Research Article

Biphasic Modulation of NOS Expression, Protein and Nitrite Products by Hydroxocobalamin Underlies Its Protective Effect in Endotoxemic Shock: Downstream Regulation of COX-2, IL-1β, TNF-α, IL-6, and HMGB1 Expression

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Background. NOS/NO inhibitors are potential therapeutics for sepsis, yet they increase clinical mortality. However, there has been no in vivo investigation of the (in vitro) NO scavenger, cobalamin’s (Cbl) endogenous effects on NOS/NO/inflammatory mediators during the immune response to sepsis. Methods. We used quantitative polymerase chain reaction (qPCR), ELISA, Western blot, and NOS Griess assays, in a C57BL/6 mouse, acute endotoxaemia model. Results. During the immune response, pro-inflammatory phase, parenteral hydroxocobalamin (HOCbl) treatment partially inhibits hepatic, but not lung, iNOS mRNA and promotes lung eNOS mRNA, but attenuates the LPS hepatic rise in eNOS mRNA, whilst paradoxically promoting high iNOS/eNOS protein translation, but relatively moderate NO production. HOCbl/NOS/NO regulation is reciprocally associated with lower 4h expression of TNF-α, IL-1β, COX-2, and lower circulating TNF-α, but not IL-6. In resolution, 24h after LPS, HOCbl completely abrogates a major late mediator of sepsis mortality, high mobility group box 1 (HMGB1) mRNA, inhibits iNOS mRNA, and attenuates LPS-induced hepatic inhibition of eNOS mRNA, whilst showing increased, but still moderate, NOS activity, relative to LPS only. experiments (LPS+D-Galactosamine) HOCbl afforded significant, dose-dependent protection in mice Conclusions. HOCbl produces a complex, time- and organ-dependent, selective regulation of NOS/NO during endotoxaemia, corollary regulation of downstream inflammatory mediators, and increased survival. This merits clinical evaluation.

1. Introduction

Cobalamin, C_{63}H_{88}O_{14}N_{14}PCO (Figure 1), participates in only two known mammalian enzymatic reactions. Yet, these two Cbl-dependent enzymes, cytosolic methionine synthase (MS) [EC 2.1.1.13], requiring methylcobalamin (MeCbl), and mitochondrial methylmalonyl-CoA mutase (MU) [EC 5.4.99.2], requiring adenosylcobalamin (AdoCbl) [1, 2], are critically involved in key metabolic pathways essential for gene expression and regulation, via formation of S-adenosylmethionine (SAM) and methylation, and in protein synthesis and catabolism, cellular respiration, and energy. Activation of methionine synthase also ensures key antioxidant defense status, as it triggers concurrent activation of cystathionine β-synthase (CβS), the pivotal enzyme at the homocysteine junction in the trans-sulfuration pathway to glutathione (GSH) [3].
Cobalamin is the standard treatment for autoimmune “pernicious” anaemia, and macrocytic or megaloblastic anaemia, as well as for subacute combined degeneration of the spinal cord. However, an increasing body of work suggests that Cbl may also play a central role in the regulation of immunity and inflammation (reviewed in [4]). Cbl confers significant protection in various animal models of shock, from anaphylaxis to trauma and sepsis [5–7], and has remarkable organ/tissue protective effects when used clinically for the treatment of analogous inflammation in CN poisoning (reviewed in [8]). Amongst Cbl’s known immunological effects are an augmentation of the CD8+/CD4+ T-cell subset and a significantly reduced expression in peripheral blood monocytes [12], whilst Cbl deficiency raises circulating IL-6 in humans [13] and Cbl physiological status regulates IL-6 levels in rat cerebrospinal fluid [14]. Moreover, in both rodents and humans there appears to be an inverse relation between Cbl physiological levels and tumour necrosis factor alpha (TNF-α) serum levels [15]. In vitro, neuronal Cbl deficiency is also associated with increased expression of two TNF-α-converting enzyme secretases [16]. A reasonable hypothesis is that such Cbl/TNF-α/IL-6 regulation may be partly effected via Cbl indirect regulation of the central immune regulatory transcription factor, nuclear factor kappa B (NF-κB) [8]. Normal physiological levels of Cbl in spinal fluid appear to correlate with NF-κB quiescence, at least, in a non-inflammatory/non-immune challenge model [17]. Recently, a kinetic study reported that Cob(II)alam reacts with superoxide at rates approaching superoxide dismutase [18]. CNCbl protects human aortic endothelial cells, and neuronal cells, in vitro, against superoxide induced injury [19, 20]. Given that oxidative stress is a major trigger of NF-κB activation, this potential antioxidant effect of Cbl could theoretically lead to NF-κB inhibition. It may also be of critical local importance in vivo, as the phagocytic burst includes release of the Cbl carrier, haptocorrin (HC/TC 3), in the immediate vicinity of NADPH oxidase [8, 21] one of the major biochemical sources of superoxide in immune challenge and inflammation [22]. HC/TC3, moreover, is upregulated by IL-1β, itself expressed within fifteen minutes of inflammatory challenge [23]. Nevertheless, though antioxidant effects of Cbls have been observed in vitro [24] and may, indeed, be important in vivo [20], no systematic analysis of the in vivo mechanisms of Cbl conferred protection against inflammation during acute immune challenge has hitherto been done.

We wondered if a more comprehensive explanation for Cbl effects on inflammation and immunity, and thence beneficial outcomes in sepsis and other forms of shock, may lie in a potential direct/indirect regulation by Cbl of one or more of the several actions of nitric oxide (‘NO) as a ubiquitous, cell-signal transduction molecule and second messenger for post-translational modification, whose targets include soluble guanylate cyclase [25]. ‘NO is the product of three nitric oxide synthases (NOS): two constitutive, nNOS (neuronal NOS; NOS I) and eNOS (endothelial NOS; NOS III), and one inducible, iNOS (NOS II), at much higher levels of expression, with the potential to produce 1000-fold higher than normal amounts of ‘NO, during gestation, growth, and the immune response [26]. Cobalamins are known to have effects on ‘NO [27–29], but these have hitherto been thought to be a consequence of Cbl/‘NO scavenging effects [7, 30–35] demonstrable chemically and in vitro [36, 37], but biologically unproven in vivo and still controversial [38–40].

