

# Extracellular haemoglobin upregulates and binds to tissue factor on macrophages: Implications for coagulation and oxidative stress

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## Summary

The mechanisms of crosstalk between haemolysis, coagulation and innate immunity are evolutionarily conserved from the invertebrate haemocyanin to the vertebrate haemoglobin (Hb). In vertebrates, extracellular Hb resulting from haemolytic infections binds bacterial lipopolysaccharide (LPS) to unleash the antimicrobial redox activity of Hb. Because bacterial invasion also upregulates tissue factor (TF), the vertebrate coagulation initiator, we asked whether there may be functional interplay between the redox activity of Hb and the procoagulant activity of TF. Using real-time PCR, TF-specific ELISA, flow cytometry and TF activity assay, we found that Hb upregulated the expression of functional TF in macrophages. ELISA, flow cytometry and immunofluorescence microscopy showed binding between Hb and TF, in isolation

and *in situ*. Bioinformatic analysis of Hb and TF protein sequences showed co-evolution across species, suggesting that Hb $\beta$  binds TF. Empirically, TF suppressed the LPS-induced activation of Hb redox activity. Furthermore, Hb desensitised TF to the effects of antioxidants like glutathione or serum. This bi-directional regulation between Hb and TF constitutes a novel link between coagulation and innate immunity. In addition, induction of TF by Hb is a potentially central mechanism for haemolysis to trigger coagulation.

## Keywords

Haemolysis, lipopolysaccharide, sepsis, oxidative stress, co-evolution of haemoglobin and tissue factor

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## Introduction

Microbial invasion can simultaneously activate two remarkably complex systems – the blood coagulation and the innate immune response. These highly integrated systems work in collaboration towards host defense against pathogens (1, 2). The functional liaison between coagulation and innate immunity has been conserved over 500 million years of evolution, existing in both humans and horseshoe crabs (1). The coagulation cascade of the horseshoe crab is activated by microbial cell wall components such as lipopolysaccharide (LPS), thus participating in pathogen recognition (3). The coagulation system further contributes to immune defense by activating the latent pro-phenoloxidase activity (4) of

the respiratory protein, haemocyanin. The oxidase function of haemocyanin may also be triggered upon direct contact with microbial proteases and LPS. The activated phenoloxidase results in the production of quinones that kill the pathogen (5). Vertebrates employ a similar mechanism, converting their respiratory protein haemoglobin (Hb) into a functional oxidase upon microbial assault (5). The redox activity of Hb (6) is tightly regulated within erythrocytes, but haemolytic infection and sepsis cause extensive haemolysis, elevating plasma Hb levels up to 2.5 mg/ml (7, 8), far exceeding the binding capacity of the Hb scavenger, haptoglobin (9). Extracellular Hb rapidly auto-oxidises to metHb and participates in a catalytic pseudoperoxidase cycle to generate reactive oxygen species (ROS) (10). Binding with LPS on the bacterial

surface enhances the pseudoperoxidase activity of Hb, thus generating harmful ROS, which kills the pathogen (5, 11). In addition to the direct microbicidal action, Hb further plays a role in the immune response by enhancing the pathophysiological effects of LPS (12). For example, Hb boosts the coagulation cascade by augmenting the LPS-induced production of tissue factor (TF) in endothelial cells (13).

TF, a transmembrane glycoprotein, is the primary physiological initiator of the extrinsic coagulation cascade in vertebrates (14). TF forms a complex with factor VIIa (15) that initiates the activation of factor X to Xa, ultimately resulting in thrombin generation and fibrin clot formation. Under normal physiological conditions, negligible levels of functional TF are expressed by intravascular cells (16). Upon pathogen invasion, a variety of pro-inflammatory stimuli such as LPS, cytokines and immune complexes may induce the expression of TF in monocytic cells (17). TF and the activated coagulation proteases are themselves pro-inflammatory (18, 19), creating a positive feedback loop between coagulation and inflammation. Studies in animal models of sepsis have shown that inhibition of TF attenuates coagulopathy and improves survival (20). These exemplify the central role of TF in the interplay between coagulation and inflammation. Another positive feedback loop connects coagulation with haemolysis, as intravascular coagulation increases the mechanical shear on red blood cells, and haemolysis releases factors that promote coagulation (21, 22). Hb constitutes 97% of the dry content of erythrocytes (23) and its impact on TF merits closer study. In invertebrates, the respiratory protein haemocyanin binds to factor B, a serine protease in the coagulation cascade (24), which triggers ROS production from haemocyanin, but whether a similar effect occurs in the vertebrates is unknown.

