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Haptoglobin improves acute phase response and endotoxin tolerance in response to bacterial LPS

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ABSTRACT

Sepsis is characterized by delayed acute phase response and lowered immune tolerance in patients. Acute phase serum proteins, like Haptoglobin (Hp), have been associated with increased mortality in bacteria mediated acute lung inflammation and sepsis in neonates. However, it's direct role in modulating the immune response by regulating pro-inflammatory mediators leading to immune tolerant state and if gender affects its expression levels during bacterial infection, especially in blood has not been fully explored. To understand its specific role in endotoxin-mediated immune response, we investigated the correlation between the rise in Hp levels on bacterial infection and its influence on the expression of pro-inflammatory mediators in male and female Whole blood (WHB) and PBMCs. Here, we observed pathogen-specific and gender-specific expression of Hp. Gonadal steroid hormones differentially influenced the Hp expression in LPS-induced WHB, where the addition of Estrogen increased Hp expression, with suppression of TNFα, in both genders. Further on evaluating, the influence of Hp on TNFα expression in endotoxin tolerance (ET), we show that increased Hp levels directly reduced TNFα expression in ET models. Interestingly, blockade of secreted Hp significantly reversed the (ET) state, confirmed by a significant rise in TNFα expression in both *ex vivo* and *in vitro* ET models, indicating a possible feedback inhibition by Hp on inflammatory mediators like TNFα. We also investigated the role of PKCδ in the regulation of LPS induced secretion of acute phase proteins (Hp) in serum, where inhibition of PKCδ, reduced secretion of anti-microbial proteins in response to LPS shown by restored bacterial growth. These findings clearly highlight the crucial role of Hp in maintaining immune tolerance via suppressing the pro-inflammatory mediators and also in preventing bacterial proliferation in blood during infection.

1. Introduction

Acute phase reaction (APR) initiated due to rise in cytokine levels by monocytes and other tissue macrophages in response to bacterial infection, tissue injury, trauma or post-surgical infection plays a critical role in counteracting sepsis progression. Physiological response of the immune system to sepsis may vary with the patient, causative organism, site of infection and in some cases even with gender making it difficult to devise a strategy for diagnosis and treatment [1]. One of the major reasons for failure of standard antimicrobial treatment is the inability to clear bacterial toxins or particles from blood, leading to a rise in pro-inflammatory immunological response. The early death of sepsis patient has been attributed to either drastic rise in serum pro-inflammatory cytokines or delayed anti-inflammatory response [2]. Moreover, interactions between different leukocyte subsets are important for the coordinated response to septic stimuli and to T-cell immunosuppression in particular. The cytokine network and its regulation of APR are central in this respect. Hepatic expression of acute phase proteins(APP) is initiated by increased TNFα, IL-1 in response to infection, leading to rise in the second wave of cytokines which activates release of the stored APP from blood monocytes and neutrophils during APR [3,4].

Proteomic studies on serum protein profiles of sepsis patients have identified several positive and negative acute phase proteins (APP) to be associated with sepsis progression [5–9]. Elevated levels of one such positive acute phase protein, serum Haptoglobin (Hp) showed decreased in-hospital mortality in sepsis patients, whereas an increased risk of mortality was associated with elevated levels of free-Hemoglobin in circulation due to low levels of Hp [10,11]. IL-6 produced in response to TNFα and IL-1β is reported to be the major inducer for Hp

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expression. Hp protein is a tetrameric glycoprotein consisting of two α-(18–20 kDa) and two β-chains (40–45 kDa). Three different phenotypes of Hp have been identified among the human population - Hp1-1, Hp2-2, and the heterozygous Hp2-1. The polymorphism is related to heterogeneity in the α-chain [12]. Anti-inflammatory role of Hp has been attributed to its antioxidant activity, where Hb binding to Hp prevents heme and iron release, thus reducing the generation of ROS [13]. We had earlier reported that a decreased level of serum Hp and delayed APR at early stages is associated with an increased risk of mortality in males with *K. pneumoniae* induced bacterial sepsis [14].

Hematological analysis of sepsis patients showed that impaired immune responses are characterized by enhanced apoptosis and dysfunction of lymphocytes, impaired phagocyte functions and decreased *ex vivo* cytokine production. Studies by Nikolaos *et al*provided evidence that decreased cytokine production in LPS stimulated PBMCs lead to immunosuppressed state and was associated with increased risk of death in sepsis patients [15]. Although there is convincing evidence that proinflammatory cytokines play a major role in rapid progression of the disease, the exact mechanisms by which APP regulate the immune reactions that trigger the cytokine release via PBMCs in blood are not fully understood. Several therapeutic approaches have been reported to use animal models to study the pathophysiological changes and hematological alterations of sepsis. However these attempts to mimic the sepsis-like conditions targeting the pro-inflammatory mediators in animal models have failed to correlate with the *in vivo* conditions of sepsis patients. One of the reasons reported is the difference in the pro- and anti-inflammatory cytokines between animals and humans [16]. Human blood models used for virulence and host-immune response studies have reported to be successfully correlated with the *in vivo* human conditions and patients. Hence, *ex vivo* human whole blood model (WHB) of infection has been the best used alternative to an animal model for studying sepsis immunological response to understand the role of blood proteins [17,18].

Elevated Hp levels were reported to be an early prognostic marker in neonatal sepsis [19,20]; however, its efficiency as a sepsis marker and how it modulates the immune response by decreasing pro-inflammatory cytokine levels during bacterial infection and/ or inflammation needs to be evaluated. In the present study, we addressed these questions to understand if Hp expression is influenced by different bacterial pathogens and steroid hormones. For instance, variations in APR have been previously reported between male and female patients, but the effect of estrogens and testosterone on Hp expression in response to bacterial infection in blood and its influence on cytokine levels have not been investigated. We replicated immunosuppression state in PBMCs and hepatic cells by creating endotoxin tolerance to reevaluate the immunomodulatory role of Hp on pro-inflammatory cytokine levels (TNFα) in bacterial infection. Further, we also studied the role of PKCδ in the secretion of APP such as Hp and regulating secretion of antimicrobial proteins on LPS stimulation. Although this *ex vivo* system cannot accurately correlate with inflammatory processes in the whole body, employing WHB to a major extent mimics and evaluates the capacity of PBMCs in whole blood to synthesize and secrete Hp in response to LPS stimulation, especially in early stages providing substantial evidence for Hp as one of the major anti-inflammatory and antibacterial proteins secreted during APR in response to bacterial infection.

2. Materials and methods

2.1. Blood culture

Blood was collected from healthy volunteers (n = 6) with informed consent. All the blood samples collected were age, gender and blood group matched to eliminate any heterogeneity in the study. Exclusion criteria were lack of informed consent, age younger than 18 years, pregnancy, fever, and infection, intake of antibiotics or anti-inflammatory medication during the study.

Undiluted whole blood (WHB) was cultured in flat-bottomed 24-well culture plates (Thermo Scientific™ Nunc™, USA) at a volume of 900 μL per well prior to the addition of test agents. All test agents were diluted with RPMI 1640 medium (Invitrogen, USA) supplemented with 0.1% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 mg/mL streptomycin (all chemicals were purchased from Sigma Aldrich, India) and prepared freshly from the stock. Blood Cultures were incubated at 37 °C in a 5% CO_2 atmosphere. At each indicated incubation time point, cultured cells were harvested, centrifuged at 1500 × g for 5 min. Subsequently, peripheral blood mononuclear cells (PBMC) were collected for RNA isolation, whereas the supernatants/ serum were stored at −80 °C for further western blot analysis.

2.2. Exposure to differential bacterial LPS and gonadal steroid hormones

Gram-negative bacteria *Klebsiella pneumoniae* (ATCC700603), *Escherichia coli* (ATCC25922), *Salmonella typhi (ATCC25567), Proteus mirabilis* (ATCC25933) *and Pseudomonas aeruginosa* (ATCC27853) were obtained from ATCC. The bacterial cultures were cultured and maintained in nutrient agar/broth (Himedia, India). Cold ethanol magnesium precipitation procedure developed by "Darveau and Hancock", additional steps modified by "Eugene *et al"* using Tri-reagent for isolation of whole LPS from bacterial cells was employed [21,22]. The concentration of isolated LPS was determined by Standard KDO method [23,24] (Sigma, India). For WHB treatments, 900 μL blood was cultured separately in 24 well plates, 10 ng of each isolated bacterial LPS diluted in RPMI were added to per mL blood and incubated for 12 h. For steroid hormone exposure, 900 μL of male and female WHB was cultured separately in 24 well plates. Estrogen (50 nM) and Testosterone (50 nM) diluted in RPMI were added to the blood and incubated for 6 h. LPS 10 ng/mL *K. pneumoniae* (Sigma-Aldrich, India) was added to the hormone pretreated WHB and incubated for an additional 12 h. RNA was extracted from both the treatments and analyzed by qRT-PCR. Serum was subjected to western blotting.