Nitrosylcobalamin has not been detected, to date, in vivo or in vitro, amongst naturally occurring intracellular Cbls [41]. Nevertheless, if the hypothesis that Cbl is involved in NOS catalysis has any substance [42, 43], then it is conceivable that, in analogy to previously observed ferric-heme-NO complex formation at the conclusion of NOS catalysis [44], NOCbl might be transiently formed, just prior to release of free ‘NO by the NOS [43]. Such a theoretical transience and discrete localisation might account for the failure to detect NOCbl in vivo to date. Ubiquitous and continuous Cbl scavenging of ‘NO, on the other hand, may pose biochemical hazards. For ‘NO has important antioxidant and cell-signalling actions [25] which might be obstructed by HOCbl’s previously proposed, indiscriminate ‘NO scavenging, or even just by a recently proposed, Cbl structural-based, direct inhibition of the NOS tout court and nothing else [45]. There is some evidence that HOCbl can discriminate between exogenous ‘NO donors and the natural endogenous donor, S-nitrosothiol, GSNO, actually prolonging only GSNO-induced, gastric fundus relaxations [46]. This hints at a more complex Cbl/‘NO regulatory relationship.
Moreover, there are also diverse indications that positive Cbl status is allied to beneficial \( ^\ast \)NO activity: in diabetic rats, high cobalamin levels correlate with high NOS protein levels, \( ^\ast \)NO activity, and increased erectile function [47]; Cbl supplementation of vegetarians with low Cbl status significantly increases eNOS \( ^\ast \)NO release in the brachial artery [48]; in the digestive tract of endotoxemic rats, the highest expression of iNOS in the ileum, precisely where Cbl is internalized [49], and both Cbl and \( ^\ast \)NO are known to mediate cell protective effects via ERK1/2 and Akt [50–54]. These protective effects of \( ^\ast \)NO and Cbl include induction and regulation of heme oxygenase-1 (HO-1) [52, 55–58], which converts biliverdin to the powerful antioxidant bilirubin, and carbon monoxide. CO can then in turn decrease \( ^\ast \)NO [59].

(For a more comprehensive list of coincidences of Cbls/\( ^\ast \)NO's positive actions, see Table 1 and its related discussion in [43]).

Thus, in these studies we explored an alternative hypothesis to that of Cbl as just an \( ^\ast \)NO, or, indeed, superoxide, mop. We posited that the principal mechanism behind Cbl's beneficial, pleiotropic effects in inflammation may involve a biphasic regulation of NOS expression and protein translation and the ensuing \( ^\ast \)NO synthesis, during the two distinct pro- and anti-inflammatory phases of the immune response.

2. Materials and Methods

2.1. Animals. Male C57BL/6 mice, weighing 20 to 25 g, were purchased from Harlan, UK, and maintained on a standard chow pellet diet, containing standard amounts of Cbl (50 \( \mu \)g/kg vitamin B12/CNCbl), with tap water supplied ad libitum. Animals were kept in a 12:00 h light/dark cycle, and all were housed for 7 days prior to experimentation. All experiments were performed in accordance with UK Home Office regulations (Guidance On the Operation of Animals: Scientific Procedures Act, 1986).

2.2. Cobalamins. The coenzymes, \( 5^{'}, \)deoxyadenosycobalamin (vitamin B12), and methylcobalamin; Vitamin B12a, cyanocobalamin, and hydroxocobalamin (CAS 78091-12-0) were purchased from Sigma-Aldrich (UK). Glutathionylobalamin and N-acetyl-cysteinyl-cobalamin were synthesized and supplied by Professor Nicola Brasch (Kent State University, Ohio, USA). All Cbls (and Cbl-treated animal samples) were protected from light during storage and handing, and were 98% to 99.5% pure.

2.3. Drug Treatment and Experimental Design. \( 5^{'}, \)deoxyadenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), hydroxocobalamin (HOClb), glutathionylobalamin (GSCbl), and N-acetyl-cysteinyl-cobalamin (NAC-Cbl) were all stored at \(-20^\circ \)C, and fresh solutions of them were made using sterile, pyrogen-free, phosphate-buffered saline (PBS; Gibco), prior to the experiments. For the in \( \text{vivo} \), experiments, cobalamins were diluted at 10 mL/kg prior to treatments (with PBS used as vehicle). Cobalamins were administered according to the protocol summarized in Table 1.

2.4. Effects of Endogenous Cobalamins on NF-\( \kappa \)B Promoter Activity. RAW 264.7 macrophage cells, stably transfected with NF-\( \kappa \)B luciferase reporter construct (Stratagene), were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) fetal bovine serum, 2 mM l-glutamine, 1 \( \mu \)g/mL Geneticin, and 50 \( \mu \)g/mL G418. Cells (2 \( \times \)10\(^5\) cells) were seeded in 96-well plates and then preincubated for 1 h with increasing concentrations (1–10–100 \( \mu \)M) of the five principally occurring, intracellular Cbls. Thereafter, at time 0 h, cells were stimulated with E. coli LPS (0111:B4; 1 \( \mu \)g) for 4 h and then processed for measurement of luciferase activity in a luminometer (Luminometer TD-20/20; Turner Designs Instruments).

2.5. Non-Lethal and Lethal Endotoxemia. Endotoxemia was induced by the intraperitoneal injection of LPS (0.1 mg/kg), alone (non-lethal) or, in the lethal endotoxemia protocol, in combination with 1 g/kg D-Galactosamine (Table 1). Sample collection in non-lethal endotoxemia was carried out at both 4 and 24 h after LPS challenge. Animal survival, in all lethal endotoxemia experiments, was monitored for a total of 5 days, and all data were analysed using Chi-squared or Kaplan-Meier tests.

Times shown are in relation to time 0 h, when either LPS alone or LPS+D-Gal was administered by intraperitoneal injection. Individual cobalamins were injected into the peritoneum at the doses and times reported in Table 1.

2.6. Sample Preparation for Real-Time Reverse Transcriptase-PCR. Blood (500 \( \mu \)L) was centrifuged (for 5 min, at 2500 rpm), and the plasma then collected for ELISA analysis. 500 \( \mu \)L of TRIZOL reagent (Invitrogen) was added to the remaining fraction. RNA purification was performed as recommended by the manufacturer. Following extraction, RNA (20 \( \mu \)L) was treated with 2U (1 \( \mu \)L) of TURBO DNase (Ambion, Austin, TX), as described by the manufacturer, to remove any contaminating genomic DNA. An aliquot of the DNA-free RNA (7.6 \( \mu \)L) was then transferred to a new RNase-free tube and reverse-transcribed into complementary DNA (cDNA), using Superscript III Reverse Transcriptase (Invitrogen), as described by manufacturer. The following reagents were used: Oligo dT primers (Invitrogen); 1 \( \mu \)L, 10 mM dNTP (Bioline); 4 \( \mu \)L of 5X first-strand buffer; 1 \( \mu \)L, 0.1 M DTT; 1 \( \mu \)L (40 U) RNaSeOUT; and 1 \( \mu \)L (200 U) of Superscript III Reverse Transcriptase (Invitrogen). After synthesis, cDNA was quantified using a Nanodrop ND-1000 and diluted (80 ng/\( \mu \)L) in molecular biology grade water and then loaded into 384-well plates for real-time PCR.

