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J Clin Cell Immunol. Author manuscript; available in PMC 2014 August 27.

Published in final edited form as:
J Clin Cell Immunol. ; 5(218): . doi:10.4172/2155-9899.1000218.

Endogenous Tetrapyrroles Influence Leukocyte Responses to Lipopolysaccharide in Human Blood: Pre-Clinical Evidence Demonstrating the Anti-Inflammatory Potential of Biliverdin

Kavita Bisht[1,*], **Jens Tampe**[2], **Cecilia Shing**[3], **Bhavisha Bakrania**[1], **James Winearls**[4], **John Fraser**[5], **Karl-Heinz Wagner**[6], and **Andrew C. Bulmer**[1,4,*]

[1]Heart Foundation Research Centre, Griffith Health Institute, Griffith University, Gold Coast, QLD, Australia

[2]Griffith Enterprise, Griffith University, Nathan, QLD, Australia

[3]School of Health Sciences, University of Tasmania, Launceston, Tasmania, Australia

[4]Gold Coast University Hospital Intensive Care Unit and Gold Coast University Hospital Critical Care Research Group, Gold Coast, QLD, Australia

[5]Critical Care Research Group, The Prince Charles Hospital, University of Queensland, QLD, Australia

[6]Emerging Field Oxidative Stress and DNA Stability and Research Platform Active Aging, Department of Nutritional Science, University of Vienna, Vienna, Austria

Abstract

Sepsis is associated with abnormal host immune function in response to pathogen exposure, including endotoxin (lipopolysaccharide; LPS). Cytokines play crucial roles in the induction and resolution of inflammation in sepsis. Therefore, the primary aim of this study was to investigate the effects of endogenous tetrapyrroles, including biliverdin (BV) and unconjugated bilirubin (UCB) on LPS-induced cytokines in human blood. Biliverdin and UCB are by products of haem catabolism and have strong cytoprotective, antioxidant and anti-inflammatory effects. In the present study, whole human blood supplemented with BV and without was incubated in the presence or absence of LPS for 4 and 8 hours. Thereafter, whole blood was analysed for gene and protein expression of cytokines, including IL-1β, IL-6, TNF, IFN-γ, IL-1Ra and IL-8. Biliverdin (50 μM) significantly decreased the LPS-mediated gene expression of IL-1β, IL-6, IFN-γ, IL-1Ra and IL-8 ($P<0.05$). Furthermore, BV significantly decreased LPS-induced secretion of IL-1β and IL-8 ($P<0.05$). Serum samples from human subjects and, wild type and hyperbilirubinaemic Gunn rats were also used to assess the relationship between circulating bilirubin and cytokine

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[*]**Corresponding authors:** Ms. Kavita Bisht, Heart Foundation Research Centre, Griffith Health Institute, Griffith University, Parklands Drive, 4222, Southport, Queensland, Australia; Tel: +61 7 55528372; Fax: +61 7 55528908; kavita.nic@gmail.com. Dr. Andrew C. Bulmer, Heart Foundation Research Centre, Griffith Health Institute, Griffith University, Parklands Drive, 4222, Southport, Queensland, Australia; Tel: +61 7 55528215; a.bulmer@griffith.edu.au.

Conflict of interest: The authors declare no conflict of interests.

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expression/production. Significant positive correlations between baseline UCB concentrations in human blood and LPS-mediated gene expression of IL-1β (R=0.929), IFN-γ (R=0.809), IL-1Ra (R=0.786) and IL-8 (R=0.857) were observed in blood samples (all $P<0.05$). These data were supported by increased baseline IL-1β concentrations in hyperbilirubinaemic Gunn rats ($P<0.05$). Blood samples were also investigated for complement receptor-5 (C5aR) expression. Stimulation of blood with LPS decreased gene expression of C5aR (P<0.05). Treatment of blood with BV alone and in the presence of LPS tended to decrease C5aR expression ($P=0.08$). These data indicate that supplemented BV inhibits the *ex vivo* response of human blood to LPS. Surprisingly, however, baseline UCB was associated with heighted inflammatory response to LPS. This is the first study to explore the effects of BV in a preclinical human model of inflammation and suggests that BV could represent an anti-inflammatory target for the prevention of LPS mediated inflammation *in vivo*.

Keywords

Cytokine; Inflammation; Tetrapyrroles; Lipopolysaccharide

Introduction

Sepsis, caused by systemic microbial infection, is a potentially life-threatening condition and characterised by uncontrolled inflammation [1]. The pathogenesis of sepsis involves several factors that interact in a chain of events from pathogen recognition to an overwhelming host response [2]. Among the molecules involved, toll like receptor (TLR) and complement receptor 5a (C5aR) are major contributors leading to septic shock, coagulation abnormalities, tissue hypoperfusion and organ failure [3,4]. Activation of TLRs and C5aR promote the production of pro and anti-inflammatory cytokines by immune cells, contributing to the ‘cytokine storm’ of acute inflammation [1,5]. Several studies implicate the involvement of both pro- and anti-inflammatory cytokines in initiation and aggravation of infectious and inflammatory disorders, including sepsis, arthritis, and atherosclerosis [6,7]. Septic patients and animals often experience increased circulating concentrations of tumour necrosis factor (TNF), interleukin (IL)-1β, IL-6 and interferon (IFN)-γ, resulting in exacerbated inflammation and, ultimately, organ dysfunction [7,8].

Discovery of new treatments for sepsis and the application of such treatments to patients presenting with sepsis poses significant challenges to both researchers and clinicians. Despite many years of exhaustive research and clinical trials the pathophysiology of sepsis remains incompletely understood and specific anti-inflammatory and immuno-modulatory therapies have not been translated into improved patient outcomes [9]. A number of therapies (activated protein C (APC), steroids and cytokine blockade) have been investigated in both preclinical and clinical trials to target the host response factors thought to play a significant role in the inflammatory response to sepsis. None of these therapeutic approaches have translated into improved patient outcomes despite promising early results [9]. Activated protein C has antithrombotic, anticoagulant, anti-inflammatory and antifibrinolytic effects. Initial data suggested a significant mortality benefit associated with the use of APC in severe sepsis and septic shock [10]. However, in a recent Cochrane

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Review the use of APC found no evidence to support the use of APC in severe sepsis and in fact showed a trend to significant haemorrhagic complications [10]. A number of trials investigating therapeutic targets against TNF and IL-1 showed promise in experimental models of sepsis, but again these effects were not translated into beneficial outcomes for patients in clinical trials [11-13]. The use of systemic steroids in severe sepsis and septic shock remains controversial despite almost 50 years of research into the area. Again there is strong biological rationale to support the use of steroids in severe sepsis but this has yet to be translated into improved patient outcomes [14]. Therefore, the discovery of new and effective anti-inflammatory therapeutics to reduce morbidity and mortality due to sepsis and septic shock are necessary.

Endogenous tetrapyrroles, including biliverdin (BV) and unconjugated bilirubin (UCB) are haem catabolites and are formed by the sequential action of haem oxygenase and biliverdin reductase (BVR) forming BV and UCB, respectively, within cells of the reticuloendothelial system [15,16]. Unconjugated bilirubin is water insoluble and must be conjugated by uridine diphosphate glucuronosyltransferase (UGT1A1) in hepatocytes forming bilirubin mono and diglucuronides, which are then excreted into the bile [16,17]. Several *in vivo* and *in vitro* studies report strong cytoprotective effects of these compounds in various animal models of ischaemia-reperfusion injury (IRI), transplantation, sepsis and endotoxic shock [18-21]. It is suggested that these compounds induce cytoprotection via attenuation of inflammation and free radical induced macromolecule oxidation [22,23].

Biliverdin inhibits the expression of TLR-4 and C5aR *in vitro* [24,25]. Our groups and others have also shown that BV and UCB modulate the expression and production of TNF, IL-6 and IL-1β in cell culture and animal models [25-28]. However, whether anti-inflammatory effects of BV/UCB exist in human models, remains unknown. Therefore, the primary aim of this study was to investigate the effects of supplemented BV and baseline UCB on cytokine expression and release after lipopolysaccharide (LPS) activation of whole human blood. Similar *ex vivo* models have been applied to investigate the efficacy of lead anti-inflammatory compounds with this system providing some advantages over *in vitro* assays, including culture of isolated peripheral blood mononuclear cells (PBMCs) [8,29]. To reveal whether UCB accumulation influences baseline cytokine production, we also obtained baseline serum samples from Gunn rats (an animal model of hyperbilirubinaemia due to autosomal recessive deficiency of UGT1A1) and control rats. We hypothesised that BV/UCB would demonstrate anti-inflammatory effects by mitigating LPS-mediated cytokine expression and release into whole blood.

Material and Methods

Human blood sample collection and *ex vivo* incubation with LPS and BV

To assess the effects of BV on *ex vivo* cytokine expression, fasting blood was collected from healthy male volunteers (25-52 years). Exclusion criteria for the subjects included current smoking, recent (within two weeks) bacterial infection and/or consumption of antioxidant supplements, consumption of >8 standard alcoholic drinks/week, elevated glucose or serum liver enzyme activities or presence of hyperlipidaemia. We also excluded subjects who showed less than a 10 fold increase in IL-1β expression in response to LPS to ensure the

homogeneity of responder phenotype in participating subjects. The study was approved by the Human Ethics Research Committee of Griffith University (MSC/02/10/HREC).