2.3. PKCδ inhibition and anti-microbial assay

WHB (900 μL) and PBMCs (3×10^6) freshly harvested by Histoplaque density gradient centrifugation (Sigma Aldrich, India), and washed twice with DPBS (Dulbecco's phosphate buffered saline) (Himedia, India) were cultured in separate 24 well plates. WHB was pretreated with 5μM rottlerin (PKCδ inhibitor) for 1 h followed by LPS 10 ng and 100 ng/mL K. *pneumoniae* (Sigma-Aldrich, India) for 12 h. RNA was extracted and analyzed by qRT-PCR. Whereas PBMCs were pretreated with rottlerin (5μM) for 1 h, followed by 2 ng, 10 ng, 100 ng LPS / mL of RPMI culture media for 6 h. Cell-free media was collected. Both, serum from WHB treatments and PBMCs free media supernatants were subjected to western blotting for Hp.

For the anti-microbial assay, 20 μL of LPS and rottlerin treated serum from WHB treatments was incubated with 20 μL of anti-Hp antibody (1: 1000 diluted ∼1 μg) on ice for 30 min. In parallel serum was incubated with the anti-β-Actin antibody (∼1 μg/mL) and anti-Lipocalin antibody (∼1 μg/mL) as controls. Both sera with/without antibody treatment were added to the wells on nutrient agar plates spread with overnight cultures of *K. pneumoniae* (ATCC 700603) and *S. aureus* (ATCC 25923). Growth was observed after 16 h of incubation at 37 °C. Zone of inhibition was measured.

2.4. Resazurin dye assay and Agar plate colony count (CFU/mL) method

WHB (900 μL) was treated with LPS (100 ng/mL) and Heat killed *Staphylococcus aureus* in presence or absence of rottlerin (5μM). For heat killed inoculums, *S. aureus* used for treatments was grown until log phase and heat-killed at 60 °C for 1 h. The cells were pelleted down and washed with PBS without Ca^{++}. Each 0.1 unit at O.D 600 nm $= 10^8$

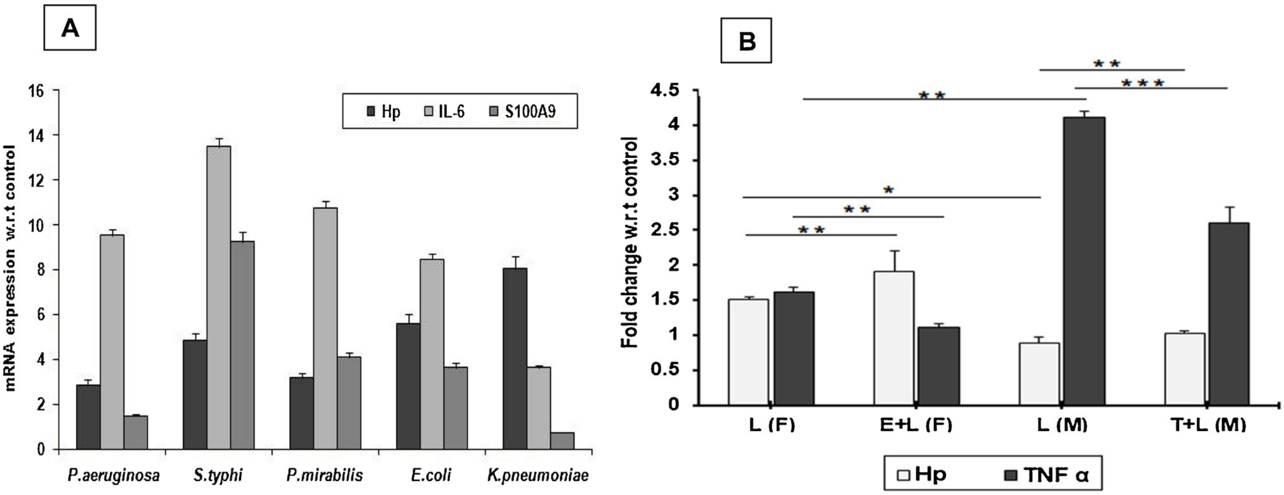

Fig. 1. Effect of differential bacterial LPS and gonadal hormones on Hp mRNA expression in whole blood. (A) The graph represents mRNA expression of Hp, IL-6 and S100A9 in presence of LPS isolated from different Gram-negative bacteria. Whole blood was stimulated with LPS (10 ng) from *P. aeruginosa, S. typhi, P. mirabilis, E. coli* and *K. pneumoniae* Gram -negative bacteria for 12 h ($p < 0.05$). (B) Male (**M**) and Female (**F**) whole blood (1 mL n = 6) was pretreated (6 h) with Estrogen (**E**) /Testosterone (**T**) followed by LPS (**L**)stimulation (10 ng/mL) (K. *pneumoniae, sigma*) for 12 h. mRNAexpression of the genes were detected by real-time PCR. The graph shows the relative mRNA levels normalized to GAPDH. ***$p < 0.0001$, ** $p < 0.01$, * $p < 0.05$ represent significant difference between treatments by Tukeys test.

cells/mL, 100 µL from aliquot containing 2×10^9 cells/mL was used for whole blood treatments. Serum was collected from the above treatments for antimicrobial assay. The overnight activated cultures of *K. pneumoniae* and *S. aureus* were inoculated in fresh nutrient broth and allowed to grow until Log phase; O.D at 600 nm of the culture media was taken to observe the growth phase. Cultures giving an O.D of 0.5-0.6 were taken and serial diluted. 10^{-4} dilution for *K. pneumoniae* (2.4×10^6 CFU/mL) and *S. aureus* (2.1×10^5 CFU/mL) was then taken for further experiments. The dilutions were done to maintain a uniform number of cells and get countable colonies. The cultures were then added to sterile 24 well plates. The experiments were carried out in two sets, in the first set cultures were inoculated and treated with serum from above mentioned whole blood cultures. In the second set, cultures were inoculated and incubated with treated serum in the presence or absence of anti-Hp antibody (~ 1 µg/mL) to neutralize the secreted Hp in the serum. The experiments were done both for *K. pneumoniae* and *S. aureus* and the cultures were incubated at 37 °C for 6 h. At the end of incubation 50 µL of culture from each well was spread plated on nutrient agar plates and incubated at 37 °C for 16 h. To test the viability by Resazurin indicator, both the cultures of *K. pneumoniae* and *S. aureus* were diluted from 10^{-3} dilution to get 10^{-4} dilution in each well of microtitre plate, the cultures were then treated with serum in presence or absence of anti-Hp antibody (˜1 µg/mL). 20 µL of Resazurin dye (0.02%) was added to each well to get a final volume of 200 µL and incubated at 37 °C for 6 h. The color change in the well was then observed visually and O.D was taken at 570 nm and 600 nm respectively using BioTek microplate reader (BioTek, USA). Any color change observed from purple/blue to pink or colorless was measured by A570/600 to indicate the reduction of Resazurin (blue) to Resorufin (pink) indicating the microbial growth.

2.5. Western blotting analysis

At the end of incubation after hormonal and rottlerin treatments blood was spun at 1500 × g for 5 min and serum was collected. The serum was then subjected to albumin depletion by modified TCA-Acetone precipitation as described previously [25].The pellet obtained after acetone precipitation was then dissolved in rehydration buffer (4 M urea, 2 M Thiourea, 65 mM DTT, 0.1% glycerol, 4% w/v CHAPS). The total protein content of serum and PBMCs cell-free media supernatants was estimated by Bradford's protein assay (Sigma-Aldrich, India) and equal amounts of protein (10–20 µg) were separated on 12% SDS-PAGE and subjected to western blot [26]. Nonspecific binding sites were blocked by incubating the nitrocellulose membrane in Tris-buffered saline containing 0.1% Tween-20 (TTBS) with 3% Bovine serum albumin. Blotted membranes were then incubated in (1:5000) primary antibody, anti-rabbit -Haptoglobin antibody (Abcam, USA) (BioVision, San Francisco, USA), anti-rabbit -TNFα antibody (Santa Cruz Biotechnology, USA) and anti-rabbit-β-Actin antibody (Santa Cruz Biotechnology, USA), anti-rabbit -PKCδ antibody(Cell Signaling Technology, USA), after subsequent washing the blotted membranes were incubated in Goat-anti-rabbit-(IgG)- ALP secondary antibody (Merck, India). The bands were then visualized by adding BCIP-NBT substrate (Sigma-Aldrich, India). The image was scanned using Bio-rad scanner (Bio-Rad, India) and analyzed by Li-Cor western analysis software Image studio Lite (version 5.2). All the protein band intensities were normalized with β-Actin to represent final graphs.