Previous work found that Hb did not induce TF expression in endothelial cells, but other cell types were not tested (13). The importance of non-endothelial pathways of TF induction was underscored in recent work with an animal model of sickle cell disease (SCD), where multiple indicators of disease burden depended on TF, and these effects could be abrogated by anti-TF antibody, but not by endothelium-specific knockout of TF (25). This implies that clinically important aspects of SCD depend on an endothelium-excluded source of excessive TF. In sepsis and endotoxaemia, aberrant TF expression by monocytes and macrophages has been implicated in coagulopathy (26). In xenograft transplantation with disseminated intravascular coagulation and haemolysis, the cells responsible for excessive TF expression were found to be circulating cells, not endothelial cells (27). Many pathological states exhibit dysregulated feedback loops with coagulation, but the pathological excess of TF is not necessarily endothelial.

Here, we examined the potential interaction or regulatory influence between Hb and TF. A TF-expressing macrophage model system was used for *in situ* assays. Macrophages derived by stimulation of THP-1 monocytes with phorbol 12-myristate 13-acetate (PMA) (28) were used because these macrophages share greater similarity with human primary monocyte-derived macrophages, as compared to those of U937, HEL or HL-60 cells (29). We found that Hb induced the expression of functionally active TF in THP-1

macrophages. Hb-TF interaction was demonstrated empirically using ELISA, flow cytometry and immunofluorescence microscopy. Co-evolutionary analysis of Hb and TF predicted interactions between the Hb $\beta$  subunit and TF, indicating functional significance of their interaction. The TF-Hb interaction dampened the LPS-mediated redox activity of Hb. Hb induced an unexpected stabilisation of TF activity, abrogating its sensitivity to environmental antioxidants. Specifically, Hb boosted TF activity in the presence of glutathione and serum, but attenuated TF activity when the environment lacked antioxidants. We propose that Hb-TF interaction would protect host cells from oxidative damage, would upregulate coagulation in most instances of haemolysis, and could potentially dampen excessive TF activity during an oxidative crisis.

## Materials and methods

### Reagents

For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### Cell culture, differentiation and stimulation

THP-1 monocytic cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 50 U/ml penicillin with 50  $\mu$ g/ml streptomycin (Pen-Strep, Invitrogen, Carlsbad, CA, USA) in 5% CO<sub>2</sub> at 37°C. To prepare THP-1 macrophages, cells were seeded in 12-well plates at a density of  $4 \times 10^5$  cells/ml, and treated with 10 ng/ml PMA for 24 hours (h). Cells were rinsed and cultured in fresh RPMI 1640 containing 10% FBS and Pen-Strep for a further 48 h. THP-1 macrophages were treated with 10 ng/ml LPS for 6 h to upregulate TF expression. These cells are referred to as 'TF<sup>+</sup> macrophages'. Stimulation time was determined on the basis of results obtained in preliminary time-course experiments (Suppl. Figure 1, available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)). For some experiments, TF<sup>+</sup> macrophages were treated with anti-TF antibody (4  $\mu$ g/ml) for 1 h to block the availability of TF. These are referred to as 'TF<sup>-</sup> macrophages'.

### Analysis of TF procoagulant activity

THP-1 macrophages were grown in 96-well plates and treated with various doses of Hb for 6 h in serum-free RPMI 1640. Macrophages treated with LPS were positive controls. After 6 h of treatment, cells were either left untreated or lysed in a buffer of 50 mM Tris-HCl, 100 mM NaCl, 0.1% Triton X-100, pH 7.4, for 30 minutes (min) at 37°C. TF functional activity was measured using ACTICHROME® Tissue factor activity assay kit, as per manufacturer's instructions.