Whole blood was drawn from each subject into ethylenediaminetetra-acetic acid (EDTA) (Becton and Dickinson, Australia; total 50 mL). Two millilitres of EDTA blood was centrifuged at 1500 g for 15 min at 4°C using a benchtop centrifuge (Beckman Coulter, Australia) to obtain plasma for the measurement of UCB concentration. The remaining EDTA blood samples were kept in the dark and were prepared for *ex vivo* incubation with LPS and BV/control within one hour.

Two millilitres of EDTA blood was supplemented with BV (10 and 50 μM; Frontier Scientific, Logan UTA, USA) dissolved in DMSO (solvent control), in the presence or absence (control) of LPS (3 μg/mL) from *Escherichia coli* (K235, Sigma-Aldrich, Australia). Lipopolysacchraide was chosen as a stimulant because it is a specific TLR-4 ligand and stimulates cytokine release from immune cells [30]. Blood samples were then incubated in closed eppendorff tubes, in a water bath at 37°C for 4 and 8 hours, with hourly mixing. Samples were continuously protected from light using aluminium foil. Samples were collected for RNA extraction at 4 h from EDTA blood samples, which is an appropriate time point for cytokine expression analysis within whole blood [31]. Gene/ mRNA expression was assessed using quantitative real-time quantitative polymerase chain reaction (qRT-PCR) using cytokine primers as reported in Table 1. Thereafter, blood was centrifuged at 4 and 8 h as previously described. Plasma samples were then stored at −80°C until the analysis of cytokine concentrations.

Animal experiments

Breeding pairs of heterozygote Gunn rats were imported from the Rat Research and Resource Centre (Columbia, MO, USA) and non-jaundiced Wistar rats were used as wild type controls. Animals were housed at Griffith University Animal Facility (12 h light:dark cycle, constant temperature (22°C) and humidity (60%)). All the animals had continuous access to standard laboratory food pellets (Speciality Feeds, Glen Forest, Australia) and fresh water. Male homozygous Gunn rats (jaundiced) were sourced from an internal colony by breeding male homozygote Gunn rats with heterozygote females (non-jaundiced) after weaning. Concentrations of UCB were measured in blood collected from the tail tip of pups at the age of 21 days to confirm the presence of jaundice. The pups were kept under brief isofluorane anaesthesia (3% in O_2; 1-2 L/min) until blood collection was complete. Serum UCB was analysed using HPLC (see below). For the present study, male rats were used (10 wild type controls and 17 Gunn rats). Animals at 12 months of age were anaesthetised using an intraperitoneal injection of thiobutabarbital sodium (concentration 60 mg/mL; 1 mL/kg). A mid-line laparotomy was performed and ~ 5 mL of blood was collected from thoracic cavity as previously described [32]. Serum samples were stored at −80°C. All the animal experiments were conducted after approval by Griffith University Animal Ethics Research Committee (MSC/06/12).

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RNA extraction and qRT-PCR

Total RNA was isolated from whole blood using QIAamp® RNA Blood Mini Kit (Qiagen, Australia) and qRT-PCR was performed as previously described [25]. Primers for human HPRT-1, IL-6, IL-1β, TNF, IFN-γ, IL-1Ra IL-10, IL-8 and C5aR were designed using Primer Quest Software (Table 1; Integrated DNA technologies, Australia). Quantitative real time PCR was performed with Applied Biosystems Stepone™ and Stepone Plus™ Real-Time PCR Systems (AB Applied Biosystem, USA) using EvaGreen master mix (Integrated Biosciences, Australia). The relative quantification of gene expression was analysed using 2^{-} C_T method [33], normalised to the housekeeping gene (HPRT) and expressed as fold expression.

Cytokine analysis

Cytokines in plasma samples were analysed using a Milliplex® Human Cytokine Magnetic Panel kit for IL-6, IL-1β, TNF, IFN-γ, IL-10 and IL-1Ra and Rat Cytokine Magnetic Panel kit for IL-6, IL-1β and TNF (Abacus, Australia) according to manufacturer's instructions. The plasma concentration of each cytokine was detected and quantified using a Bio-plex Multiplex system (BioRad, USA). Human IL-8 concentration was measured using a high sensitivity ELISA kit (R & D Systems, Australia).

Cell count, haem and bilirubin analysis

Total blood cell counts were performed in fresh human EDTA blood samples using a Beckman Coulter Counter (Beckman Coulter Inc. USA). Plasma UCB and haem concentrations were quantified using HPLC and a photodiode array detector (Waters, Australia) as previously described [15]. A C18 reverse-phase HPLC guard and analytical column (4.6 × 150 mm, 3 μM; Phenomenex, Australia) was perfused at 0.7 mL/min using methanolic 0.1 M di-*n*-octylamine acetate (methanol:H_2O 95:5 v/v) mobile phase. The extracted samples were injected with a run time of 18 min and were analysed in duplicate. Haem (λmax 400 nm) and UCB (λmax 450 nm) eluted at 8 and 13 mins, respectively. Haemin and UCB (Frontier Scientific, Logan UTA, USA) at a concentration of 0-100 μM were used for external standards.

Statistical analysis

To detect any effect that varying BV concentrations (0, 10 and 50 μM) had on LPS induced cytokine gene and protein expression, one way of analysis of variance (ANOVA; post-hoc Tukey; Sigmastat, Ver. 11.0) was used. A repeated measure ANOVA (post-hoc Bonferronni t-test) was used to determine the effects of incubation time and BV treatment on haem and UCB concentrations. The relationship between baseline UCB and cytokine expression was analysed using Pearson correlation coefficient, or Spearman's rank correlation coefficient in data sets lacking normal distribution. Furthermore, unpaired t-tests were performed to detect differences in UCB concentration, body weight and IL-1β concentration between Gunn and wild type animals. When data was non-normally distributed, a Mann Whitney U-test was used. A P-value of <0.05 was considered significant. Data is expressed as either mean ± S.E. or median (25-75% interquartile range), as appropriate.

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Results

Clinical parameters, haem and UCB concentration

Healthy male subjects were recruited (37.1 ± 8.5 years old) for this study. All total blood cell counts were within the normal range (Table 2). Haem and UCB concentrations at both baseline and after 4 and 8 h of incubation were assessed in all conditions. All samples underwent minor haemolysis after 4 and 8 h of incubation (Table 3). The average UCB concentration of the subjects was 5.23 ± 1.41 μmol/L at baseline and significantly increased after 4 and 8 h of incubation with 50 μM BV only (Table 3, $P<0.05$). Furthermore, control samples showed a non-significant increase in baseline UCB concentration after 4 and 8 h of incubation (Table 3).

Biliverdin and cytokine expression

The mRNA expression of pro- and anti-inflammatory cytokines from blood samples incubated with BV ± LPS were assessed. Individual subjects' response to LPS-mediated cytokine expression can be found in Figures S1. Biliverdin treatment alone had no effect on cytokine mRNA abundance (Supplementary Figure S2). However, a dose dependent decrease in the mRNA expression of IL-1β, IL-6, IFN-γ and IL-1Ra occurred when blood was stimulated with LPS and BV. Fifty micromolar BV was required to significantly reduce the expression of these cytokines (Figures 1A, 1B, 1D and 1E, $P<0.05$). Biliverdin had no effect on the expression of TNF in the presence of LPS (Figure 1C).

Plasma cytokine concentrations were measured 8 h after LPS incubation in accordance with previous studies, which show a robust increase in IL-1β at this time point [6,34]. Similar to the gene response, subjects showed variation in their response to cytokine protein expression after LPS exposure (Supplementary Figure S3). Therefore, inhibition of cytokine release by BV is presented relative to each individual's LPS response (Figure 2). Biliverdin alone did not affect cytokine release into plasma (Supplementary Figure S4). However, BV dose dependently and significantly decreased IL-1β plasma concentration in the presence of LPS (Figure 2A, $P< 0.05$). Biliverdin did not significantly affect LPS-induced IL-6, TNF, IFN-γ, IL-1Ra and IL-10 cytokine release into plasma (Figures 2B-2F).

Association between baseline UCB concentration and cytokine expression

We have previously shown that increasing concentrations of UCB *in vivo* are associated with increased circulating IL-1β concentrations [35]. Therefore, we sought to investigate whether baseline UCB concentration in our cohort study impacted upon the gene and protein expression of cytokines in response to LPS (i.e. in solvent control samples not treated with BV). A significant positive correlation between UCB and LPS-mediated IL-1β ($R = 0.929$; $P<0.001$), IFN-γ ($R=0.809$; $P=0.027$) and IL-1Ra ($R=0.786$; $P=0.025$) gene expression (Figures 3A, 3D and 3E) existed. However, no significant correlation between baseline UCB concentration and gene expression of IL-6 and IL-10 after LPS exposure occurred (Figures 3B and 3F). Furthermore, there were no significant correlations between baseline UCB concentrations and LPS-mediated cytokine (IL-1β, IL-6, IFN-γ, IL-1Ra and IL-10) release into plasma (Figure 4). Interestingly, increasing concentration of UCB tended to be associated with increases gene and protein expression of TNF (Figures 3C and 4C, $P<0.1$).