2.6. Endotoxin tolerance (ET) conditions

PBMCs were obtained from fresh whole blood by Histoplaque density gradient centrifugation (Sigma Aldrich, India) and washed twice with sterile DPBS. PBMCs were adjusted to 5×10^6 cells/mL of RPMI medium (Invitrogen, USA) and cultured in low adhesion 24-well plates at 37 °C and 5% CO_2. All the experiments were done in triplicate. HepG2 and Jurkat cells were procured from NCCS, Pune, India and maintained according to their respective cell culture conditions. HepG2 cells (1×10^6 cells/mL) were seeded in 6 well plates and grown to full confluence in Eagles MEM, 10% FBS, 100 units/mL penicillin and 100 mg/mL streptomycin. Jurkat T cells (3×10^6 cells/mL) were grown in RPMI, 10% FBS, 100 units/mL penicillin and 100 mg/mL streptomycin (Invitrogen, USA)

Lipopolysaccharide from *K. pneumoniae* (Sigma-Aldrich, USA) was used. The treatments were divided into two parts, in this *ex vivo* model of endotoxin tolerance, PBMCs and HepG2 cells were first cultured for 12 h without (control group and unprimed cells) or with 2 ng/mL LPS (primed cells). The PBMCs were then washed twice with DPBS and subjected to second exposure for 6 h with 100 ng/mL LPS (unprimed and primed cells – Fig. 1A, B). Both the treatments were done in two sets, one with and other without Anti-Haptoglobin antibody added to the cells to block the secreted Hp.

After the first treatment, cells were centrifuged and spent culture

Table 1
List of primer sequences for analyzing mRNA expression.

Gene	Right primer	Left primer
Hp	CATAGCCATGTGCAATCTCG	AGAGGCAAGACCAACCAAGA
TNFα	AGATGATCTGACTGCCTGGG	CAGCCTCTTCTCCTTCCTGA
IL-6	CTGCAGCCACTGGTTCTGT	CCAGAGCTGTGCAGATGAGT
PKCδ	GCTCATAGGAGTTGAAGGCG	CGGGAGCCAGGACTAAGG
β-Actin	CCTTGCACATGCCGGAG	GCACAGAGCCTCGCCTT
GAPDH	AATGAAGGGGTCATTGATGG	AAGGTGAAGGTCGGAGTCAA

medium after Hp neutralization was collected. The collected conditioned medium was then diluted 1:3 (1 part spent media : 3 fresh media) and added to freshly isolated unprimed PBMCs (5×10^6 cells/mL) and Jurkat cells, both the cell lines were treated for 12 h with the diluted conditioned media. At the end of 12 h, the resulting cell pellets from both treatments were subjected to RNA extraction with Trizol (Sigma-Aldrich, USA) and analyzed by quantitative real-time polymerase chain reactions (qRT-PCR).

2.7. qRT-PCR analysis

Treated whole blood samples and respective cell pellets from treatments were subjected to RNA extraction using Trizol (Sigma-Aldrich, USA) and treated with RNase free DNase (Macherey-Nagel, Germany) and reverse transcribed using Bio-Rad cDNA synthesis kit (Bio-Rad, USA), according to the manufacturer’s instructions. qRT-PCR was performed on cDNA samples using Fast start universal SYBR Green Master (Rox) (Roche Diagnostics, USA) with 10 pmol of forward and reverse primers for respective genes (Table 1). Standard curves were used to determine cDNA concentration and melting temperatures for primer sets. Melting temperatures were set as per primer sets used with rest of PCR conditions. Thermocycling was performed in a final volume of 20 μL containing 0.5 mM of each required primer, each in triplicates. PCR was performed with an initial 5-minute denaturation step at 95 °C, followed by 40 cycles of a touchdown PCR protocol (30 s annealing at 68–58 °C, and 34 s extension at 72 °C). Gene expression was calculated using the ΔΔCt method [27], where β-Actin and GAPDH were used as reference housekeeping genes for normalization.

2.8. Statistical analysis

Results are presented as fold change relative to the control group. Data were analyzed by Students T-test, Statistical analyses were performed using Prism 7 for Windows (GraphPad Software, La Jolla, CA, USA). One way ANOVA with Tukey's HSD test was done to analyze overall significant variations between different treatment conditions. p-value of < 0.001 and < 0.05 was considered statistically significant.

3. Results

3.1. Pathogen-specific expression of Hp

The mRNA expression of anti-inflammatory (Hp) and pro-inflammatory mediators (IL-6, S100A9) in whole blood (WHB) upon stimulation with different bacterial LPS were measured to study the possible relationship between disease severity and the cytokine-inducing capacities of these strains. The pattern of Hp expression showed its levels to be elevated in response to *K. pneumoniae* > *E. coli* > *S. typhi* > *P. mirabilis* - *P. aeruginosa*, in this order. Hp expression levels were observed to be maintained high up to 12–24 h in response to *K. pneumoniae* LPS when compared to other strains (Fig. 1A). The pattern of expression of IL-6 and S100A9 showed the highest expression in *S. typhi* and lowest in *K. pneumoniae.* Results clearly point out pathogen-specific LPS induced expression of Hp and that causative microorganisms play a key role in the host response and may trigger different inflammatory responses, depending on their intrinsic properties.

3.2. Gonadal steroid hormones tend to regulate the Hp expression in whole blood (WHB)

LPS induced Hp expression on exposure to external estrogen in female WHB and testosterone in male WHB was investigated; mRNA analysis showed that addition of estrogen increased Hp expression (2 fold, $p < 0.01$) in female WHB in presence of LPS. However, TNFα expression in female WHB in presence of LPS (1.6 fold, $p < 0.01$) was lowered on estrogen exposure (1.1 ± 0.2 fold, $p < 0.01$) (Fig. 1B). Opposite results were observed for testosterone, where the addition of testosterone to male WHB showed decreased Hp expression (0.9 ± 0.12 fold); with elevated TNFα expression levels (2 ± 0.60 fold, $p < 0.01$). Immunoblotting performed for Hp and TNFα protein

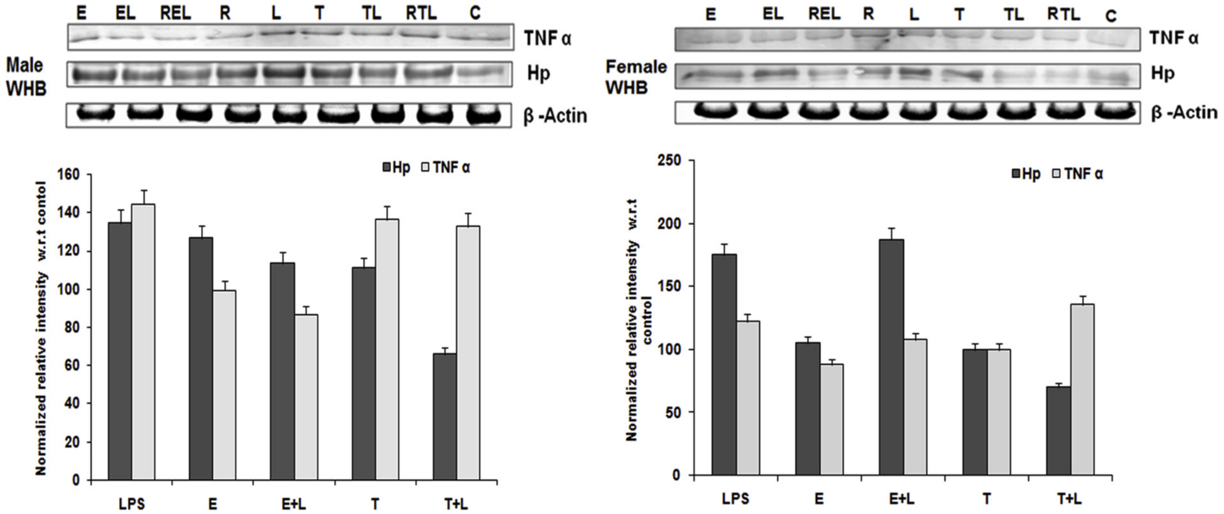

Fig. 2. The effect of Estrogen and Testosterone on LPS induced Hp and TNFα protein levels in whole blood. Blot represents [Estrogen (E), Estrogen + LPS (EL), rottlerin + Estrogen + LPS (REL), PKCδ inhibitor rottlerin (R), LPS (L), Testosterone (T), Testosterone + LPS (TL), rottlerin + Testosterone + LPS (RTL), Control (C)]. Male and Female WHB pretreated for 6 h with Estrogen /Testosterone followed by LPS stimulation for 12 h. 20 μg of albumin depleted and acetone precipitated protein was subjected to SDS-PAGE. The expression of Hp and TNFα Protein was measured by using western blot analysis. The graph shows relative Hp and TNFα protein levels normalized to β – Actin and control untreated serum.