2.7. Real-Time Reverse Transcriptase PCR. Real-time PCR assays were performed on the various samples in order to evaluate the expression of the following genes: GAPDH, RPL32, IL-1\( \beta \), COX-2, iNOS, eNOS, TNF-\( \alpha \), and HMGB1 (Table 2). For each gene analyzed, reactions were performed using 1 \( \mu \)L of the Qiagen QuantiTect Primer Assay, added to 5 \( \mu \)L Power SyBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and then diluted with 2 \( \mu \)L molecular grade water. A final volume of 8 \( \mu \)L was dispensed into each
PCR reactions were performed using ABI Prism 7900 real-time PCR equipment. The thermal profile consisted of 95°C for 15 min, then 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. This was plotted as a melting curve. The comparison between samples was performed using GAPDH and RPL32 as internal standards. REST MCS software was utilized for the calculation of the relative difference between the test groups.

2.8. iNOS and eNOS Western Blotting. Liver tissues were harvested from (n = 5) animals, after LPS endotoxaemia, with or without HOCbl treatment and then homogenized in lysis buffer, which contained a cocktail of protease inhibitors. Protein concentrations prior to loading were determined using the Bradford assay (Sigma): samples were mixed with 6x Laemmlil sample buffer, and equal protein amounts (100 μg) then underwent electrophoresis on a 10% polyacrylamide gel in running buffer (0.3% Tris base, 1.44% glycerine, and 0.1% SDS in distilled water). This was followed by transfer of the proteins onto PVDF membranes in transfer buffer (using 0.3% Tris base, 1.44% glycerine, and 20% methanol, in distilled water). Membranes were blocked for 1 h with 5% nonfat milk solution in TBS containing 0.1% Tween 20. iNOS expression was assessed using a specific monoclonal antibody (1:1000; Santa Cruz, USA). The signal was amplified with HRP-linked anti-mouse secondary antibody (1:2000) and visualized by ECL (Western blotting detection reagent; Amersham Biosciences, USA). Densitometric analysis was performed using NIH ImageJ software and normalised to tubulin loading controls in the same sample.

2.9. NOS Activity: Nitrate/Nitrite Production Assays. Animals (n = 5) were challenged with LPS and treated with Cbls as described above. At 4 h and 24 h after LPS challenge, lung and liver tissue samples were harvested, homogenized, and processed for determination of NOS activity, as measured by nitrate/nitrite end-products of NO. The ultrasensitive NOS assay used (Oxford Biomedical Research, Oxford, MI, USA: ultrasensitive colorimetric NOS assay; cat no. NB78) employs an NADPH recycling system—NADP⁺, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and the substrate, L-arginine, but not the cofactor, BH₄— to ensure that NOS operate linearly for up to 6 hours, as NO-derived nitrate and nitrite accumulate. The assay kit can accurately measure as little as 1 pmol/millil. (~1 nM) NO produced in aqueous solution. In these studies, the assay was run for 5 h at 37°C. The enzyme nitrate reductase was used to convert all nitrate to nitrite, then Griess reagent employed to quantify nitrite levels, with the generation of a nitrite standard, as recommended by the supplier. The completed reaction was read at 540 nm in a Microtiter plate reader. Data are expressed as mmol nitrite/μg protein.

2.10. Determination of Tumour Necrosis Factor Alpha (TNF-α) and Interleukin (IL-6) Levels. After collection, blood was centrifuged and the plasma separated, under low lighting conditions, then stored at −80°C until performance of the analyses. For determination of circulating TNF-α and IL-6 levels, using ELISA assays, samples were diluted 1:10 in the assay diluent, as specified by the manufacturer (R&D, UK). Absorbance was plotted in a standard curve, and data expressed as the content of TNF-α (ng) or IL-6 (pg) per mL of plasma.

2.11. Reagents. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, Poole, UK.

2.12. Statistics. Data are shown as a mean ± S.E. of 5 animals per group for the analyses in non-lethal endotoxaemia and, initially, 7–9 per group, then 12 per group, for lethal endotoxaemia survival, series I and II, respectively. Statistical differences were determined by ANOVA, following the Student Newman Keuls test. Chi-square and Kaplan-Meier tests were used for the lethality studies. In all cases, a P < 0.05 was taken as significant.

Table 1: Protocol for cobalamin treatment of LPS-induced endotoxaemia.

| Experimental protocols | Inflammatory stimulus | Cbl dose | Treatment | Time of Cbl treatment |
|------------------------|-----------------------|----------|-----------|----------------------|
| 4 h non-lethal         | LPS (0.1 mg/kg)       | 0.2 mg/kg| ~1h, +1 h, +2 h |
| 24 h non-lethal        | LPS (0.1 mg/kg)       | 0.2 mg/kg| ~1, +1, +2, +6, +22 h |
| Lethal series I        | LPS (0.1 mg/kg) + D-Gal (1 g/kg) | 40 mg/kg | ~1, +1, +2, +6, +22 h |
| Lethal series II       | LPS (0.1 mg/kg) + D-Gal (1 g/kg) | 80 mg/kg HOCbl | +2, +4 h |

Table 2

| Assay code          | Gene and accession number of detected transcripts |
|---------------------|--------------------------------------------------|
| QT00100275          | NOS2; NM_0090927                                 |
| QT00152754          | NOS3; NM_008713                                  |
| QT00247786          | HMGB1; NM_010439                                 |
| QT00104006          | TNF-α; NM_013693                                 |
| QT01048555          | IL-1β; NM_008361                                 |
| QT01658692          | GADPH; NM_008084                                 |
| QT01752387          | RPL32; NM_72086                                  |

For the lethality studies, Meier tests were used. In all cases, a P < 0.05 was taken as significant.
3. Results