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To confirm a possible effect of physiologically, severely elevated UCB (beyond that seen in our human subjects) on physiological IL-1β concentrations in blood; serum samples were collected from wild type and hyperbilirubinaemic Gunn rats. Gunn rats had significantly reduced body mass compared to control animals (Figure 5A, $P<0.001$) and had significantly increased UCB concentrations compared to their wild type counterparts (Figure 5B, $P<0.05$), as reported previously [32]. Gunn rats also had a significantly elevated plasma IL-1β concentration compared to wild type controls (Figure 5C, $P<0.001$). Furthermore, a significant and positive relationship existed between UCB and IL-1β concentrations (Figure 5D, R=0.488 and $P=0.01$).

Unconjugated bilirubin, biliverdin and chemokine IL-8 expression

Interleukin-8, the most abundant chemokine secreted by neutrophils, promotes the migration of neutrophils towards the site of inflammation, encouraging the acute phase of tissue damage/pathogen destruction [36,37]. Blood samples incubated with BV ± LPS were analysed for IL-8 gene and protein expression. Biliverdin alone significantly decreased IL-8 gene expression (Supplementary Figure S5A). When BV was co-incubated with LPS, IL-8 gene and protein expression also were decreased in a dose dependent manner, with 50 μM BV being most effective (Figures 6A-6B; $P<0.05$).

We also analysed whether baseline UCB concentration affected IL-8 expression in leukocytes after LPS activation. A positive correlation existed between UCB and IL-8 gene expression (R=0.857, $P=0.006$; Figure 6C); however, no significant relationship existed between UCB and IL-8 release (Figure 6D).

Biliverdin and C5aR expression

We have recently shown that stimulation using LPS induces C5aR expression in RAW 264.7 and bone marrow derived macrophages after 24 and 48 h incubation [25]. Biliverdin at 50 μM significantly reduced the LPS-mediated increase in C5aR in both primary and immortalised macrophages [25]. Therefore, we investigated whether incubation of whole blood with LPS would induce C5aR and whether BV would mitigate this increase. Stimulation of whole blood with LPS significantly decreased C5aR gene expression ($P<0.05$; Supplementary Figure S6A). However, BV+LPS failed to show any additional significant reduction in C5aR expression (Supplementary Figure S6A). The effect of BV treatment alone on C5aR expression was also assessed. Biliverdin treatment tended to decrease C5aR expression (ANOVA effect; $P=0.08$; Supplementary Figure S6B).

Discussion

The present study shows novel immuno-modulatory effects of supplemented BV and physiological UCB concentrations on both proand anti-inflammatory cytokine gene and protein expression in human blood, in response to whole blood LPS exposure. Biliverdin, the precursor of UCB, mitigated *ex vivo* LPS-induced expression of IL-1β, IL-6, IFN-γ and IL-1Ra at the transcriptional level. Biliverdin also attenuated LPS-mediated IL-1β and IL-8 release into plasma. Increasing baseline concentrations of UCB in human samples were associated with increased IL-1β, IFN-γ, IL-1Ra and IL-8 gene expression. Furthermore,

increased baseline IL-1β concentrations in severely hyperbilirubinaemic rat blood samples were positively correlated with bilirubin concentrations.

Biliverdin and cytokine response

A significant body of evidence shows the anti-inflammatory potential of BV in cell culture and in animal models of organ transplantation and sepsis. For example, investigations in cardiac, lung, liver transplantation and sepsis models show that BV treatment improves tissue graft survival, function and tissue injury by inhibiting pro-inflammatory cytokine expression [26,38-41]. Furthermore, a recent study in a rat model of haemorrhagic shock and resuscitation reported that pre-treatment with BV attenuated lung injury via decreased expression of IL-6, TNF and iNOS in lung tissue [42]. Although these studies show great promise, they have all been conducted in animal models, which have limitations when predicting human responses. For example, Seok et al. [43] recently demonstrated that mouse models of inflammation poorly correlate with human inflammatory responses. Therefore, we conducted the first in human *ex vivo* assay to assess the effect of exogenous BV on leukocyte responses to LPS exposure. We adopted the whole blood *ex vivo* model of LPS stimulation without any culture media as used in other studies [29,44]. Whole blood retains all blood components and provides a normal working environment for cell to cell interactions [29].

The data presented here further strengthens the argument for an anti-inflammatory role of BV, as reported in animal studies, by showing inhibitory effects of BV (50 μM) on LPS-mediated mRNA abundance of IL-1β and IL-6. However, when cytokines were analysed in plasma, BV only decreased LPS-mediated IL-1β release. The enhanced production of cytokines, particularly IL-1β during acute inflammation is important for resolution of inflammation/infectious diseases, including sepsis [5]. Furthermore, animal studies show that the beneficial effects of anti-IL-1β neutralising antibody (XOMA 052) in several acute and chronic inflammatory diseases, including type 2 diabetes, gout and ischaemia [45,46]. XOMA 052 antibody blocked the IL-1β induced expression of IL-6 and IL-8 in human lung fibroblast cell line, suggesting the importance of IL-1β in inflammation [45]. Therefore, BV's inhibitory effect on IL-β appears to be a very important finding and provides preliminary evidence in support of BV's anti-inflammatory potential in humans. These data are in agreement with BV's effects in experimental and *in vitro* studies [26,38-41]. Moreover, experiments performed in animal models of transplantation and sepsis investigated BV's effects on pro-inflammatory cytokine gene expression only and reported that BV consistently reduced the expression of pro-inflammatory cytokines. For example, BV treatment prior to endotoxin shock or caecal ligation and puncture (CLP) or organ transplantation significantly decreased the mRNA expression of pro-inflammatory cytokines, including IL-1β, IL-6, TNF and monocyte chemoattractant protein (MCP)-1 [26,27,41] in injured tissues. In contrast to this, BV decreased both the gene and protein expression of IL-6 in LPS-stimulated RAW macrophages; however, protein expression of TNF remained unchanged [25,26]. We also report here suppression of LPS-induced IFN-γ and IL-1Ra gene expression. Biliverdin at a higher concentration (100 μM) also suppresses IFN-γ release in anti-CD3 stimulated mice splenocytes [38]. The mechanism to explain the differential effects of BV on pro-inflammatory cytokine expression remains unknown.

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However, the data presented here are valuable, in that they document 1) inhibitory effects of BV on gene expression in human leukocytes and 2) confirm that some of these responses are accompanied by reductions in cytokine release, which is rarely documented in cell culture and animal studies.

We suggest that BV's inconsistent capacity to decrease the release of cytokines into plasma may be mediated by the variations in human cytokine kinetics and release after LPS stimulation. For example, the maximum mRNA levels for TNF and IL-6 in whole blood are reported between 2-4 h after LPS exposure and protein levels were rapidly increased at 4-6 h after LPS stimulation and, thereafter, start to decrease [34,47]. In contrast to this, IL-1β gene expression decreases slowly and protein levels peak after 8 h after whole blood LPS stimulation [34]. Unfortunately, it was beyond the scope of this manuscript to measure each cytokine at each of their optimal time points, however, the data do provide very interesting and novel evidence to suggest that BV can reduce IL-1β and IL-8 expression and their release in a human blood *ex vivo* LPS model of inflammation.

In the present study, total cell counts showed that neutrophils represented the major cell population of white blood cells in blood. Therefore, we also investigated BV's effects on IL-8. We reported a significant decrease in LPS-induced IL-8 gene and protein expression by BV (50 μM). This is the first study to show an effect of BV on IL-8. Supportive data presented by Andria et al. [21] recently showed that BV treatment prevented IRI-induced cell death and reduced infiltration of neutrophils by >50 % in the pig livers [21]. In addition, rats pre-treated with BV showed reduced neutrophil recruitment into bronchoalveolar lavage fluid and intestine after LPS and CLP exposure, respectively [26,41], suggesting that BV might reduce the severity of sepsis in various organs via inhibition of IL-8 mediated neutrophil infiltration. We suggest that BV exerts these effects by suppressing leukocyte IL-8 expression and release, as documented here.

Unconjugated bilirubin and cytokine response

A surprising finding of this study was that in humans, higher baseline UCB concentrations were significantly associated with greater LPS-mediated cytokine gene expression. Furthermore, serum samples from hyperbilirubinaemic Gunn rats had increased baseline IL-1β concentrations. A previous report indicates that baseline IL-1β concentration is elevated in hyperbilirubinaemic humans [35]. We sought to determine whether elevated IL-1β in humans and rats might be caused by increased IL-1β gene expression in whole blood. A positive correlation between UCB concentration and expression of IL-1β in addition to IFN-γ, IL-1Ra and IL-8 was found after LPS exposure; however, no significant correlation between UCB concentration and LPS-induced IL-6, TNF and IL-10 gene expression occurred. Furthermore, when cytokines were measured in plasma samples, no significant correlation existed between UCB concentrations and LPS-induced cytokine release. Similar observations were reported in Gunn rats and RAW macrophages, in which, UCB showed no effect on IL-6, TNF and IL-10 concentrations after LPS exposure; however UCB decreased the expression of LPS-mediated inducible nitric oxide synthase (iNOS) [48]. These findings are supported by an *in vivo* study by Dorresteijn et al. (recently presented in abstract form), showing no change in LPS-induced pro-inflammatory cytokine

concentrations in subjects receiving atazanavir (300 mg twice daily for four days). Atazanavir induces hyperbilirubinaemia by inhibiting the enzyme UGT1A1 [49]. However, in the same study, Dorresteijn et al. showed that atazanavir significantly decreased LPS-mediated IL-10 concentration, suggesting immuno-modulatory activities of UCB in humans.