in serum (Fig. 2) showed, ≥ 2 fold rise of Hp expression in female (175 ± 6.7% of control, $p < 0.001$) than in male (134 ± 5.61% of control, $p < 0.001$) samples on LPS stimuli when compared to control/ unstimulated blood. However, TNFα expression levels were observed to be (1.5 fold) higher in male samples (Fig. 2). Estrogen pretreatment showed marked rise in Hp expression in females (187 ± 12.3% of control, $p < 0.001$). In males however, Hp levels remained same as in LPS treated samples (113 ± 4.2% of control), whereas TNFα levels were significantly decreased in presence of estrogen in females (Hp vs. TNFα – 79 ± 5.2% of control decrease in TNFα levels) and males (Hp vs. TNFα -27 ± 3.4% of control decrease). TNFα was observed to be strongly expressed in testosterone pretreated male samples (136 ± 12% of control; $p < 0.001$), whereas in female samples no change was observed in this condition. However, the addition of LPS in presence of testosterone showed a decrease in Hp level in female (107 ± 9.5%) and male (68 ± 4.8%) WHB when compared to respective controls/ unstimulated blood in both male and female. Estrogen addition was observed to increase Hp levels with a decrease in TNFα levels, whereas Testosterone addition showed decreased Hp levels with the rise in TNFα levels in both male and female WHB on LPS stimuli.

3.3. Endotoxin tolerance models show increased Hp expression

We studied the expression of Hp during endotoxin tolerant state in HepG2 cells and PBMCs. Hp expression was observed to increase in LPS (2 ng + 100 ng) primed cells by 1.5 fold, while TNFα expression remained unchanged when compared to unstimulated or control cells. Whereas unprimed cells (100 ng LPS) showed ≥ 2.2 fold elevation in the expression of TNFα with fall in Hp levels by ≤ 0.86 fold when compared to unstimulated or control cells ($p < 0.01$) (Fig. 3). Expression pattern was observed to be similar in PBMC where LPS primed cells (2 ng + 100 ng) showed ≥ 1.7 fold rise in Hp expression associated with fall in TNFα levels by ≤ 0.85 when compared to control cells ($p < 0.001$) (Fig. 4). Higher Hp expression correlated with low TNFα expression in LPS primed cells when compared to unprimed cells in both the cell types during endotoxin tolerance.

3.4. Blocking secreted Hp in LPS treated conditioned media reversed the endotoxin tolerant state

We investigated if the conditioned media from the endotoxin tolerance treated cells were able to produce the ET state in cells without priming them with LPS. When the conditioned media from HepG2 cells was added to Jurkat T cells, we observed that unprimed cells directly entered ET state, which was shown by ≥ 2 fold rise in Hp levels, with fall in TNFα levels by ≤ 0.60 in conditioned media from the primed cells (Fig. 3). PBMC too showed a similar pattern where high Hp levels (≥ 2 fold) were associated with low TNFα (≤ 0.70) in primed cells when compared to the unstimulated or control cells ($p < 0.0001$). Whereas unprimed cells (100 ng) showed ≥ 3 fold rise in TNFα levels ($p < 0.001$) (Fig. 4).

Secreted Hp in the conditioned media was blocked by anti-Hp antibody added during treatment and/ or during incubation. Blocking of Hp did not seem to have much effect on Hp and/ or TNF α expression when anti-Hp-Antibody was added during treatment to HepG2 and PBMC (first treatment) (Figs. 3 and 4). However when the Hp neutralized conditioned media from first treatment was added to Jurkat cells and PBMCs (second Treatment), TNFα expression was observed to rise ≥ 2 fold in Jurkat cells and ≥ 3 fold in PBMC. This rise in TNFα expression was associated with fall in Hp levels by ≤ 0.26 in Jurkat cells, whereas not much variation was observed in PBMCs when compared to unstimulated or control cells ($p < 0.0001$). Neutralization/ blocking of Hp in conditioned media from endotoxin-tolerant cells showed a rise in TNFα levels indicating the reversal of tolerant state in both Jurkat cells and PBMCs. Hp may thus play a role in maintaining endotoxin tolerant state.

3.5. Effect of PKCδ inhibitor rottlerin on LPS stimulated Hp mRNA and protein expression in whole blood

We investigated changes in Hp, IL-6 and PKCδ (PKCD) levels in WHB when treated with rottlerin in presence/ absence of LPS (Fig. 5). We observed that pretreatment of WHB with rottlerin for 1 h followed by LPS (*K. pneumoniae* and *E. coli)* showed a decrease in Hp, IL-6 and PKCδ gene expression after 12 h when compared to only LPS treated WHB ($p < 0.005$). Rottlerin decreased the Hp expression in presence of both *K. pneumoniae* and *E. coli* LPS in a time-dependent manner. However, as the expression was higher in presence of *E. coli* LPS, hence the decrease in the expression of all genes in presence of rottlerin was observed more clearly on *E. coli* LPS treatment when compared to *K. pneumoniae* LPS.

Culture supernatants of PBMC subjected to western blot for Hp showed decreased secretion of Hp on rottlerin pretreatment in presence of LPS in media (Fig. 7B). Western blotting of LPS treated WHB serum showed two fragments of PKCδ- 65–72 kDa and 40–45 kDa catalytic fragment (CF unit) (Fig. 7A). The CF unit has been associated to be formed on cleavage of PKCδ by Caspase 3 in response to the apoptotic signal. The CF unit was observed in LPS treated serum, whereas rottlerin treated serum showed the absence of CF unit, indicating that rottlerin inhibits PKCδ, by inhibiting the cleavage of CF unit. This may be also related to decreased expression of Hp and pro-inflammatory mediator IL-6 in presence of rottlerin.

3.6. Inhibition of PKC δ and blocking of Hp reduces the anti-microbial activity of serum

LPS treated serum was shown to reduce the bacterial growth, indicated by 1 mm zone of inhibition in *K. pneumoniae* and 1.2 mm in *S. aureus* (Fig. 6A). However, rottlerin pretreated WHB serum restored bacterial growth shown by reduction in the zone of inhibition from 1 mm (LPS) to 0.4 mm (rottlerin + LPS) for *K. pneumoniae* and from 1.2 mm (LPS) to 0.5 mm (rottlerin + LPS) for *S. aureus*. Rottlerin is hence indirectly resulting in the decreased anti-microbial activity of serum, as observed by lower Hp levels. To demonstrate the anti-microbial activity of Hp, secreted Hp was neutralized in the stimulated serum by the anti-Hp antibody, which restored the bacterial growth from 1 mm zone of inhibition (LPS) to 0.1 mm (after neutralizing Hp) and 0.4 mm (rottlerin + LPS) to no zone or complete restoration of *K. pneumoniae* bacterial growth. Indicating that rottlerin only partially inhibits Hp secretion. Similar results were observed for *S. aureus*; however, blocking of Hp did not completely restore *S. aureus* growth. Further, for control experiments incubation of serum with anti- β-Actin antibody and anti- NGAL (Lipocalin) antibody showed that NGAL which is also a known anti-microbial protein was observed to inhibit bacterial growth shown by 1 mm zone of inhibition when compared to β-Actin shown by 1.2 mm zone of inhibition. However, when compared to Hp blocking, both β-Actin (1.2 mm) and NGAL (1 mm), showed minimal / no effect on the antibacterial activity of LPS treated serum (Table 2).