### Analysis of TF procoagulant activity in the presence of Hb

Ten pM of lipidated TF was treated with increasing doses of Hb, with or without pre-treatment with GSH (20 mM), human serum (1:10 diluted) or TFPI-depleted serum (1:10 diluted) for 30 min. The procoagulant activity was then measured using ACTICHROME® Tissue factor activity assay kit. Anti-TF antibody was added to lipidated TF and to macrophages to confirm the specificity of the assay. Additionally, TF<sup>+</sup> macrophages were subjected to the same treatments as above. Following treatment, the surface procoagulant activity was measured as described.

### In vitro chemiluminescence assay for Hb redox activity

The effect of TF on the redox activity of Hb was assayed using a chemiluminescent probe, Cypridina-Luciferin Analog (CLA), 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3(7H)-one. For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### In situ chemiluminescence assay for Hb redox activity

TF<sup>+</sup> and TF<sup>-</sup> macrophages were incubated with Hb for 30 min, washed thrice and then subjected to CLA assay, either with or without pre-treatment with LPS. The reaction mixture consisted of  $2 \times 10^5$  cells, 5 µg LPS, 10 µM CLA and 5 mM H<sub>2</sub>O<sub>2</sub> in a reaction volume of 100 µl. Untreated cells served as negative controls.

### Analysis of TF mRNA

For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### Analysis of TF protein

For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### Flow cytometry for cell surface TF expression and binding of Hb to TF<sup>+</sup> macrophages

For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### Immunofluorescence staining

For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### ELISA for Hb-TF interaction

For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### Measurement of intracellular ROS

Intracellular ROS levels were measured using a cell-permeable probe, DCFH-DA. For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### Cell viability assay

The effect of various treatments on cell viability was measured using CellTiter™ Blue. For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### Analysis of the co-evolution of Hb and TF sequences

ComplexCorr (30) (<http://webclu.bio.wzw.tum.de/complexcorr>) was used to perform co-evolutionary analysis of TF and Hb. For details, see Suppl. Materials and Methods (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### Statistical analysis

Data are expressed as mean ± standard deviation (SD) of three independent experiments with triplicates each. Two group comparisons were performed using a two-tailed Student's t-test. P-values of less than 0.05 and 0.01 were considered significant (\*) and very significant (\*\*), respectively.