Although studies have shown that individuals with mildly elevated concentration of UCB in Gilbert's Syndrome (GS) have low prevalence of cardiovascular disease [50,51], excessive accumulation of UCB (>200 μM) in newborn infants causes jaundice [52,53]. Elevated UCB concentrations are clearly toxic to neuronal tissues, promoting apoptosis in astrocytes and in brain endothelial cells via induction of pro-inflammatory cytokines (IL-1β, IL-6 and TNF) [54,55]. Our human *ex vivo* and *in vivo* data from rat serum samples both support a hypothesis that UCB increases IL-1β expression in leukocytes, which then excrete IL-1β into plasma. IL-1β is synthesised as pro IL-1β and requires activation by caspase-1. Caspase-1 together with caspase-3 and -9 induce apoptosis and DNA fragmentation [1]. Studies show that UCB increases caspase-3 and caspase-9 activities in hepatocytes and cardiomyocytes [56,57]. However, UCB's effect on caspase-1 (which is strongly associated with septic responses) [1] remains unknown, clearly warrants future investigation and represents a potentially very exciting area of future research.

Unconjugated bilirubin's effects on cytokine expression, reported here, are interesting because Gunn rats and mice treated with UCB (8.5 μmol/kg) show improved survival of cardiac and islets grafts, respectively via attenuation of mRNA expression of TNF, IL-6, MCP-1, iNOS and cyclooxygenase (COX)-2 [19,58]. However, none of the above studies showed a relationship between baseline UCB concentrations and pro-inflammatory cytokine expression. This suggests that UCB may have dichotomous effects in rodents and humans. Importantly, the data presented here show that increasing concentration of UCB is positively correlated with IL-1β expression and are in agreement with a previous study that showed elevated circulating IL1β concentrations in hyperbilirubinaemic humans [35]. We suggest that UCB concentration plays an important role in modulating inflammatory responses with very low (<5 μM) and mildly elevated UCB (>17 μM) concentrations associated with increased IL-1β concentrations [35]. Furthermore, in support of our findings, human neutrophils treated with UCB alone (10-300 μM) for 24 h showed increased IL-1β and IL-8 concentration in media [59], implying that UCB at elevated concentrations may heighten inflammation. However, no studies have thus far reported effects of UCB on LPS-mediated cytokine gene and protein expression. We reported significant, positive correlations between increasing baseline UCB concentration (up to 12 μM) and LPS-driven gene expression of IL-1β, IFN-γ, IL-1Ra and IL-8. However, the baseline UCB concentrations were not associated with the release of cytokines into plasma. These data are in agreement with an *in vitro* study showing that UCB concentration (10-300 μM) did not influence IL-1β and IL-8 release into media after LPS activation of human neutrophils [59]. We suggest that a higher concentration (compared to 12 μM studied here) of UCB is required to increase synthesis and release of baseline IL-1β [35,59], which was confirmed in our hyperbilirubinaemic Gunn animals (UCB ~ 100 μM).

It is possible that BV could be infused into Gunn rats and IL-1β concentrations assessed. It should be noted, however, that BV is rapidly reduced to UCB [15,20], which will result in

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further increase in the UCB concentration in Gunn rats and may promote inflammation. However, a recent study by Kosaka et al. [42] have shown that Sprague–Dawley rats administrated various doses of BV (0-100 mg/kg) were protected haemorrhagic shock induced lung injury, further supporting the cytoprotective potential of BV.

These data suggest that both BV and UCB induce differential effects on inflammatory mediators expression after LPS exposure, which is interesting because BV is rapidly reduced to UCB [15,20,26]. Our data confirms that leukocytes are capable of such reduction, showing ~ a three-fold increase in UCB concentration after addition of 50 μM BV. However, all the samples showed mild increase in haemolysis, which resulted in a small increase in UCB concentrations in control samples. We suggest that the BV (50 μM)-induced increase in UCB concentration is a consequence of both haem and BV metabolism. This data is in agreement with *in vivo* data showing that UCB increases by approximately 33% of the exogenously administered circulating BV concentration [15]. Cell culture and animal studies provide an insight into the differential effects on BV and UCB. For example, BV inhibits the activation of nuclear factor kappa B (NF-κB) in HEK293A cells and in animal models of sepsis and transplantation [26,38,41,60]. On the contrary, UCB does not affect NF-κB expression both *in vivo* and *in vitro* [61]. Therefore, we suggest that in humans, BV via activation of transcription factor NF-κB may counter-regulate inflammation in the acute phase (Figure 7). Accumulating evidence suggests that UCB is the potential activator and ligand of transcription factor aryl hydrocarbon receptor (AhR) [22,62]. AhR was first discovered as a mediator of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin) toxicity and over the last decade it has emerged as a potential regulator of immune system [63]. We suggest that UCB, similar to AhR agonist TCDD [64], may increase the gene expression of IL-1β in the presence/absence of LPS stimulation (Figure 7). Therefore, it is likely that BV and UCB induce their effects on inflammatory mediators via different mechanisms.

Biliverdin and C5aR expression

Having established that BV decreases LPS-dependent C5aR expression in primary and immortalised macrophages [25], we aimed to assess whether LPS/BV would also modulate C5aR gene expression in human blood. Lipopolysaccharide significantly inhibited C5aR expression at 4 h. Although, blood leukocytes from patients with severe septic shock show a remarkable increase in C5aR gene expression compared to healthy individuals [65], incubation of human monocytes with LPS (6-12 h) significantly decreased C5aR mRNA expression [66,67], suggesting counter-regulatory role of LPS on C5aR in human leukocytes. However, BV+LPS had no additional significant effect on C5aR expression vs. LPS control. Biliverdin treatment alone tended to decrease C5aR expression (ANOVA effect; P=0.08) in agreement with our previous published reports indicating that BV treatment of macrophages decreases C5aR expression [25]. Assessment of C5aR expression in a larger group of individuals may be necessary to reveal a statistically significant effect of BV.

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Limitations

Although we recruited a relatively small sample of volunteers, we reduced between subject responses by investigating healthy individuals and limited recruitment to male subjects, eliminating possible variation introduced by the oestrous cycle in women [68]. By investigating human subjects who showed >10 fold IL-1β expression our findings are limited to those individuals with a strong host response to LPS. In addition, all samples experienced mild haemolysis at 4 and 8 h, which contributed to a non-significant increase in UCB in control samples. Haemolysis is frequently observed in patients with sepsis after acute infection [69]. However, haemolysis did not contribute to inflammation in the present study as indicated by low levels of cytokines in non-LPS treated samples. Only one time point was used to measure the release of cytokines in this study (8 h) and it is possible that measurement at other time points (4-6 h) [34,47] might reveal additional significant effects of BV and UCB. Despite this, the 8 h time point was appropriate for LPS-mediated IL-1β and IL-8 release into plasma, which was decreased after BV treatment. Clearly, the kinetics of cytokine release differs between targets and, therefore, this likely accounted partly for the lack of congruence between gene and protein data. Although previous *in vitro* studies showed suppressive effects of BV on both gene and protein expression of pro-inflammatory cytokines (IL-6), these studies were performed using a single cell type. The present study has benefit of studying inflammatory responses in a complex, yet appropriate matrix composing of multiple cell lineages and, most importantly, these responses were tested in human cells.

Summary

Collectively, these data show that BV inhibits whole human blood responses to LPS, by reducing mRNA expression of IL-1β, IL-6, IFN-γ, IL-1Ra and IL-8. Biliverdin also attenuated the LPS-induced excretion of IL-1β and IL-8 into plasma. Interestingly, UCB at increasing baseline concentrations was correlated with greater transcription of cytokines in response to LPS, suggesting UCB has pro-inflammatory potential. In summary, in this report we demonstrate that both BV and UCB are immuno-modulatory compounds and that BV could represent potential therapeutic target against inflammatory disorders, including sepsis, based upon its potent ability to potently inhibit IL-1β and IL-8 transcription and release in leukocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Ms Connie Boon for assistance with HPLC experiments. This project was funded by the FWF-Austrian Science Fund (P21162-K-H.W. and A.B.)