These observations were further confirmed by Resazurin dye-based assay (6B) and agar plate colony counting (Fig. 6C), where A570/600 values showed lower % reduction of Resazurin (blue) to Resorufin (pink) indicating negative microbial growth or non-viable cells as observed for LPS (15 ± 2.4% reduction) and heat-killed *S. aureus* (14% reduction) treated sera. This was confirmed by plating the treated cultures, which also showed a decreased number of colonies for *K. pneumoniae* (Log 6 CFU/ mL) and *S. aureus* (Log 4.8 CFU/ mL) in presence of LPS treated sera when compared to untreated cultures (Log 7.6–8.7 CFU/mL). Rottlerin pretreated sera indicated increased reduction to Resorufin, for *K. pneumoniae* (21 ± 3.2%) and *S. aureus* (63 ± 4.21), showing a rise in the bacterial viability. Increased colony count by Log 6.9 CFU/mL for *S. aureus* and Log 8.4 CFU/mL for *K.*

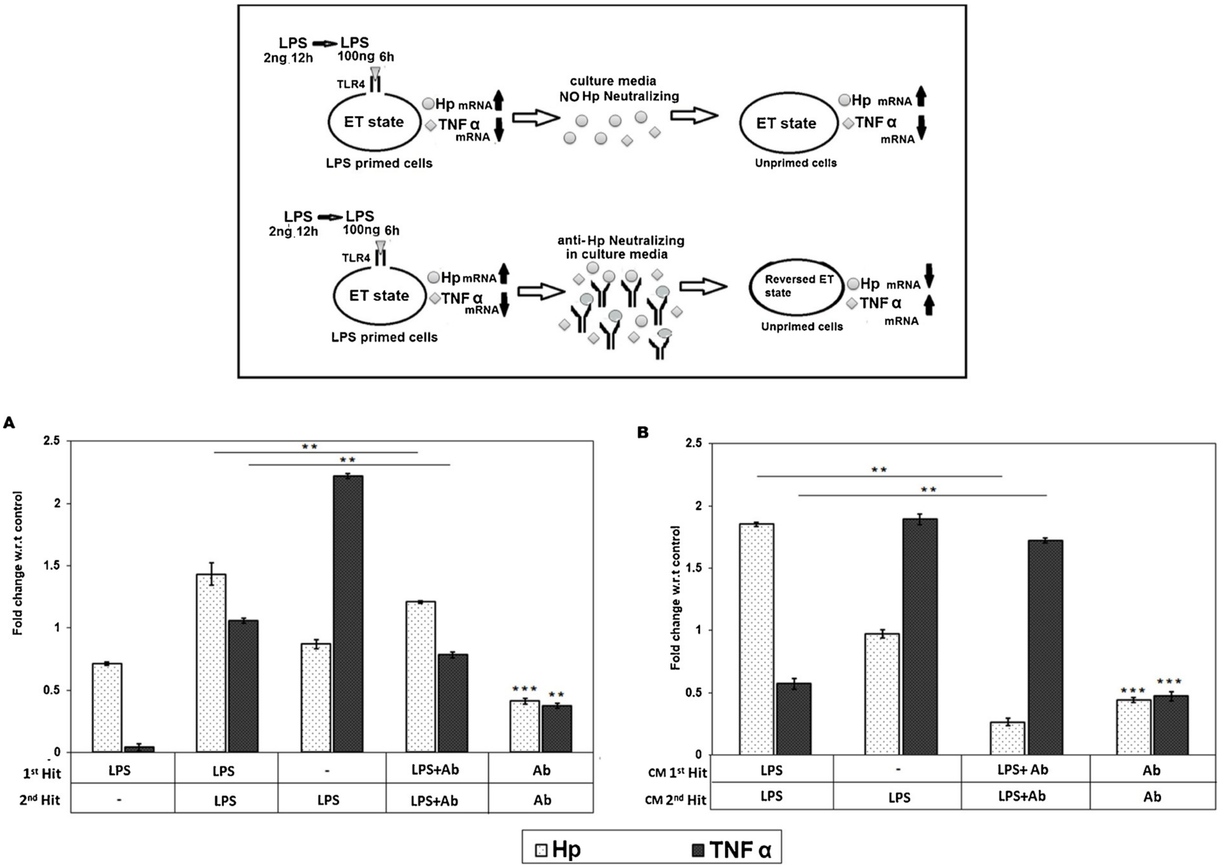

Fig. 3. Endotoxin tolerance is associated with increased Hp. Diagram represents endotoxin tolerance experimental model. Graph represents Hp and TNFα fold change in (A) ET in LPS primed cells (1st Hit- LPS 2 ng/mL, 2nd Hit- LPS 100 ng/mL)(B) ET via Hp neutralized HepG2 ET spent media (CM). Hepg2 cells were stimulated with LPS in presence or absence of anti Hp antibody (Ab) (1 μg /mL). TNFα and Hp mRNA expression in Jurkat cells incubated with conditioned medium (1:3) derived from unstimulated and stimulated HepG2 cells. mRNA levels of TNFα and Hp in an *in vitro* model of endotoxin tolerance were analyzed after 12 h using RT-PCR. The mRNA level was normalized with reference gene GAPDH and then compared to the control group. Significant difference between different groups with internal control represented as, *p* value ** < 0.01, *** < 0.001.

pneumoniae was observed on the plates for rottlerin pretreated sera, indicating that rottlerin indeed inhibits secretion of certain anti-bacterial proteins in presence of LPS. Conversely, rottlerin pretreatment did not affect the viability for both *K. pneumoniae* and *S. aureus* in presence of serum treated with heat-killed *S. aureus* indicated by the blue color of Resazurin. Further neutralization of secreted Hp showed restored bacterial growth in both *K. pneumoniae* (LPS + anti-Hp Ab – 46 ± 1.19%, LPS + rottlerin + anti- Hp Ab – 91 ± 2.87%) and *S.*

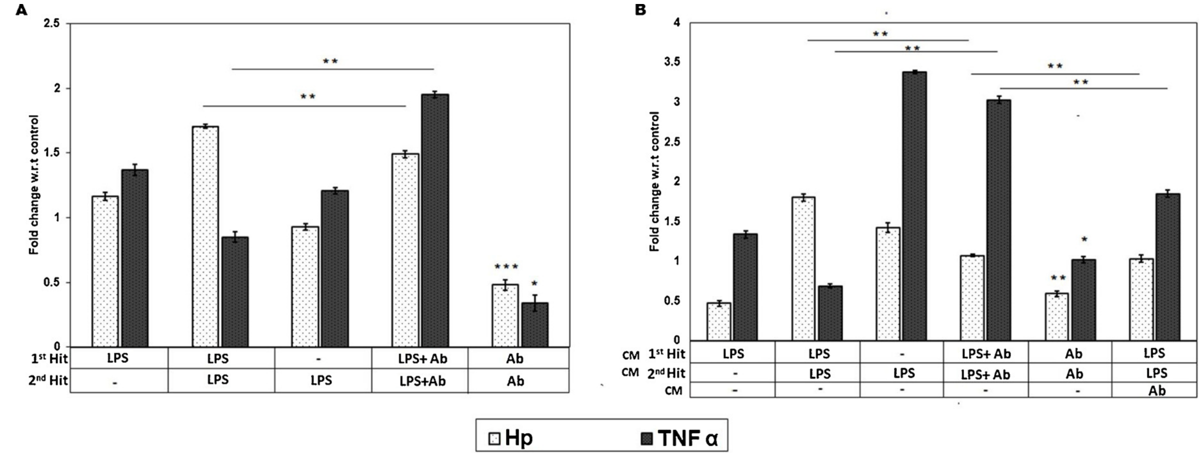

Fig. 4. Endotoxin tolerance showed increased Hp expression in PBMCs and reversed the tolerant state on neutralizing Hp. Graph represents Hp and TNFα fold change in (A) ET in LPS primed cells (B) ET via Hp neutralized PBMC spent media (CM). PBMCs were stimulated with LPS in presence or absence of anti Hp antibody (1 μg /mL). TNFα and Hp mRNA expression in unstimulated PBMCs incubated with conditioned medium (1:3) derived from LPS primed and unprimed PBMCs from first treatment. mRNA levels of TNFα and Hp in an *ex vivo* model of endotoxin tolerance were analyzed after 12 h using RT-PCR. The mRNA level was normalized to that of the reference gene (GAPDH) and then compared to the control group. Significant difference between different groups with internal control represented as, *p* value *** < 0.001, ** < 0.01, * < 0.05.