3.1. Cobalamins Do Not Inhibit LPS-Induced NF-κB Activation In Vitro. Since Cbl has been shown to prevent NF-κB activation in a non-immune challenge model [17], and activation of NF-κB leads to iNOS induction, we first looked at the effects of the five principally occurring, intracellular Cbls, (CNCbl, HOCbl, GSCbl, and the two mammalian enzyme cofactors for MU and MS, respectively, AdoCbl and MeCbl), on LPS-induced NF-κB activation in vitro, using a canonical reporter assay. Although the various incoming forms of Cbl are all reduced or dealkylated soon after cell entry, prior to MS/MU cofactor formation [60], the different incoming forms affect both the rate and ratio of formation of the two known active cofactors, AdoCbl/MeCbl [60–62]. Theoretically, this variability in Cbl cofactor formation may impact on the effects of Cbls in immune challenge with respect to NF-κB activation. Thus, it was important to make this comparison. RAW 264.7 macrophage cells, stably transfected with NF-κB luciferase reporter construct, were preincubated for 1h with increasing concentrations (1–10–100 μM) of Cbls. Upon LPS stimulation, none of the five Cbls significantly affected or inhibited LPS-driven NF-κB activation at 1h, with no significant inhibitory effect on NF-κB at a later time point (24h). CNCbl alone slightly stimulated NF-κB activity, but only at the 1h time point and when tested at the concentration of 1μM (Table 3).

In two separate experiments, each performed in triplicate, RAW246.7 cells, stably transfected with NF-κB luciferase reporter construct, were seeded in 96-well plates and preincubated for 1h with increasing concentrations (1–10–100 μM) of individual Cbls, followed by stimulation with E. coli LPS (1 μg). At 1h, and 24 h, following LPS in the respective experiments, cells were processed for measurement of luciferase activity. Basal values of fluorescence were 2.60 ± 0.38 and 3.77 ± 0.31 for 1h and 24h incubation, respectively. Data are expressed as a mean ± SEM of triplicate observations. *P < 0.05 versus LPS alone.

3.2. Endogenous Cobalamins Enhance Survival in Acute Endotoxaemia. As there was no observable difference between the effects of alkyl and non-alkyl Cbls on NF-κB in vitro, we chose to focus these first investigations in vivo principally on HOCbl, as a clinically licensed Cbl form, known to be partially converted on cell entry to the two known active cofactors, MeCbl and AdoCbl, for MS and MCM, respectively [63]. Furthermore, at supraphysiological doses of 5 g i.v., HOCbl, as a clinical cyanide antidote, has shown remarkable protection against corollary inflammation (analogous to the inflammation seen in SIRS, sepsis, and septic shock), that goes beyond merely acting as a magnet for CN [8].

We therefore next decided to see if the lethality survival protection also conferred by HOCbl in a sepsis/endotoxaemia mouse model [7] was reproducible in a different strain and in a more acute endotoxaemia model. Some groups of animals were alternatively treated with the relatively novel, intracellular Cbl, glutathionylcobalamin (GSCbl) [61, 64] whose clinical effects are untested in sepsis, or with N-acetyl-cysteinyl-cobalamin (NAC-Cbl), a synthetic cobalamin, used as a non-endogenously occurring, thiol Cbl comparison. To gain some information on potential clinical dosage, all Cbls were tested in two distinct, high dosing regimes, with or without prophylactic pretreatment.

In a severe sepsis protocol (LPS+D-Gal), using C57BL/6 mice, we administered a relatively low dose of Cbls (0.2 mg/kg i.p.), equivalent to a maximal concentration of approximately 1μM (considering a total blood volume of 2.5 mL in the mouse, this concentration being well within the range tested in vitro). Individual Cbls were administered i.p. –1h prior to LPS+D-Gal and then given in repeated doses at +1, +2, +6, and +22 h after LPS+D-Gal. Alternatively, a high dose Cbl protocol (40 mg/kg i.p.) was administered only twice, at +2 and +22 h after LPS+D-Gal, to assess its potential as a rescue regimen. The urine of all Cbl-treated animals was red, within 1h of administration, an indicator of rapid, high, systemic Cbl saturation (data not shown).

LPS+D-Gal mice rapidly reached 88.9% mortality by 8h. This did not change further up to 24 h. Animals treated with the relatively low-dose regimen of GSCbl or NAC-Cbl, were protected in the early, 4–8 h time frame (Figure 2(a)). During this period all Cbl-treated animals also exhibited less huddling and pilo-erection (data not shown).

At 8 h after LPS+D-Gal, all relatively low-dose Cbl treatments afforded 25% survival, *P < 0.05 versus LPS+D-Gal alone (Figure 2(a)). However, only low-dose HO-Cbl treatment maintained this level of protection up to 24h.

| Treatments | NF-κB reporter assay (fold increase over basal) |
|------------|-----------------------------------------------|
|            | 1h                                           |
|            | 24h                                          |
| LPS        | 5.63 ± 0.37                                  |
| CNCbl (μM) |                                               |
| 1          | *7.10 ± 0.30                                 |
| 10         | 5.53 ± 0.29                                 |
| 100        | 5.23 ± 0.62                                 |
| GSCbl (μM) |                                               |
| 1          | 6.33 ± 0.37                                 |
| 10         | 6.25 ± 0.32                                 |
| 100        | 5.98 ± 0.26                                 |
| MeCbl (μM) |                                               |
| 1          | 6.10 ± 0.10                                 |
| 10         | 6.10 ± 0.15                                 |
| 100        | 6.00 ± 0.58                                 |
| HOCbl (μM) |                                               |
| 1          | 5.67 ± 0.20                                 |
| 10         | 5.98 ± 0.26                                 |
| 100        | 7.00 ± 1.10                                 |
| AdoCbl (μM)|                                               |
| 1          | 6.23 ± 0.39                                 |
| 10         | 6.26 ± 0.90                                 |
| 100        | 6.20 ± 0.61                                 |
a consistent 28.60% survival up to 24 h, the lesser protection of the former in the first hours, offered at 24 h. In contrast, high-dose GSCbl and HOCbl, despite in the first 6 h, was not significantly different from controls NAC-Cbl, which again provided some degree of protection regimen was less protective within the first 8 h. High-dose 𝑛=9 and for GSCbl/HOCbl, when tested against LPS+D-Gal (HOCbl when tested against LPS+D = Gal (significant survival advantage of HOCbl treatment results 80 mg/kg dose, a single bolus administration at +2 h. The +2 h and +4 h only for the 40 mg/kg, and, in the case of the 80 mg/kg dose, two distinct ultra-high doses of HOCbl (40 mg/kg and 80 mg/kg) were tested, with a more concise dose/time frame, +2 h and +4 h only for the 40 mg/kg, and, in the case of the 80 mg/kg dose, a single bolus administration at +2 h. The trend towards improved survival seen at the higher HOCbl dose, two distinct ultra-high doses of HOCbl (40 mg/kg and 80 mg/kg) were tested, with a more concise dose/time frame, +2 h and +4 h only for the 40 mg/kg, and, in the case of the 80 mg/kg dose, a single bolus administration at +2 h. The significant survival advantage of HOCbl treatment results demonstrated over 5 days, in Figures 3(a) and 3(b), not only shows that our HOCbl data is consistently reproducible, but also that increasing the dose of HOCbl significantly increases survival, from 25% up to 33.33%; *𝑃 < 0.01 for HOCbl at 80 mg/kg by using the Kaplan-Meyer test. By comparison, at 24 h in the LPS-only group, there was 90% mortality.