References

1. Cinel I, Opal SM. Molecular biology of inflammation and sepsis: a primer. Crit Care Med. 2009; 37:291–304. [PubMed: 19050640]

Europe PMC Funders Author Manuscripts

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2. Annane D, Bellissant E, Cavaillon JM. Septic shock. Lancet. 2005; 365:63–78. [PubMed: 15639681]
3. Tsujimoto H, Ono S, Efron PA, Scumpia PO, Moldawer LL, et al. Role of Toll-like receptors in the development of sepsis. Shock. 2008; 29:315–321. [PubMed: 18277854]
4. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, et al. The role of the anaphylatoxins in health and disease. Mol Immunol. 2009; 46:2753–2766. [PubMed: 19477527]
5. Ward PA. New approaches to the study of sepsis. EMBO Mol Med. 2012; 4:1234–1243. [PubMed: 23208733]
6. Wang JE, Solberg R, Okkenhaug C, Jørgensen PF, Krohn CD, et al. Cytokine modulation in experimental endotoxemia: characterization of an ex vivo whole blood model. Eur Surg Res. 2000; 32:65–73. [PubMed: 10810211]
7. Ait-Oufella H, Taleb S, Mallat Z, Tedgui A. Recent advances on the role of cytokines in atherosclerosis. Arterioscler Thromb Vasc Biol. 2011; 31:969–979. [PubMed: 21508343]
8. Dorresteijn MJ, Draisma A, van der Hoeven JG, Pickkers P. Lipopolysaccharide-stimulated whole blood cytokine production does not predict the inflammatory response in human endotoxemia. Innate Immun. 2010; 16:248–253. [PubMed: 19710091]
9. Aziz M, Jacob A, Yang WL, Matsuda A, Wang P. Current trends in inflammatory and immunomodulatory mediators in sepsis. J Leukoc Biol. 2013; 93:329–342. [PubMed: 23136259]
10. Martí-Carvajal AJ, Solà I, Gluud C, Lathyris D, Cardona AF. Human recombinant protein C for severe sepsis and septic shock in adult and paediatric patients. Cochrane Database Syst Rev. 2012; 12:CD004388. [PubMed: 23235609]
11. Fisher CJ Jr, Opal SM, Dhainaut JF, Stephens S, Zimmerman JL, et al. Influence of an anti-tumor necrosis factor monoclonal antibody on cytokine levels in patients with sepsis. The CB0006 Sepsis Syndrome Study Group. Crit Care Med. 1993; 21:318–327. [PubMed: 8440099]
12. Zeni F, Freeman B, Natanson C. Anti-inflammatory therapies to treat sepsis and septic shock: a reassessment. Crit Care Med. 1997; 25:1095–1100. [PubMed: 9233726]
13. Cohen J, Carlet J. INTERSEPT: an international, multicenter, placebo-controlled trial of monoclonal antibody to human tumor necrosis factor-alpha in patients with sepsis. International Sepsis Trial Study Group. Crit Care Med. 1996; 24:1431–1440. [PubMed: 8797612]
14. National Health and Medical Research Council A, Group AaNZICSCT. ADjunctive coRticosteroid trEatment iN criticAlly ilL Patients With Septic Shock (ADRENAL). 2012.
15. Bulmer AC, Coombes JS, Blanchfield JT, Toth I, Fassett RG, et al. Bile pigment pharmacokinetics and absorption in the rat: therapeutic potential for enteral administration. Br J Pharmacol. 2011; 164:1857–1870. [PubMed: 21486273]
16. Bulmer AC, Verkade HJ, Wagner KH. Bilirubin and beyond: a review of lipid status in Gilbert's syndrome and its relevance to cardiovascular disease protection. Prog Lipid Res. 2013; 52:193–205. [PubMed: 23201182]
17. Fevery J. Bilirubin in clinical practice: a review. Liver Int. 2008; 28:592–605. [PubMed: 18433389]
18. Nakao A, Neto JS, Kanno S, Stolz DB, Kimizuka K, et al. Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both. Am J Transplant. 2005; 5:282–291. [PubMed: 15643987]
19. Wang H, Lee SS, Dell'Agnello C, Tchipashvili V, d'Avila JC, et al. Bilirubin can induce tolerance to islet allografts. Endocrinology. 2006; 147:762–768. [PubMed: 16254033]
20. Wegiel B, Baty CJ, Gallo D, Csizmadia E, Scott JR, et al. Cell surface biliverdin reductase mediates biliverdin-induced anti-inflammatory effects via phosphatidylinositol 3-kinase and Akt. J Biol Chem. 2009; 284:21369–21378. [PubMed: 19509285]
21. Andria B, Bracco A, Attanasio C, Castaldo S, Cerrito MG, et al. Biliverdin protects against liver ischemia reperfusion injury in swine. PLoS One. 2013; 8:e69972. [PubMed: 23922878]
22. Jangi S, Otterbein L, Robson S. The molecular basis for the immunomodulatory activities of unconjugated bilirubin. Int J Biochem Cell Biol. 2013; 45:2843–2851. [PubMed: 24144577]
23. Bulmer AC, Blanchfield JT, Toth I, Fassett RG, Coombes JS. Improved resistance to serum oxidation in Gilbert's syndrome: a mechanism for cardiovascular protection. Atherosclerosis. 2008; 199:390–396. [PubMed: 18155709]

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24. Wegiel B, Gallo D, Csizmadia E, Roger T, Kaczmarek E, et al. Biliverdin inhibits Toll-like receptor-4 (TLR4) expression through nitric oxide-dependent nuclear translocation of biliverdin reductase. Proc Natl Acad Sci U S A. 2011; 108:18849–18854. [PubMed: 22042868]
25. Bisht K, Wegiel B, Tampe J, Neubauer O, Wagner KH, et al. Biliverdin modulates the expression of C5aR in response to endotoxin in part via mTOR signaling. Biochem Biophys Res Commun. 2014; 449:94–99. [PubMed: 24814708]
26. Sarady-Andrews JK, Liu F, Gallo D, Nakao A, Overhaus M, et al. Biliverdin administration protects against endotoxin-induced acute lung injury in rats. Am J Physiol Lung Cell Mol Physiol. 2005; 289:L1131–1137. [PubMed: 16155084]
27. Nakao A, Otterbein LE, Overhaus M, Sarady JK, Tsung A, et al. Biliverdin protects the functional integrity of a transplanted syngeneic small bowel. Gastroenterology. 2004; 127:595–606. [PubMed: 15300591]
28. Kadl A, Pontiller J, Exner M, Leitinger N. Single bolus injection of bilirubin improves the clinical outcome in a mouse model of endotoxemia. Shock. 2007; 28:582–588. [PubMed: 17577133]
29. Myrianthefs P, Karatzas S, Venetsanou K, Grouzi E, Evagelopoulou P, et al. Seasonal variation in whole blood cytokine production after LPS stimulation in normal individuals. Cytokine. 2003; 24:286–292. [PubMed: 14609570]
30. Shiratsuch H, Basson MD. Differential regulation of monocyte/macrophage cytokine production by pressure. Am J Surg. 2005; 190:757–762. [PubMed: 16226954]
31. Rainen L, Oelmueller U, Jurgensen S, Wyrich R, Ballas C, et al. Stabilization of mRNA expression in whole blood samples. Clin Chem. 2002; 48:1883–1890. [PubMed: 12406972]
32. Boon AC, Hawkins CL, Bisht K, Coombes JS, Bakrania B, et al. Reduced circulating oxidized LDL is associated with hypocholesterolemia and enhanced thiol status in Gilbert syndrome. Free Radic Biol Med. 2012; 52:2120–2127. [PubMed: 22521902]
33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]
34. Netea MG, Drenth JP, De Bont N, Hijmans A, Keuter M, et al. A semi-quantitative reverse transcriptase polymerase chain reaction method for measurement of MRNA for TNF-alpha and IL-1 beta in whole blood cultures: its application in typhoid fever and exentric exercise. Cytokine. 1996; 8:739–744. [PubMed: 8932986]
35. Wallner M, Bulmer AC, Mölzer C, Müllner E, Marculescu R, et al. Haem catabolism: a novel modulator of inflammation in Gilbert's syndrome. Eur J Clin Invest. 2013; 43:912–919. [PubMed: 23865893]
36. Remick DG. Interleukin-8. Crit Care Med. 2005; 33:S466–467. [PubMed: 16340423]
37. Neubauer O, Sabapathy S, Ashton KJ, Desbrow B, Peake JM, et al. Time course-dependent changes in the transcriptome of human skeletal muscle during recovery from endurance exercise: from inflammation to adaptive remodeling. J Appl Physiol. 2014; 116:274–287. [PubMed: 24311745]
38. Yamashita K, McDaid J, Ollinger R, Tsui TY, Berberat PO, et al. Biliverdin, a natural product of heme catabolism, induces tolerance to cardiac allografts. FASEB J. 2004; 18:765–767. [PubMed: 14977878]
39. Wang J, Zhou HC, Pan P, Zhang N, Li WZ. Exogenous biliverdin improves the function of lung grafts from brain dead donors in rats. Transplant Proc. 2010; 42:1602–1609. [PubMed: 20620483]
40. Fondevila C, Shen XD, Tsuchiyashi S, Yamashita K, Csizmadia E, et al. Biliverdin therapy protects rat livers from ischemia and reperfusion injury. Hepatology. 2004; 40:1333–1341. [PubMed: 15565657]
41. Overhaus M, Moore BA, Barbato JE, Behrendt FF, Doering JG, et al. Biliverdin protects against polymicrobial sepsis by modulating inflammatory mediators. Am J Physiol Gastrointest Liver Physiol. 2006; 290:G695–703. [PubMed: 16537973]
42. Kosaka J, Morimatsu H, Takahashi T, Shimizu H, Kawanishi S, et al. Effects of biliverdin administration on acute lung injury induced by hemorrhagic shock and resuscitation in rats. PLoS One. 2013; 8:e63606. [PubMed: 23667646]