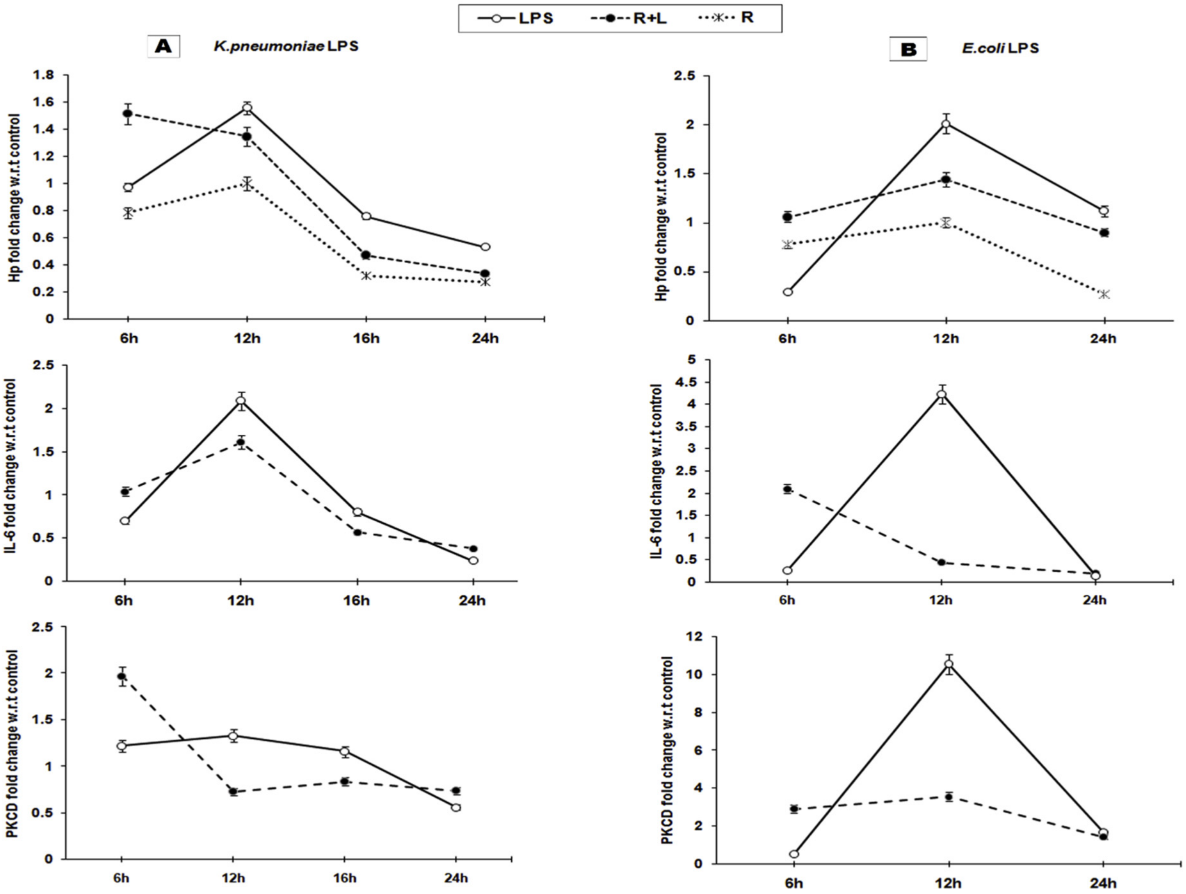

Fig. 5. Effect of PKCδ inhibitor rottlerin on *K. pneumoniae* and *E. coli* LPS stimulated Hp expression in whole blood culture. The graph represents the mRNA expression of **(A)** *K. pneumoniae* -Hp, IL-6, PKCD, **(B)** E. *coli*- Hp, IL-6, and PKCD in presence or absence of PKCδ inhibitor rottlerin on LPS stimulation. Whole blood (1 mL n = 6) were incubated with 5μM rottlerin for 1 h followed by LPS (*K. pneumoniae* and *E. coli* 10 ng/mL) for 6,12,16,24 h. mRNA was analyzed by qRT-PCR. Graphs show that rottlerin addition reduced mRNA expression of Hp and IL-6 by inhibition of PKCδ in presence of LPS ($P < 0.005$).

*aureus (*LPS + anti-Hp Ab – 63 ± 2.4%, LPS + rottlerin + anti- Hp Ab – 90 ± 6.9%) indicated by pink color of Resorufin and increased number of colonies on plating by Log 8.2 CFU/mL for *K. pneumoniae*and Log 7.1 CFU/mL for *S. aureus*. Results thus confirm that PKC*δ* plays a major role in regulating secretion of anti-microbial proteins in serum. Where Hp is one of the major anti-microbial proteins secreted in the serum on LPS stimulation, shown by complete restoration of gram-negative and partially, gram-positive bacterial growth on blocking Hp in sera. This response is conspicuous in case of LPS rather than heat killed *S.aureus*.

4. Discussion

Immune alteration especially decreased or delayed acute phase response leads to increased mortality in patients with bacterial sepsis. Previously, we identified Hp as an acute phase protein to be lowered during initial stages in non-survivors of sepsis. [14]. Antibacterial role of Hp has been attributed to its function in host-pathogen interactions especially by inhibiting pathogen proliferation via sequestering Iron, making it unavailable to the invading bacteria in blood [28]. Increased Hp expression in PBMCs is known to be influenced by TNFα in neutrophils. Although pro-inflammatory cytokines released in response to primary bacterial stimulus and its influence on the hepatic rise of APR is known, how these Acute phase proteins, especially Hp once released, stabilize the immune response and also inhibit bacterial proliferation needs to be evaluated in detail. Low levels of Hp in non-survivors of sepsis may be associated with an uncontrolled rise in pro-inflammatory cytokines. In the present study, we show gender and pathogen-specific role of Hp in modulating immune response via regulation of endotoxin tolerant state, making it a suitable candidate to predict the severity of bacterial sepsis.

The immune system responds differently to different causative pathogens. Previous studies have reported that the clinical features of sepsis are mainly due to the host response to circulating endotoxin, when compared to the intact bacteria. LPS is the major active component of endotoxin, and is employed as model of sepsis. When administrated LPS mimics the host response, producing sepsis- like pathophysiological responses, especially elevated cytokine levels (TNFα, IL-1β), correlating with sepsis patients. *Ex vivo* human whole blood models (WHB) stimulated with endotoxin (LPS) have been reported to mimic the *in vivo* complexity of the immune response of blood components as in human models when compared to other animal models, especially to monitor the hematological alterations [29]. When we compared the WHB acute phase protein levels in response to different gram-negative bacteria LPS, Hp indicated maximum expression in response to *K. pneumoniae* LPS (Fig. 1A). This indicates that acute phase response varies with infectious agents; hence APP candidates like Hp could be effective classifiers for discriminating among different infectious agents and their clinical manifestations.

Previous studies have shown gender-based variation in the pattern of expression of acute phase proteins and sepsis-associated mortality [30,31], wherein mutually opposing observations have been made, which make adjustments with concurrent data while generalizing observations. Significantly elevated levels of pro-inflammatory markers (i.e., IL-6, procalcitonin) have been reported in male compared with female sepsis patients [32,33]. Studies suggest that low DHT and/or