To gain information about the mechanisms behind the consistent protection afforded by HOCbl, and to observe any potential impact on the NOS, a non-lethal protocol was next deployed. Then the expression of inflammatory mediator genes in liver and lung was analysed, in both the pro- and anti-inflammatory phases of the immune response, at the 4 h and 24 h time points.

3.3. HOCbl Selective Promotion/Modulation of eNOS/iNOS mRNA, Inhibition of IL-1β, and Cox-2 Expression: 4 h Time Point. The early effects of HOCbl treatment on eNOS mRNA appeared organ dependent, with significant promotion of eNOS mRNA in the lung and attenuation in the liver (Figures 4(a) and 4(b)). For eNOS, in LPS-only animals we observed a decrease in the lung of ∼2.9 ± 0.1, whereas there was an increase of 2.1 ± 0.1-fold change in LPS+HOCbl-treated animals (Figure 4(a)). Paradoxically, in the liver of LPS-only treated animals, there was an increase of eNOS expression of up to ∼15-fold compared to up to ∼4-fold change only in LPS+HOCbl—treated animals (Figure 4(b)).

Liver and lung iNOS and COX-2 gene expression levels were increased in LPS-only treated animals when compared to that of PBS-only injected mice, whose value was set as 1. However, as for eNOS, the effects of HOCbl on iNOS expression were once more organ selective, failing to inhibit the rise in iNOS mRNA in the lung, but attenuating...
it in the liver (Figures 4(c) and 4(d)). Strikingly, in spite of HOCbl’s failure to completely inhibit iNOS expression, HOCbl was a consistent inhibitor of COX-2 mRNA in both liver and lung, bringing its degree of expression back to and below that of PBS-injected mice (Figures 4(e) and 4(f)). HOCbl treatment also had a consistent regulatory effect on IL-1β expression, which was moderately and significantly decreased in lung and completely inhibited in liver (Figures 4(g) and 4(h)).

3.4. HOCbl Has Early Promotional Effects on Translation of eNOS/iNOS Protein. To determine efficiency of translation of the post-LPS increased NOS mRNA, we assessed eNOS and iNOS protein expression by Western blot in 4h liver samples. As predicted by our hypothesis that sepsis may involve a failure in translation of the NOS, this revealed that whilst in the LPS-only challenged group there was a significant depression of eNOS protein translation, that was at odds with its high mRNA expression, HOCbl significantly promoted eNOS protein translation, above the levels of both PBS control and LPS-only treatment groups (Figure 5(a)). A similar paradoxical pattern was observed in hepatic iNOS protein translation, with significant depression of iNOS protein translation in the LPS-only challenged group, and promotion of iNOS protein in the HOCbl+LPS treated group (Figure 5(b)). We confirmed that these effects of HOCbl on NOS protein promotion were not random or artifactual, but were specific to Cbl, by repeating the LPS non-lethal endotoxaemia experiment using either GSCbl or NAC-Cbl treatment and performing Western blots for eNOS/iNOS protein. Once again, we observed a significant early promotion of eNOS/iNOS protein by these other Cbls, when compared to LPS only (Figures 5(a) and 5(b)).

3.5. HOCbl Moderates High *NO Synthesis at 4h and 24h after LPS. However, when NOS activity was measured (using a nitrite production assay) both in the early post-LPS challenge, pro-inflammatory phase and in the late anti-inflammatory, resolution phase, a further paradoxical result emerged, suggesting that HOCbl may exert some post-translational modification of NOS activity. Levels of nitrite at 4h showed an inverse relation to levels of NOS protein, with significantly higher levels of nitrite in the LPS-only eNOS/iNOS-depressed group and significantly lower levels of nitrite being generated in the HOCbl/eNOS/iNOS-promoted group (Figure 6(a)). (That this was a general, reproducible Cbl effect was confirmed, as stated previously, by also doing the Western blot with 4h GSCbl/NAC-Cbl-treated liver samples and also running the NOS activity assay with both thiol-Cbl treated samples.) Here we again observed a correlation between GSCbl/NAC-Cbl promoted high NOS protein in the Western blots and decreased nitrite in the NOS activity assay (Figure 7).

At 24h following LPS, levels of NOS-derived nitrite, as measured in tissue samples, were even higher than at 4h in both the LPS-only and HOCbl-treated groups. Nevertheless, HOCbl consistently showed relatively less nitrite production than LPS only, in both lung and liver tissue samples (Figures 6(b) and 6(c)).

3.6. HOCbl Regulates TNF-α Expression and Protein but Leaves Circulating IL-6 Protein Levels Unchanged. Since high
Figure 4: HOCbl selectively modulates NOS enzymes and inhibits COX-2, IL-1β mRNA, in lung and liver, at 4h following LPS-induced endotoxaemia. Mice (n = 5) were treated with HOCbl, at the low dose protocol (0.2mg/kg i.p.) and compared to LPS only (0.1mg/kg i.p. at time 0h). Organs (lung and liver) were harvested at 4h after LPS and gene expression was quantified in tissue extracts by real-time PCR, using GAPDH and RPL32 as internal standards. ((a), (b)) eNOS mRNA data; ((c), (d)) iNOS mRNA data; ((e), (f)) COX-2 mRNA data; ((g), (h)) IL-1β mRNA data. Values are a mean ± SEM of triplicate observations. *P < 0.05 versus vehicle (PBS-treated) control; †P < 0.05 versus LPS-only group.

iNOS expression/protein and •NO activity in the sepsis literature are associated with high TNF-α, IL-6, and ensuing toxicity, we evaluated how HOCbl might impact upon systemic levels of TNF-α and IL-6 triggered by LPS. Plasma levels of these cytokines were quantified using ELISA. Levels of IL-6 protein were not significantly lower in the HOCbl-treated group (Figure 8(a)). However, HOCbl treatment significantly (P < 0.05) attenuated the post-LPS-induced increase in circulating plasma TNF-α, as measured at the 4h time point, (~50% reduction: Figure 8(b)). Consistent with its effects on plasma TNF-α, HOCbl also showed some protection from the inhibitory effects of LPS on TNF-α mRNA in the lung and significant attenuation of TNF-α mRNA in liver (Figures 8(c) and 8(d)).
3.7. **HOCbl Late Effects on NOS and Cox-2 mRNA: 24 h Time Point.** In the resolution phase of the immune response to LPS, the effects of HOCbl treatment on NOS expression displayed a degree of organ selectivity, though most notable with respect to iNOS expression. Whilst HOCbl did not change LPS-induced eNOS mRNA inhibition in the lung, it significantly attenuated its inhibition in the liver, from −75- to −58 fold-change, respectively, for LPS only and LPS+HOCbl (Figures 9(a) and 9(b)).