Europe PMC Funders Author Manuscripts

43. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A. 2013; 110:3507–3512. [PubMed: 23401516]
44. Marie C, Fitting C, Muret J, Payen D, Cavaillon JM. Interleukin 8 production in whole blood assays: Is interleukin 10 responsible for the downregulation observed in sepsis? Cytokine. 2000; 12:55–61. [PubMed: 10623443]
45. Owyang AM, Issafras H, Corbin J, Ahluwalia K, Larsen P, et al. XOMA 052, a potent, high-affinity monoclonal antibody for the treatment of IL-1Î²-mediated diseases. MAbs. 2011; 3:49–60. [PubMed: 21048425]
46. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood. 2011; 117:3720–3732. [PubMed: 21304099]
47. DeForge LE, Remick DG. Kinetics of TNF, IL-6, and IL-8 gene expression in LPS-stimulated human whole blood. Biochem Biophys Res Commun. 1991; 174:18–24. [PubMed: 1989598]
48. Lanone S, Bloc S, Foresti R, Almolki A, Taillé C, et al. Bilirubin decreases nos2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxic shock in rats. FASEB J. 2005; 19:1890–1892. [PubMed: 16129699]
49. Rodríguez-Nóvoa S, Martín-Carbonero L, Barreiro P, González-Pardo G, Jiménez-Nácher I, et al. Genetic factors influencing atazanavir plasma concentrations and the risk of severe hyperbilirubinemia. AIDS. 2007; 21:41–46. [PubMed: 17148966]
50. Novotný L, Vítek L. Inverse relationship between serum bilirubin and atherosclerosis in men: a meta-analysis of published studies. Exp Biol Med. 2003; 228:568–571.
51. Lin JP, O'Donnell CJ, Schwaiger JP, Cupples LA, Lingenhel A, et al. Association between the UGT1A1*28 allele, bilirubin levels, and coronary heart disease in the Framingham Heart Study. Circulation. 2006; 114:1476–1481. [PubMed: 17000907]
52. Dennery PA, Seidman DS, Stevenson DK. Neonatal hyperbilirubinemia. N Engl J Med. 2001; 344:581–590. [PubMed: 11207355]
53. Watchko JF. Neonatal hyperbilirubinemia--what are the risks? N Engl J Med. 2006; 354:1947–1949. [PubMed: 16672708]
54. Fernandes A, Falcão AS, Silva RF, Gordo AC, Gama MJ, et al. Inflammatory signalling pathways involved in astroglial activation by unconjugated bilirubin. J Neurochem. 2006; 96:1667–1679. [PubMed: 16476078]
55. Akin E, Clower B, Tibbs R, Tang J, Zhang J. Bilirubin produces apoptosis in cultured bovine brain endothelial cells. Brain Res. 2002; 931:168–175. [PubMed: 11897102]
56. Seubert JM, Darmon AJ, El-Kadi AO, D'Souza SJ, Bend JR. Apoptosis in murine hepatoma hepa 1c1c7 wild-type, C12, and C4 cells mediated by bilirubin. Mol Pharmacol. 2002; 62:257–264. [PubMed: 12130676]
57. Kim DS, Chae SW, Kim HR, Chae HJ. CO and bilirubin inhibit doxorubicin-induced cardiac cell death. Immunopharmacol Immunotoxicol. 2009; 31:64–70. [PubMed: 18756386]
58. Lee S, Yamada T, Osako T, Stolz DB, Abe M, et al. Recipient hyperbilirubinaemia protects cardiac graft in rat heterotopic heart transplantation. Eur J Cardiothorac Surg. 2014; 45:481–488. [PubMed: 23946500]
59. Weinberger B, Archer FE, Kathiravan S, Hirsch DS, Kleinfeld AM, et al. Effects of bilirubin on neutrophil responses in newborn infants. Neonatology. 2013; 103:105–111. [PubMed: 23182920]
60. Gibbs PE, Maines MD. Biliverdin inhibits activation of NF-kappaB: reversal of inhibition by human biliverdin reductase. Int J Cancer. 2007; 121:2567–2574. [PubMed: 17683071]
61. Wang WW, Smith DL, Zucker SD. Bilirubin inhibits iNOS expression and NO production in response to endotoxin in rats. Hepatology. 2004; 40:424–433. [PubMed: 15368447]
62. Bock KW, Köhle C. Contributions of the Ah receptor to bilirubin homeostasis and its antioxidative and atheroprotective functions. Biol Chem. 2010; 391:645–653. [PubMed: 20370320]
63. Beamer CA, Shepherd DM. Role of the aryl hydrocarbon receptor (AhR) in lung inflammation. Semin Immunopathol. 2013; 35:693–704. [PubMed: 23963493]
64. Wong PS, Vogel CF, Kokosinski K, Matsumura F. Arylhydrocarbon receptor activation in NCI-H441 cells and C57BL/6 mice: possible mechanisms for lung dysfunction. Am J Respir Cell Mol Biol. 2010; 42:210–217. [PubMed: 19372248]

65. Kalkoff M, Cursons RT, Sleigh JW, Jacobson GM. The use of real time rtPCR to quantify inflammatory mediator expression in leukocytes from patients with severe sepsis. Anaesth Intensive Care. 2004; 32:746–755. [PubMed: 15648982]

66. Raby AC, Holst B, Davies J, Colmont C, Laumonnier Y, et al. TLR activation enhances C5a-induced pro-inflammatory responses by negatively modulating the second C5a receptor, C5L2. Eur J Immunol. 2011; 41:2741–2752. [PubMed: 21630250]

67. Seow V, Lim J, Iyer A, Suen JY, Ariffin JK, et al. Inflammatory responses induced by lipopolysaccharide are amplified in primary human monocytes but suppressed in macrophages by complement protein C5a. J Immunol. 2013; 191:4308–4316. [PubMed: 24043889]

68. Evans J, Salamonsen LA. Inflammation, leukocytes and menstruation. Rev Endocr Metab Disord. 2012; 13:277–288. [PubMed: 22865231]

69. Goyette RE, Key NS, Ely EW. Hematologic changes in sepsis and their therapeutic implications. Semin Respir Crit Care Med. 2004; 25:645–659. [PubMed: 16088507]

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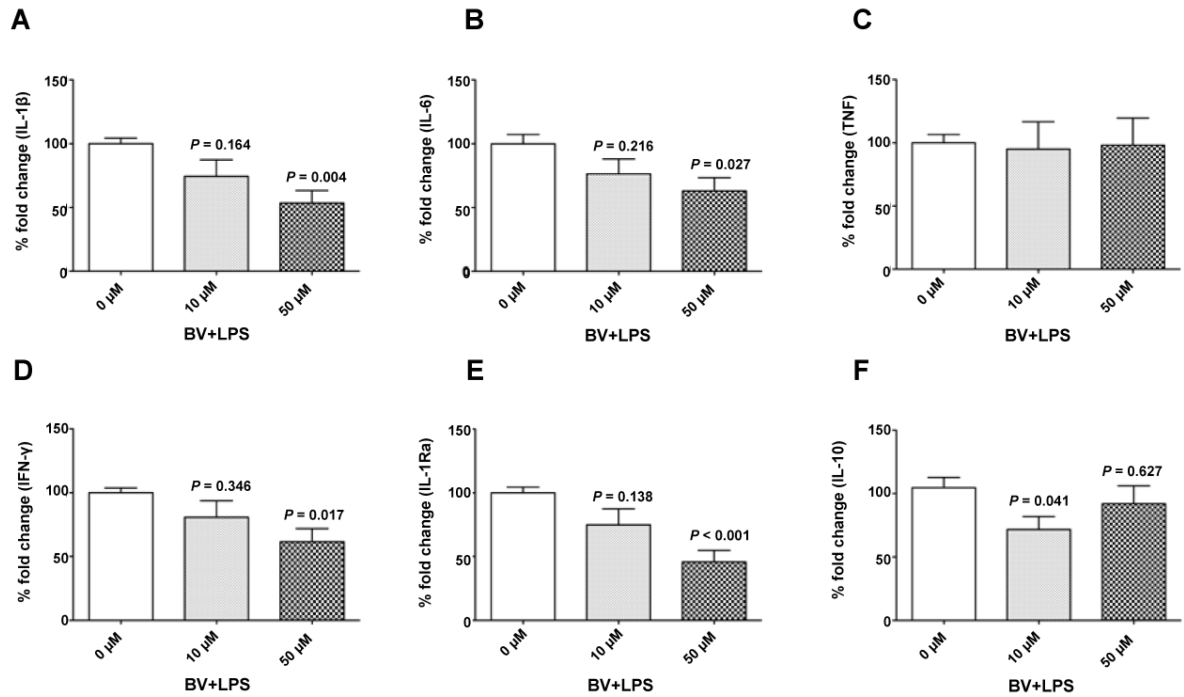

Figure 1. Cytokine gene expression in response to LPS and BV

Whole blood was incubated with BV and LPS for 4 h and the mRNA expression was assessed. The relative fold change of each cytokine **(A-F)** was analysed using $2^{-\Delta\Delta C_T}$ method. Data are presented as mean ± S.E. n=7, P<0.05 vs. sample treated with LPS only (0 μM).