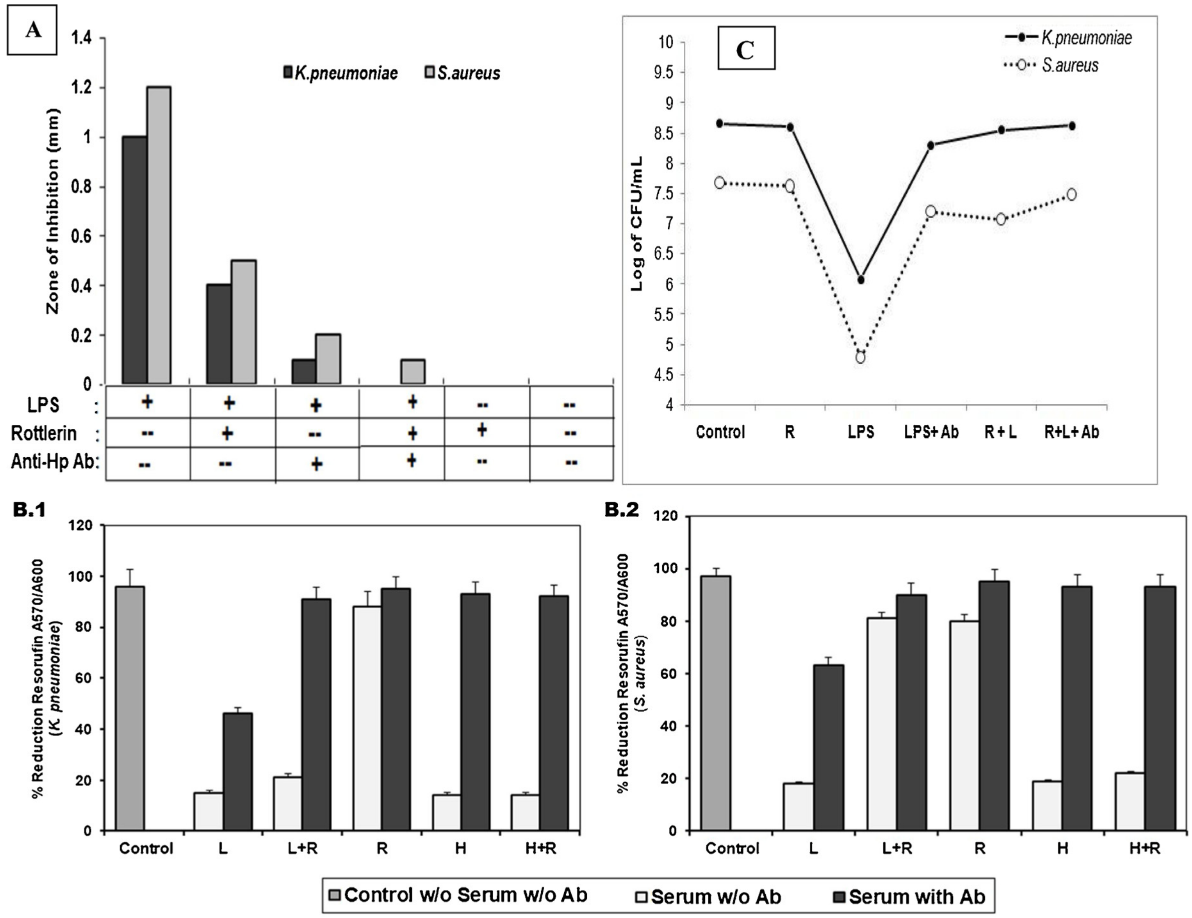

Fig. 6. Antimicrobial assays for Hp and role of PKCδ inhibition on Hp secretion in serum: Whole blood (1 mL n = 3) was treated with rottlerin 5μM for 1 h followed by LPS (100 ng), heat killed *S. aureus* (H) stimulation for 12 h and serum was collected and anti-microbial assays were performed. (**A**) Agar well assay: Graph represents zone of inhibition (mm) for *K. pneumoniae* and *S. aureus* in response to LPS treated serum in +/- of rottlerin and Anti-Hp antibody in agar well assay. Zones of inhibition for both *K. pneumoniae* and *S. aureus* were measured after plates were incubated for 16 h with treated serum. (**B**) Resazurin dye assay: (B.1) *K. pneumoniae* and (B.2) *S.aureus* were grown in nutrient broth for 6 h with / without LPS and anti-Hp antibody treated serum, cell viability was checked with Resazurin dye. Graphs represent % reduction of Resazurin to Resorufin for *K. pneumoniae* and *S. aureus* growth calculated by A570/A600. (**C**) Colony count assay: Graph represents Log CFU/ mL of bacterial growth showing antimicrobial activity of Hp and PKCδ inhibition by rottlerin determined by agar plate colony count assay. *K. pneumoniae* (2.4×10^6 CFU/mL)and *S. aureus* (2.1×10^5 CFU/mL) were grown in nutrient broth incubated with +/- LPS (L) with+/- rottlerin (R) pretreated serum and cultured in presence or absence anti-Hp neutralizing antibody (Ab) for 6 h, followed by plating on nutrient agar plates. CFU was calculated from colony count after 16 h.

Table 2
Zone of inhibition showing effect of Ab neutralization on anti-bacterial serum proteins in blood.

Zone of inhibition (mm)					
Pathogen	LPS (100 ng)	LPS + anti-Hp Ab	LPS + anti-Ngal Ab	LPS + anti-β -actin Ab	Control
K. pneumoniae	1	0.1	NA	NA	No zone
S. aureus	1.2	0.2	1	1.2	No zone

Note: K. pneumoniae and *S. aureus* were spread plated and incubated with +/- LPS serum, in presence or absence anti-Hp neutralizing antibody. For controls, anti-Ngal / Lipocalin antibody and anti-β- actin antibody were used for neutralization. Zone of inhibition was measured after 16 h.Ab- antibody, NA- not applicable.

high estradiol appear to be protective for the host following adverse circulatory conditions, i.e., septic shock. We observed that Hp mRNA expression showed a dramatic rise on estrogen addition in female WHB, whereas testosterone showed a rise in TNFα levels in male WHB in presence of LPS (Fig. 1B). However, on a comparison of protein expression, we found that high Hp expression was associated with low TNFα levels irrespective of gender in presence of estrogen (Fig. 2), suggesting that estrogen may indeed modulate innate immune response by reducing pro-inflammatory mediators like TNFα. Protective role of estrogen in females is shown in this study. Low levels of Hp in males may be one of the contributing factors for high susceptibility to infection and may be due to the paucity of estrogen in males. Our results are supported by studies showing the protective role of estrogen. Where it was demonstrated that the higher expression of TLR-4 in macrophages was observed in females leading to enhanced phagocytic ability and NADPH oxidase-mediated bacterial killing [34].On the other hand, the addition of testosterone to isolated mouse macrophages decreased TLR-4 expression [35]. Although the exact underlying mechanism(s) for the immunomodulatory effects of gonadal steroid hormones on immune responses following sepsis remain unknown, one possible explanation can be found in a study by Scotland *et al.* According to their observations, the number of resting resident leukocytes, comprising more T and B lymphocytes occupying the peritoneal and pleural cavities were reported to be higher in female than in male rats. This altered immune cell composition of the female peritoneum was found to be controlled by elevated tissue chemokines expression [36–39]. Despite these findings; gender-specific differences in the activation of lymphocytes remain controversial. Our results, however, correlate with reports showing, gender dimorphism of chronic inflammatory diseases to be associated with lower circulating levels of estrogen in men, unlike women where high estrogen and lower androgen levels are associated with increased protection from infectious diseases, but also prone to autoimmune diseases.

Our results provide evidence suggesting that Hp acts as a major anti-inflammatory mediator, and its expression to be regulated by gonadal steroid hormones. Wherein up-regulation of Hp has shown to reduce pro-inflammatory mediators, it is likely that Hp may be involved in maintaining a balance between pro and anti-inflammatory cascade in sepsis. Thus, it may also play a role in regulating endotoxin tolerance which is crucial for patient survival. TNFα has been reported to be a major mediator for initiating the inflammatory cascade of other cytokines, however, septic shock and multiple organ failure is attributed to overproduction of TNFα, which has also shown to impair the endotoxin tolerance (ET) produced by repeated exposure to LPS [40]. Development of ET state has been reported in patients with bacterial sepsis, [41] where first exposure to bacteria leads to exponential rise in inflammatory cytokine storm, which is then followed by upregulation of anti-inflammatory proteins, thus leading to development of ET state on second exposure via suppression of pro-inflammatory mediators like TNFα, IL-6, IL-12, IL-23 [42,43]. Previously we reported that sepsis survivors showed not only high Hp levels but also maintained the levels throughout recovery when compared to non-survivors suggesting that Hp proteins and/or mRNA expression may be involved in regulation of the development of immune tolerance in response to bacterial infection.

Earlier studies mainly focused on elucidating the anti-inflammatory role of Hp, where external doses of Hp dampened endotoxin-induced pro-inflammatory response, however, its individual role in the initiation of ET and inhibition of pro-inflammatory cytokine release has not been addressed in these reports [44]. It is known that primary rise in cytokine levels in response to bacterial stimuli, either elevates or decreases hepatic APR, which in turn further regulates cytokine release from monocytes and neutrophils thus leading to either hypo or hyper-immune response. To understand this, we studied role of Hp in maintaining ET state in both *ex vivo* (PBMCs) and *in vitro* (HepG2 cells) conditions. First, we worked to block secreted Hp in LPS primed hepatic HepG2 culture conditioned media, blocking the secreted Hp via Hp Neutralizing antibody caused reversal of endotoxin tolerance when conditioned media was added to Jurkat T cells (Fig. 3). T cells have CR3 (CD11b) as a receptor for Hp, where specific binding of Hp to T cells has been reported to cause influence on T cell cytokine release [45]. In our study, we observed that when Hp neutralized conditioned media was added to T cells it caused a reversal of ET state shown by the drastic rise in TNFα levels associated with fall in Hp levels. Similar results were observed in PBMCs, when subjected to the same experimental conditions, indicating that Hp indeed plays a major role in pathways leading to maintaining ET state of immune cells in response to bacterial infection. It has been reported that monocytes from sepsis patients showed down-regulation in TNFα levels when exposed to LPS. Interestingly we also observed that conditioned media from primary LPS primed cells both HepG2 and PBMCs caused the direct development of the tolerant state in secondary unprimed cells. Where elevated Hp levels were associated with low TNFα levels, even though the secondary cells (Jurkat T cells and PBMC) were not exposed to LPS challenge twice (Figs. 3 and 4). This could mean that cytokines and other feedback mediators secreted in conditioned media can cause the direct development of the tolerant state in unprimed cells without additional LPS; especially by inhibition, TNFα mediated positive feedback initiation of inflammation, though further investigation is needed to evaluate the exact mechanism and contributing factors. Overall the results from the present study showed that Hp maintains endotoxin tolerant state via lowering TNFα, a major mediator of cytokine storm, and hence may reduce the progression of bacterial sepsis.

