nm after addition of chromogenic substrate.

Cytokine Assays. Cytokine assays were performed at 2 and 6 h time points following LPS or pyrogen free saline injection. At 2 h the levels of proinflammatory cytokines IL-6, MCP-1, IFNγ, TNFα, IL-12p70 and anti-inflammatory cytokine Il-10, were quantified in the plasma sample using BD Cytometric Bead Array (CBA) Mouse Inflammation Kit and BD FACSCantoll according to manufacturer's instructions.

At 6 h the levels of various cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α) were quantified by Bio-plex Pro™ mouse cytokine 23-plex Group I kit according to the manufacturer's instructions. Briefly, 50 μl of diluted samples were incubated with 50 μl of the magnetic beads covalently coated with Abs specifically reacting with each of the analytes. Discrimination of individual analytes within a multiplex suspension was achieved by fluorescence with a distinct spectral address. The suspension was incubated for 30 min at RT in the dark on a shaker at 850 rpm. After washing 3 times, 25 μl of biotinylated detection Abs were added to create a sandwich complex and incubated at RT for 30 min in the dark on a shaker at 850 rpm. After washing a further 3 times, 50 μl of streptavidin phycoerythrin (SA-PE) conjugate was added and incubated for 30 min in the dark with agitation followed by 3 washes and 125 μl washing buffer was added into each

well to resuspend the sandwich complex. Data were acquired and analyzed on a Bio-Plex 200 System (Bio-Rad Laboratories). The minimum detection level for each analyte was 1 pg/ml.

Statistics. Graph Pad Prism 6 evaluation software (Graph Pad, San Diego, CA) was used for data processing and analysis. Values for all measurements are expressed as mean ± standard error of the mean (SEM). One-way ANOVA with the Tukey's multiple comparison test was used when there were more than 2 groups to compare. The unpaired two tailed Student's t test was used to evaluate significant differences when two groups were being compared when the data was normally distributed. The Mann-Whitney test was used to evaluate for significant differences between 2 groups when the data was non-parametric. p values of <0.05 were considered statistically significant.

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Author Contributions

S.A.K. and B.G. conceived the project, designed experiments and analyzed data. S.Z., M.Q., F.E.-A., Y.W., S.D., G.C., J.C.W. and J.B. designed, performed experiments and analyzed data. S.Z., M.Q., F.E.-A., Y.W., S.D., G.C., L.C., D.Y., J.C.W., J.B., S.A.K. and B.G. had input in writing the paper.

Additional Information

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