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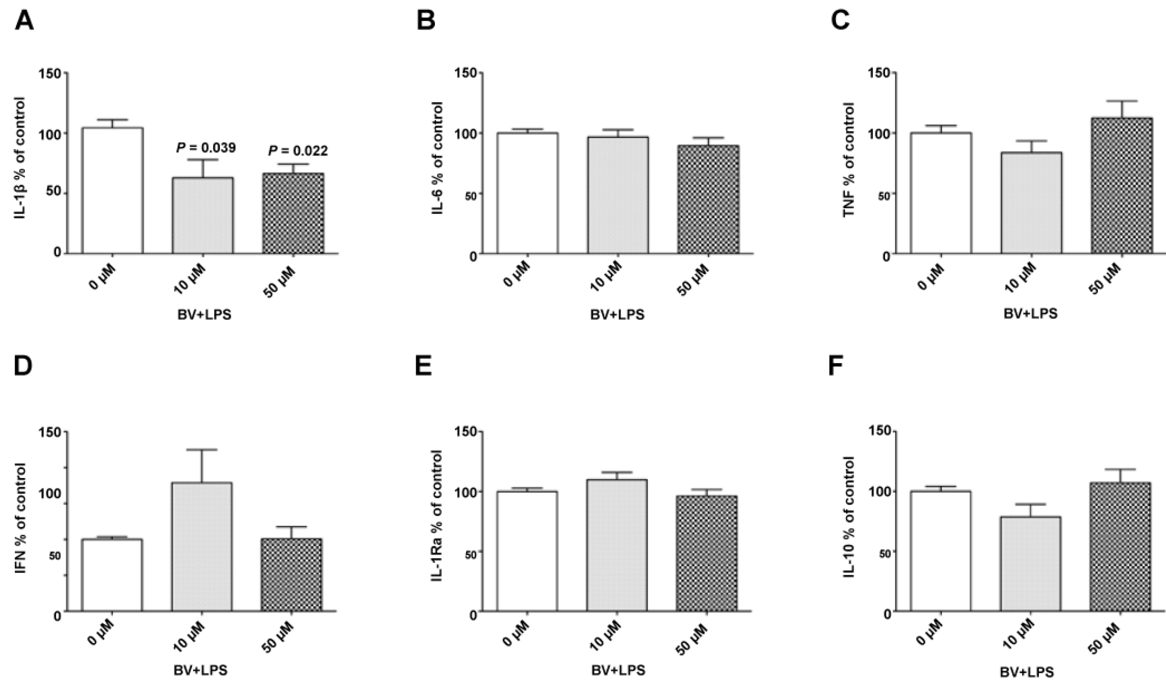

Figure 2. Cytokine concentration in response to LPS and BV

Whole blood was incubated with BV and LPS for 8 h and cytokine concentration was measured using a Milliplex human cytokine kit. The relative change in each cytokine **(A-F)** concentration is presented. Data are presented as mean ± S.E. n=7, $P<0.05$ vs. sample treated with LPS only (0 μM).

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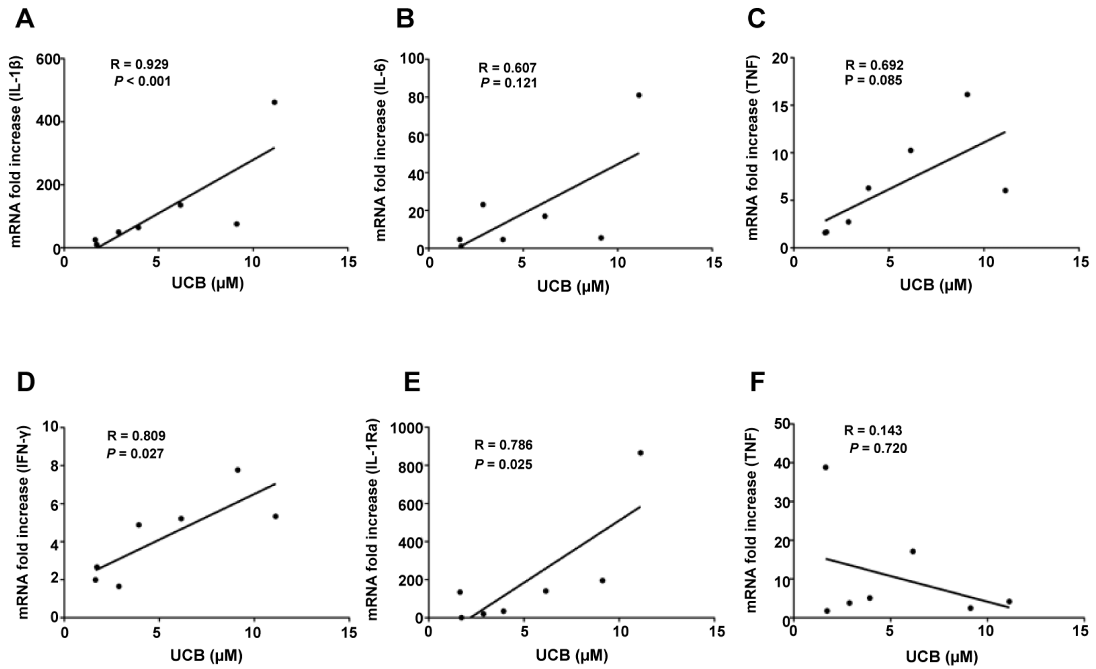

Figure 3. UCB concentration and cytokine gene expression in response to LPS

Whole blood was incubated with BV and LPS for 4 h and mRNA expression was assessed. Figure shows scatter plots and the correlation between baseline UCB concentration and cytokine gene expression **(A-F)**, n=7.

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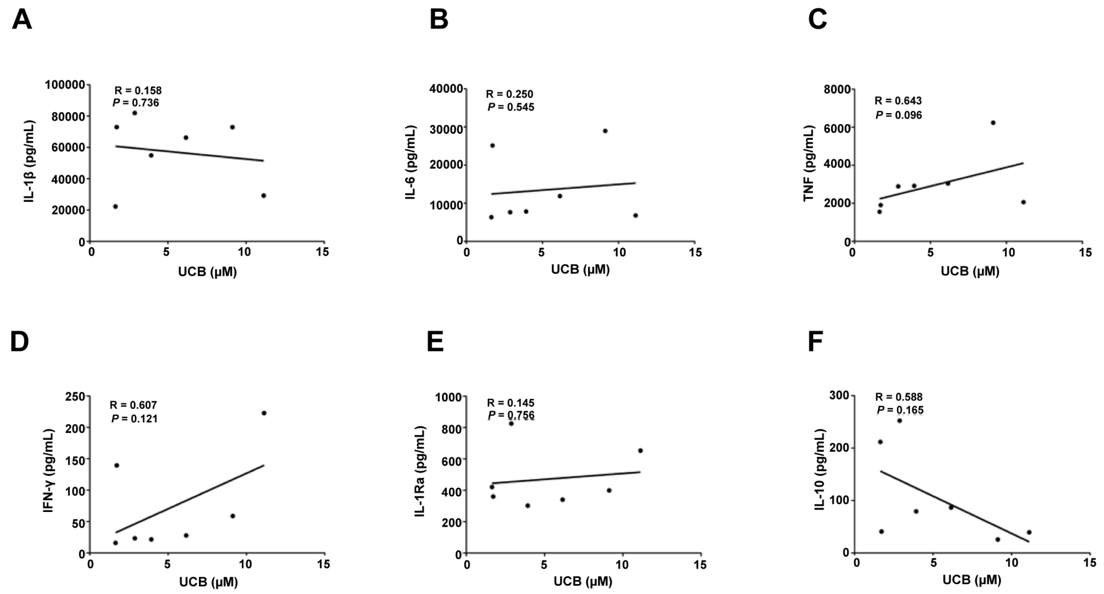

Figure 4. UCB concentration and cytokine concentration in response to LPS

Whole blood was incubated with BV and LPS for 8 h and plasma cytokine concentration was measured using a Milliplex human cytokine kit. Figure shows scatter plots and the correlation between baseline UCB concentration and plasma cytokine concentrations (**A-F**), n=7.