Anti-microbial peptides are known to be released in serum on bacterial infection in the blood. Since it has been reported that haptoglobin acts as an anti-bacterial protein due its iron sequestering / heme scavenging functions inhibiting the gram-negative bacterial growth reported in *E. coli.* Iron utilizing ability is much higher in gram-negative than gram-positive bacteria [46]. Re-evaluation of the anti-microbial role of Hp in WHB confirmed that LPS induced serum showed inhibition of bacterial growth when compared to control in both gram positive and gram negative bacteria (Fig. 6). Blocking Hp in serum restored growth completely in gram-negative bacteria; however, gram-positive bacteria on other hand did not show complete restored growth. Hence, Hp can be stated as one of the major anti-microbial proteins secreted in serum inhibiting mainly gram-negative bacterial proliferation. Studies on LPS receptor-mediated signals have shown the important role of protein kinases in regulating the cytokine release, where selective inhibitors reduced the secretion of cytokines; however the role of individual PKCs in LPS mediated immune response is still under study. PKCδ was reported to be involved in secretion of stored Hp in THP1 cells, where blocking PKCδ gene expression inhibited Hp secretion into the media; however, the expression was studied with high Hp expression vectors [47]. In contrary, our study employed WHB and PBMCs to replicate sepsis conditions in blood and showed Hp release in PBMCs is regulated by PKCδ via inhibition by rottlerin. PKCδ was reported to regulate NFκB activation and iNOS expression in mouse peritoneal macrophages, where it reduced NO synthesis [48]. PKCδ deficient macrophages demonstrated increased escape of *Listeria monocytogenes* from the phagosome into the cytoplasm and uncontrolled bacterial growth, despite elevated levels of proinflammatory cytokines and NO production [49]. It has been reported that PKCδ regulates some of the lymphocyte functions and sepsis-induced pathways, however, its effects in innate immunity and on the regulation of the expression of inflammatory genes are still under investigation. Role of PKCδ in the secretion of antimicrobial proteins has not been investigated. We for the first time report that, rottlerin inhibits the secretion of anti-microbial proteins including Hp, shown by increased bacterial growth when compared to LPS treated serum, suggesting that PKCδ is involved in secretion of anti-microbial proteins. Conversely, rottlerin pretreatment did not seem to affect viability for both *K. pneumoniae* and *S. aureus* in presence of serum treated with heat-killed *S.aureus* shown in Resazurin assay, indicating that PKCδ may not be involved in regulating the anti-microbial proteins released against heat killed gram-positive *S. aureus,* when compared to LPS treatments, where PKCδ is shown to influence the secretion of anti-microbial proteins. However blocking Hp in rottlerin treated serum showed restored bacterial growth, mainly in gram-negative bacteria, showing that Hp secretion is inhibited by rottlerin in presence of LPS. This was also observed by western blotting where the addition of rottlerin showed inhibition of Hp beta chain (45 kDa) in PBMC cell culture supernatants. Western blot for PKCδ protein revealed that PKCδ is cleaved to 40–45 kDa catalytic fragment in presence of LPS which was not observed in presence of rottlerin (Fig. 7). This confirms previous studies that report the role of PKCδ in the initiation of apoptosis. PKCδ activity increases in DNA damage and other apoptotic stimuli [50,51]. Agents such as rottlerin are shown to suppress its activity which leads to reduced caspase-3 activity and which in turn inhibits cleavage of PKCδ, and preventing DNA fragmentation [52,53]. Overall, we confirmed that PKCδ is one of the mediators involved in secretion of Hp in response to LPS stimuli which is shown by inhibition of Hp in presence of rottlerin.

In conclusion, we provide evidence that haptoglobin (Hp) plays a crucial role in balancing the inflammatory response in sepsis by preventing bacterial proliferation and reducing inflammation. We observed higher Hp expression in female blood when compared to male blood on LPS stimulation. This seems to be regulated mainly by estrogen; supporting the gender-specific expression of Hp. PKCδ was shown to regulate the secretion of anti-microbial serum proteins partly including Hp, where its inhibition by rottlerin restored bacterial growth. Also, Hp promotes endotoxin tolerance, as blocking of Hp

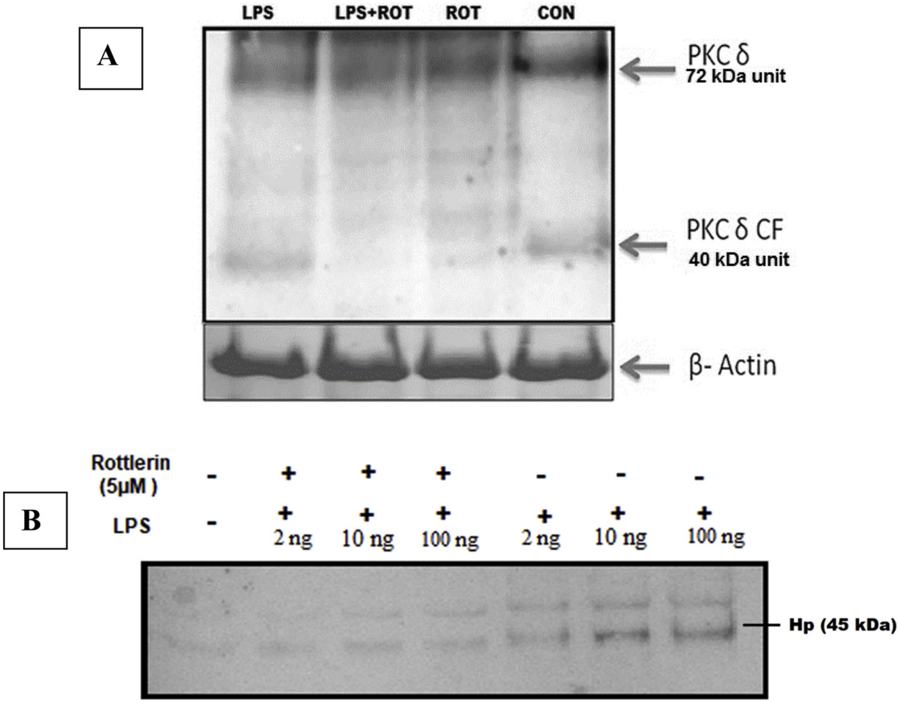

Fig. 7. Inhibition of LPS stimulated PKCδ activity by rottlerin in whole blood and PBMCs. (A) Blot represents cleavage of PKCδ into 40 kDa catalytic fragment in presence of LPS; catalytic fragment is essentially for apoptotic signaling as seen in response to LPS. Whole blood (1 mL n = 3) was treated with rottlerin 5µM (ROT) for 1 h followed by LPS (100 ng/mL) stimulation for 12 h. Serum was collected at the end of 16 h was subjected to albumin depletion by modified TCA-Acetone precipitation. 20 µg of protein was separated by SDS-PAGE and analyzed by western blotting. The blot was normalized with β-Actin gene.Cleavage is inhibited by rottlerin as demonstrated in blot by absence of CF. (B) To check role of PKCδ on Hp secretion, PBMCs were pretreated with 5µM rottlerin for 1 h followed by LPS (*K. pneumoniae* 2 ng, 10 ng, 100 ng) treatment for 6 h. Culture supernatant was subjected to western blot. Blot represent Hp band at 45 kDa.

results in reversal of endotoxin tolerant state both in *ex vivo* and *in vitro* conditions, shown by rise in TNFα levels. These results correlate with our previous patient data, where we observed that non-survivors of sepsis showed lower levels of Hp from onset to death. Overall Hp may prove to be a good prognostic sepsis marker to identify potential non-survivors among sepsis patients. This needs to be further validated by individualized trials.

Author contributions

SB and SMR designed the study. SMR performed the experiments. APK isolated bacterial LPS. ANY, helped with western blot analysis. MVSS and KR helped with antimicrobial assays. SB and SMR wrote the manuscript.

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Conflict ofinterest

Authors SB, SMR, APK, ANY, MVSS and KR declare that they have no conflicts of interest related to the manuscript.

Informed consent

Healthy volunteers for blood collection were enrolled with informed consent for participation in the study.

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