HOCbl effects on iNOS mRNA in the lung were more distinctive, with ~80% inhibition compared to LPS-only (vehicle group). In the liver, HOCbl treatment attenuated the LPS-induced inhibition of iNOS mRNA by ~40% (Figures 9(c) and 9(d)). The consistent HOCbl tissue inhibition of Cox-2 mRNA, seen at the early pro-inflammatory phase time point of 4 h, persisted at 24 h, showing a significantly greater degree of inhibition than LPS-only: 7- versus 2.5-fold for LPS-only in the lung; 115- versus 50-fold for LPS-only in liver (Figures 9(e) and 9(f)).

3.8. **HOCbl Inhibition of HMGB1 mRNA at 24 h following LPS.**

Given the early regulatory impact of HOCbl on NOS/NO activity, and COX-2, IL-1β, and TNF-α, we expected to see related downstream beneficial effects on expression of the late (≥18 h) effector of endotoxaemia, high mobility group box 1 (HMGB1). This prediction was confirmed. Where LPS-only presented an inconsistent picture, HOCbl treatment consistently inhibited HMGB1 mRNA: in the lung, from an increase of 2.5 in LPS-only to a near threefold decrease (setting levels of expression even lower than those observed in the control group, taken as a value of 1); and in the liver, to a more significant degree, even beyond the remarkable inhibitory effect of LPS-only (Figures 9(g) and 9(h)).

To conclude the 24 h gene expression analyses, tissue levels of IL-1β and TNF-α mRNA were also quantified using RT-PCR. In resolution, HOCbl treatment significantly increased inhibition of hepatic IL-1β (in line with its inhibitory effect at 4 h) and inhibition of TNF-α in the lung, whilst also, paradoxically, decreasing LPS inhibition of TNF-α in the liver (Table 4). Of note was the fact that the late effects of HOCbl on TNF-α mirrored the degree of late iNOS expression, in both lung and liver, as, indeed, did LPS-only (Figures 9(c) and 9(d) and Table 4).

4. **Discussion**

Our studies present a picture of complex and far-reaching homeostatic regulation of the activation, expression, and
translation of NOS, *NO synthesis, and inflammatory mediators by HOCbl during the immune response. We propose that this regulation accounts for the noted survivals in rodent endotoxaemia, both in our more acute, septic shock models I and II (a modest but significant 25%/28.60%, and 25%/33.333%, survival) and in a previous sub-acute, sepsis model (performed with CNCbl/HOCbl—*n = 10 animals per group—30%/40% survival [7]). The regulation we show here may also explain the observed organ/tissue-protective effects of HOCbl in the clinical treatment of CN poisoning and the ensuing shock, which appear to go beyond what may be expected from the Cbl binding of CN alone [8]. Furthermore, given the supraphysiological, saturating doses of Cbl used in our studies, if Cbl had been acting just as an *NO scavenger, or even as a NOS inhibitor, it seems unlikely that it would have permitted the increasing rise in NOS activity (as indexed by nitrite), both early and late, or that its effects would be so subtle, complicated, and ultimately beneficial.
LPS group showed no early hours protection. Moreover, in a new observation, it is noteworthy that, at the higher dose, GSCbl, as a consequence of thiol Cbl lability, might be proposed effects.

4.1. Paradoxes of Thiol Cobalamin Effects. The thiol Cbls, on the other hand, present some mysteries, about which one can only give speculative answers at this point. The survival advantage exhibited in the early hours by comparatively low dose GSCbl/NAC-Cbl treated animals, but not with HOClb treatment, may be due to the known higher reduction potential/lability of thiol Cbls [64], resulting in more rapidly available intracellular Cbl. Cbl is likely to be, at least in part, functionally deficient in sepsis, as indexed by the down-regulation of the Cbl receptors, megalin and cubilin, in the kidneys of endotoxaemic mice [65], the kidney being known, as a Cbl homeostatic regulator, to reduce its Cbl uptake in states of Cbl deficiency [66]. Pertinently, megalin is down-regulated via the LPS-induced ERK1/2 signaling pathway [67], through which, as noted earlier, both Cbl and NAC achieve their coincidentally regulatory, beneficial effects.

Although the beneficial supply of some extra GSH, as a consequence of thiol Cbl lability, might be proposed as an alternative explanation for early survival protection, it is noteworthy that, at the higher dose, GSCbl showed no early hours protection. Moreover, in a new series of endotoxaemia lethality, in vivo survival studies that compared protective effects of all endogenous Cbls against those of GSCbl and NAC-Cbl, we observed that though GSCbl/NAC-Cbl still consistently conferred the same early/pre-8-hour protection compared to the 4 other Cbls, paradoxically, both thiol Cbls repeatedly produced poor long-term survival outcomes, equivalent to or worse than LPS-only, whereas HOClb/CNCbl/MeCbl/AdoCbl all consistently showed significant protection, with CNCbl/MeCbl in particular, producing even better survival results than the current HOClb-centred study (Brancaleone, Dalli et al. 2011, unpublished data).

GSCbl is certainly known to produce a more rapid early increase in MS activity (together with fourfold greater formation of AdoCbl), when compared to HOClb [61]. Such an increase in MS activity, normally rapidly deactivated by oxidative stress [68–70], would increase synthesis of the methyl donor, S-adenosylmethionine (SAM), which inhibits LPS-induced gene expression by modulating histone methylation [71]. Whilst this may have initial short-term benefits, as observed, there are also negative long-term consequences from excessive inhibition of the necessary, pro-inflammatory gene expression at this stage. This may be why MS expression and activity are transiently decreased by 25% and 30% early on in the normal pro-inflammatory phase of the immune response to LPS [72], as well as to allow for GSH synthesis modulation [72].