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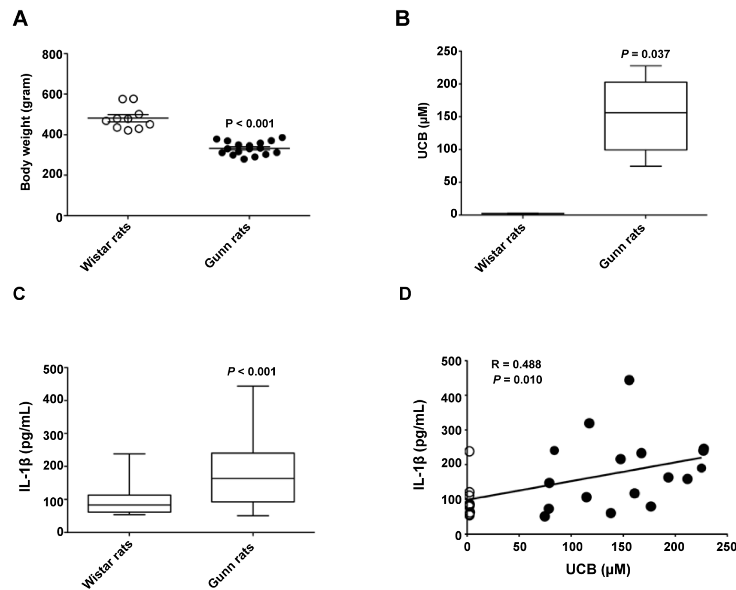

Figure 5. IL-1β concentration in blood samples of wild type control and Gunn rats

(A) Graph showing the body weight of Wistar (n=10) and Gunn rats (n=17). Data are presented as mean ± S.E; P<0.05 vs control (non-jaundiced Wister rats). Box plot showing the serum UCB concentration **(B)** and IL-1β concentration in Wistar and Gunn rats. **(C)** Data are presented as median (25-75% interquartile range); n=10 for Wister and n =17 for Gunn rats and *P*<0.05 vs. control (non-jaundiced Wister rats). **(D)** Scatter plot and the correlation between baseline UCB concentration and IL-1β concentration; n=10 for Wister and n =17 for Gunn rats.

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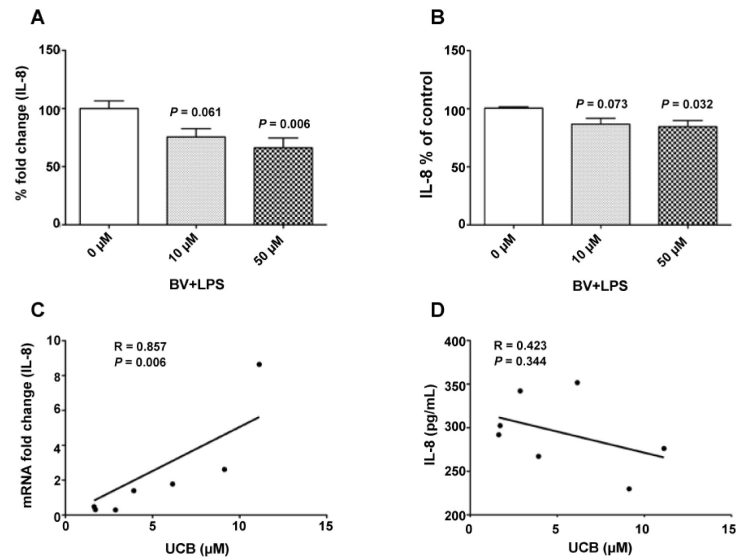

Figure 6. IL-8 concentration in response to LPS and BV

IL-8 gene and protein concentration was analysed using qPCR and high sensitivity ELISA kit, respectively in blood samples incubated with BV and LPS for 8 h. IL-8 gene expression **(A)** and plasma release **(B)** in response to BV + LPS. Data are presented as mean ± S.E. n=7 and *P<0.05* vs. sample treated with LPS only (0 μM). Scatter plot showing the correlation between baseline UCB concentration and IL-8 gene **(C)** and protein expression **(D)** in response to LPS, n=7.

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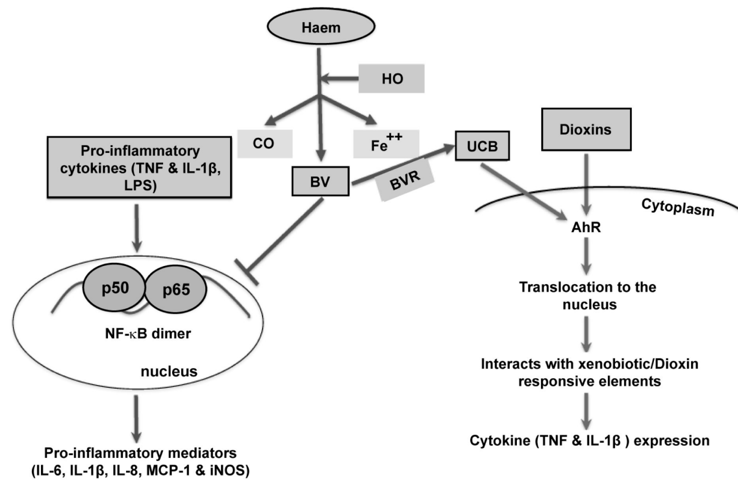

Figure 7. Possible mechanism of BV and UCB-triggered immune-modulatory effects

Haem is catabolised into BV, iron (Fe^{++}) and carbon monoxide (CO) via the action of haem oxygenase (HO). Biliverdin is rapidly reduced to UCB in the presence of BVR. Pro-inflammatory mediators and endotoxin activate NF-κB p60/p65 dimer and promote its translocation to the nucleus, where it induces the transcription and translation of pro-inflammatory genes. Biliverdin inhibits the expression of pro-inflammatory mediators via inhibition of NF-κB activation. However UCB, similar to dioxins, may promote translocation of AhR from the cytoplasm and binding to xenobiotics/dioxin responsive elements, which results in activation of AhR. Activated form of AhR then leads to increase expression of cytokines (TNF and IL-1β).

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Table 1

Primer sequences and amplicon sizes of housekeeping (HPRT) and target genes (IL-1β, IL-6, TNF, IFN-γ, IL-1Ra IL-10, IL-8 and C5aR) expressed in humans.

Gene target	Forward sequence	Reverse sequence	Amplicon (bp)
HPRT	TGGAGTCCTATTGACATCGCCAGT	AGTGCCTCTTTGCTGCTTTCACAC	197
IL-1β	AACAGGCTGCTCTGGGATTCTCTT	ATTTCACTGGCGAGCTCAGGTACT	92
IL-6	AAATTCGGTACATCCTCGACGGCA	AGTGCCTCTTTGCTGCTTTCACAC	88
TNF	TGGGCAGGTCTACTTTGGGATCAT	TTTGAGCCAGAAGAGGTTGAGGGT	128
IFN-γ	ACTAGGCAGCCAACCTAAGCAAGA	CATCAGGGTCACCTGACACATTCA	184
IL-1Ra	AATCCATGGAGGGAAGATGTGCCT	TGTCCTGCTTTCTGTTCTCGCTCA	110
IL-10	TCCTTGCTGGAGGACTTTAAGGGT	TGTCTGGGTCTTGGTTCTCAGCTT	109
IL-8	CTTGGCAGCCTTCCTGATTT	GGGTGGAAAGGTTTGGAGTATG	111
C5aR	AGACATCCTGGCCTTGGTCATCTT	TACCGCCAAGTTGAGGAACCAGAT	133

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Table 2
Clinical characteristics of recruited subjects at baseline (n=7).

Variable	Result
Age (years)	37.1 ± 8.5
BMI(kg/m^2)	24.7 ± 3.42
HGB (g/L)	148 ± 6.51
RBC (10^{12}/L)	5.14 ± 0.18
WBC (10^9/L)	6.01 ± 1.27
NE (10^9/L)	2.51 ± 0.78
LYM (10^9/L)	2.28 ± 0.51
MO (10^9/L)	0.86 ± 0.28
EO (10^9/L)	0.30 ± 0.11
BA (10^9/L)	0.05 ± 0.03

BMI: Body Mass Index; WBC: White Blood Cell; RBC: Red Blood Cell; HGB: Total Haemoglobin; NE: Neutrophil; LYM: Lymphocyte; MO: Monocytes; EO: Eosinophil; BA: Basophil

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Table 3

Unconjugated bilirubin (UCB) and haem concentrations in subjects after 0 (baseline), 4 and 8 h incubation with BV ± LPS (N=7/group).

Haem (baseline; μM)	Treatment	Haem (μM)	
		4 h	**8 h**
4.80 ± 0.63	Control	14.65 ± 0.90*	30.18 ± 3.89*#
	BV (10 μM)	16.88 ± 1.59*	27.01 ± 1.94*#
	BV (50 μM)	15.41 ± 1.38*	32.24 ± 3.43 *#
	LPS	16.69 ± 1.82*	36.25 ± 2.93*#
	LPS+BV (10 μM)	17.30 ± 2.77*	28.00 ± 3.34*
	LPS+BV (50 μM)	16.14 ± 1.62*	33.86 ± 4.84*#
UCB (baseline; μM)	**Treatment**	**UCB (μM)**	
		4 h	8 h
5.23 ± 1.41	Control	11.32 ± 2.03	9.69± 2.32
	BV (10 μM)	11.68 ± 1.98	12.85 ± 2.70*
	BV (50 μM)	14.40 ± 2.93*	15.56 ± 3.02*
	LPS	10.14 ± 3.36	9.50 ± 1.73
	LPS+BV (10 μM)	12.42 ± 4.11*	13.31 ± 4.30*
	LPS+BV (50 μM)	12.56 ± 2.35*	13.72 ± 2.90*

Note: The effect of BV and haemolysis on haem and UCB concentration was performed by repeated measures ANOVA.

* $P<0.05$ vs. baseline UCB or haem concentrations and

\# $P<0.05$ vs. haem concentrations at 4 hours.