The consequent, equally paradoxical failure of the other thiol Cbl, NAC-Cbl, to significantly increase survival beyond 8 h, at both high- and low-dose protocols, may also be attributable to the fact that NAC is particularly unstable as a Cbl ligand and may therefore have acted independently of Cbl as NAC alone. Further, though NAC can (1) act as an antioxidant by increasing GSH levels (2) it can equally act as a pro-oxidant [73], increasing disulfides, GSSG, [74] and (3) is counter-indicated in sepsis, since, whilst NAC enhances phagocytosis, it also suppresses the bactericidal respiratory burst in ICU patients, with potential negative outcomes [75]. Indeed, consistent with this independent, paradoxical observation, additional data from our in vivo endotoxaemia model shows that in the early 4 h phase of the immune response NAC-Cbl, in contrast to HOClb/GSCbl, significantly increases circulating PMN, specifically granulocytes, yet decreases the intensity of CD11b exression (see Supplementary data available online at http://dx.doi.org/10.1155/2013/741804 (and Researchgate)). The adhesion molecule/complement receptor, CD11b, is a marker of neutrophil activation, and its high expression normally correlates with a strong respiratory burst [76]. It is conceivable then that such NAC-promoted suppressive effects ultimately outweighed the very early benefits in survival protection conferred by NAC-Cbl.

4.2. Cbl, NF-κB, and iNOS. The initial in vitro observation that all major endogenous Cbls do not inhibit NF-κB activation, even at 24 h, may appear surprising, particularly in view of the Cbl beneficial outcomes in vivo. However, it has previously been shown in vivo that early inhibition of NF-κB in immune challenge increases and prolongs inflammation, and that persisting late NF-κB activation (24/48 h) permits its resolution [77]. Moreover, this failure to inhibit NF-κB by Cbl during inflammation, with positive outcomes, has now also been observed by a group who were aware of our findings [57] and, independently, in a Cbl cancer model by Marguierite et al. (2012, Marc Marten personal communication to Wheatley).

Further, since activation of NF-κB is linked to induction of iNOS, and since inadequately low levels of *NO—and, indeed, iNOS gene knockout in mice [78]—and NOS...
inhibitors in clinical trials [79, 80] have been implicated in sepsis morbidity and mortality [81], we adopted the hypothesis that NOS translation may, in contradiction to the common view, actually be depressed in sepsis and NOS catalytic activity “uncoupled” or malfunctioning. Previously observed high °NO in sepsis is believed to comprise a greater ratio of the more toxic °NO species, such as peroxynitrite, ONOO- [82], which inhibits eNOS [83], as opposed to more antioxidant/cytotoxic °NO forms, such as S-nitrosothiols/GSNO [84, 85] or °NO itself; although high levels of GSNO also have negative effects in sepsis-like inflammation [85]. Since Cbl is known to promote GSH [3, 86–88], whose synthesis is induced simultaneously with that of iNOS [89], it should theoretically alter the ratio of GSNO/°NO to ONOO- and related species [43], so that the more positive actions of °NO predominate [42] (Figure 10). Therefore, we predicted that Cbl would not inhibit iNOS expression and translation early on. This proved correct, with significant HOCbl (and GSCbl/NAC-Cbl) early promotion of both iNOS and eNOS proteins and significantly lower LPS-only iNOS/eNOS protein. Since eNOS is known to be depressed in sepsis, with adverse cardiovascular consequences [90], this early effect of Cbl may have positive clinical implications.

There is an apparent contradiction in the data showing relatively low iNOS/eNOS mRNA leading to strikingly high protein translation in the LPS+HOCbl treated animals, in direct contrast to the LPS-only group, where strikingly high iNOS/eNOS mRNA yielded much lower levels of iNOS/eNOS protein. That this is not an artefact, but a specific Cbl effect, also observed by others [47], is seen by the comparable inverse results achieved with the two, thiol Cbls. (These collective Cbl results were also mirrored by decreasing nitrite/NOS activity, in inverse proportion to the ascending levels of HOCbl/GŠCbl/NAC-Cbl iNOS protein.)

We propose that this paradox may actually be an index of Cbl/NOS regulation in endotoxaemia and that the high mRNA levels in LPS-only animals may be due to the observed phenomenon of “relaxed control of RNA synthesis” when
Figure 9: HOCbl modulates eNOS/iNOS, COX-2, HMGB1 gene expression in lung and liver at 24h subsequent to LPS-induced endotoxaemia. Mice (n = 5) were treated with HOCbl (0.2 mg/kg i.p low dose regimen (Table 1)) and compared to LPS 0.1 mg/kg (given i.p. at time 0h). Organs (lung and liver) were harvested at 24 h after LPS and gene expression was quantified in tissue extracts by real-time PCR, using GAPDH and RPL32 as internal standards, and PBS-injected group set as 1. ((a), (b)) eNOS mRNA data; ((c), (d)) iNOS mRNA data; ((e), (f)) COX-2 mRNA data; ((g), (h)) HMGB1 mRNA data. Values are a mean ± SEM of triplicate observations. *P < 0.05 versus vehicle (PBS-treated) control; †P < 0.05 versus LPS group.

SAM/methyl groups are deficient [92], as in folate or Cbl deficiency [93], thus consequential on the functional Cbl deficiency of endotoxaemia/sepsis, and more permanent MS inactivation by LPS, discussed earlier. Furthermore, it is theoretically possible that abnormal cell function in sepsis may result in much of the mRNA produced by the LPS-only group being “masked” and unavailable for efficient translation. It is possible also that the translated protein may be unstable and degrade at a faster rate. This is certainly known to be the case with eNOS mRNA in hypoxia [94] and in the presence of high TNF-α [95], both characteristic of sepsis. In contrast, given Cbl’s impact on
the two coenzymes, MS and MU, upon Cbl treatment, the septic cell should afford a degree of metabolic normality and thus economic efficiency in transcription/translation.

A remarkable study, over half a century ago, demonstrated that Cbl is capable of reactivating a diversity of key enzymes after acute oxidant stress, most of them also negatively affected in sepsis, including glucose-6-phosphate-dehydrogenase, lactate dehydrogenase, lysine, ornithine, and glutamic decarboxylase [96]. This last is critical for the supply of alpha-ketoglutarate in the Krebs cycle, which is depressed in sepsis, with consequent lower ATP production that is associated clinically with increased mortality [97]. In turn,
supply of alpha-ketoglutarate determines the availability of L-arginine, glutamate, and glutamine, all of which also decrease in sepsis, with adverse consequences, in the case of L-arginine especially for the NOS [98]. Observed low levels of L-arginine in sepsis [99] are associated with increased reactive nitrogen species, including high ONOO-, and reactive oxygen species production during NOS catalysis [98], interfering with the normal, more beneficial "NO cell signalling, necessary for the efficient resolution of the pro-inflammatory phase of the immune response.

4.3. HOClb Reciprocal Regulation of iNOS/NO and TNF-α. iNOS-derived "NO is known to have a direct regulatory correlation to levels of TNF-α [100, 101]. This iNOS/"NO/TNF-α regulation seems operative here with HOClb treatment, not LPS-only. Since HOClb permitted a moderate rise in "NO (at least, as measured by NO nitrite end-products), in tandem with moderate levels of TNF-α mRNA and protein, and since Cbl status has an inverse relation to TNF-α levels [15], it seems reasonable to conclude that such Cbl/TNF-α regulation occurs downstream of HOClb/NOS/"NO regulation. Additional evidence for this in our studies may be seen even in the resolution phase of the immune response, where HOClb-related levels of iNOS expression showed a direct correlation to those of TNF-α, even to the degree of inhibition, in both lung and liver tissues (Figures 9(c) and 9(d)/Table 3). Importantly, however, in the early pro-inflammatory phase HOClb iNOS/"NO regulation does not completely inhibit TNF-α: 50% reduction being observed in our experiments. This is a critical point as anti-TNF-α mAb treatment increases sepsis mortality in the clinic, since some degree of TNF-α production and consequent early pro-inflammatory signalling is essential for an effective immune response [102].

4.4. HOClb Regulation of IL-6, IL-1β, and Cox-2. Also noteworthy, and crucially consistent with such HOClb/iNOS/ TNF-α regulation, is HOClb's apparently selective failure to inhibit IL-6 early on. IL-6 is essential for induction of acute phase proteins, whilst simultaneously also decreasing pro-inflammatory cytokines and increasing anti-inflammatory factors [103]. IL-6 regulation of pro-inflammatory factors includes regulation of TNF-α and IL-1β [104], expression of the latter also determining that of Cox-2, involved in arachidonic acid-derived prostaglandin and leukotriene synthesis [105]. IL-1β is rapidly expressed (~15 min post LPS) [106], whereas iNOS, which may be induced by IL-1β, is not fully expressed until 6 h after LPS [107].

We have observed that treatment with high doses of endogenous Cbls (HOClb/GSCbl) promotes iNOS mRNA expression as early as 2 h following LPS (unpublished data), possibly fast forwarding the immune response. This, together with the HOClb-promoted high NOS protein, controlled rise in "NO synthesis, and thence a moderate production of TNF-α/IL-6 may form a feedback loop accounting for the tight HOClb regulation of IL-1β, and consequently also Cox-2, as seen at 4 h after LPS (Figure 10 scheme).

4.5. HOClb Inhibition of Late HMGB1 Gene Expression. Cobalamin-promoted iNOS/"NO early regulation of TNF-α/IL-6/IL-1β/Cox-2 seems also to be consistent with, and accounts for, the later inhibition of HMGB1 mRNA. If expressed at >18 h and then released extracellularly, HMGB1 can trigger further late release of TNF-α, IL-1β, and inflammatory products from COX-2, iNOS, and excessive ROS and RNI species, leading to pathology [108]. It is known that the nervous system can modulate circulating TNF-α levels via release of acetylcholine by the vagus nerve [109]. But our studies show that Cbl—essential for acetylcholine synthesis [110]—is the first known endogenous inhibitor of late HMGB1 mRNA expression. Both nicotine and ethyl-pyruvate have been used to block extracellular release of HMGB1 [109] but, in addition to the fact that HOClb appears to impact on HMGB1 much further upstream, at least in tissues, neither drug is endowed with the safety profile of Cbl [III] and, more pertinently, neither is known to exert such a central, endogenous regulation of the immune response. Further Cbl/sepsis studies should include measurement of plasma HMGB1 levels. But, on the evidence of the general anti-inflammatory regulation observed in our studies, we predict that extracellular release of HMGB1, from macrophages and PMN, should be negligible with HOClb treatment.

4.6. A New Paradigm for the Cbl/NOS/"NO Relationship? The theory that Cbl may impact on the NOS indirectly, through the contribution of its two known mammalian coenzymatic functions to NOS substrate and cofactor assembly and, indeed, to assembly of the NOS protein itself [39], may further explain our findings, including the Cbl-promoted high NOS protein (Figure 10 scheme). Furthermore, a deficiency of any of the NOS substrates and cofactors (the likely result of Cbl functional deficiency in endotoxaemia) is known to result in less tightly "coupled" NOS activity and increased free radical generation [112, 113], with a corollary increase in inflammatory mediators and prolonged period of NOS activity, indexed by our observed higher LPS-only NOS nitrite levels.

(In a forthcoming study we will also analyse more exactly how Cbl may shift the ratio of "NO/GSNO/ONOO and related species).

It may also be that Cbl, as AdoCbl and its radical, takes a direct, active part in NOS catalysis, as a third mammalian Cbl cofactor [43]. From this perspective, the high NOS protein seen with high Cbl administration may be a classical instance of the cofactor promoting coenzyme assembly. Such a central, direct Cbl/NOS, catalytic interaction would further reduce excess production of toxic forms of "NO, as well as superoxide and other related ROS and RNI species. The consequent, more precise, pro- and anti-inflammatory signalling should again result in a shorter, more effective period of NOS activity, thus lower detectable nitrite levels (as seen) with the beneficial signalling and antioxidant effects of "NO predominant. However, direct Cbl scavenger interactions, in discrete intracellular compartments, with primarily toxic RNIS, such as ONOO-/ONOOH/NO2-, with which Cbl interacts ex vivo, [114], may also play a part and cannot be
ruled out as contributing to a more complex picture behind our observed results (Figure 10).

5. Conclusions

These novel observations on the mechanism behind cobalamin protection in endotoxaemia suggest that we may be looking at the ideal natural, selective and collective regulator of the NOS, and thence of cytokines and other pivotal factors, in immune challenge and sepsis. In fact, it is now accepted that anti-inflammatory therapies (based on blocking a specific mediator) fail tout court in sepsis and that a more modulatory approach, which regulates the homeostatic inflammatory response, (in itself beneficial), could be successful. Thus, our findings may have significant clinical implications, not only for the treatment of sepsis, but also for other analogous inflammation-driven conditions, such as cancer and malaria, where NOS/NO deregulation, and consequent loss of control over key inflammatory mediators, are equally pathogenic [115, 116].

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors’ Contributions

The authors Mauro Perretti and Carmen Wheatley share senior authorship.

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