## Results

### TF expression in macrophages is induced by Hb

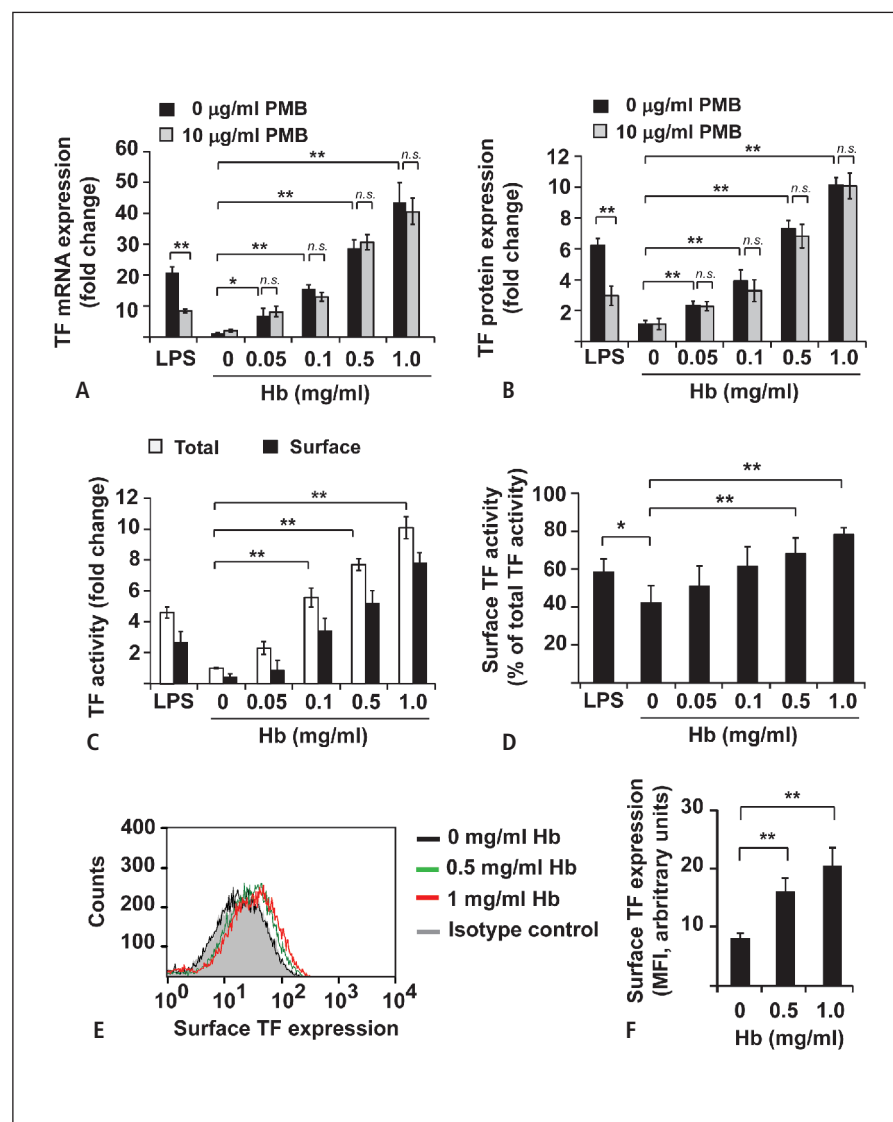
We first investigated whether extracellular Hb, resulting from haemolytic infections, could induce TF expression in macrophages. Stimulation of macrophages with increasing concentrations of Hb led to an upregulation of TF mRNA and protein expression in a dose-dependent manner (► Figure 1A and B, *black bars*). LPS is known to induce TF expression in monocytes (31), and this was used as a positive control. As expected, LPS-treatment led to an increase in the levels of TF mRNA and protein. To exclude the possibility that endotoxin contamination of Hb resulted in the upregulation of TF, the levels of endotoxin present in the Hb solutions were measured using PyroGene Assay (32). The level of endotoxin present was minimal (3 EU per mg protein) as compared to the LPS solution used as positive control (10 ng/ml, ~100 EU/ml). Furthermore, to confirm that these contaminating levels of endotoxin did not result in the Hb-mediated TF expression, we used polymyxin B (PMB), a cyclic peptide capable of neutralising LPS (33). Hb or LPS were pre-treated with PMB (10 µg/ml) for 30 min, before addition to the cells. While there was a reduction in the LPS-mediated TF expression in the presence of PMB, the Hb-mediated TF expression was not significantly affected (► Figure 1A and B, *grey bars*). This suggested that the upregulation of TF expression was mediated solely by Hb, and not due to endotoxin contamination.

Treatment of cells with a protein synthesis inhibitor, cycloheximide (CHX), prior to addition of Hb abolished the upregulation mediated by Hb (Suppl. Figure 2, available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)). This showed that *de novo* protein synthesis was required for the upregulation of TF by Hb. We next investigated whether there is a positive feedback loop, wherein binding of Hb to the cell surface TF is required to further induce TF expression. For this, THP-1 macrophages were treated with anti-TF antibody prior to addition of Hb, so as to mask available TF. We found no difference between the TF mRNA expression levels in antibody-treated versus untreated cells (Suppl. Figure 3, available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)), indicating that binding of Hb to TF does not play a role in Hb-mediated TF expression.

To examine whether the expressed TF was functionally active, the procoagulant activity of cells treated with Hb was measured. A dose-dependent increase in the procoagulant activity of TF was observed in Hb-treated cells (► Figure 1C). Additionally, we assessed the proportion of total TF activity that was expressed on the

surface of intact cells. As shown in ► Figure 1D, the surface-expressed fraction of total cellular TF increased with increasing doses of Hb. This indicates that stimulation of cells with Hb results in an increase in the expression of functional TF protein on the cell surface. At any given dose of Hb, the surface TF activity in intact cells was lower than the total TF activity of the lysed cells. This may be attributable to decryption of TF, i.e. activation of surface-located cryptic TF, which occurs as a result of membrane alterations upon cell lysis (34). The expression of TF on the cell surface in response to Hb stimulation was further confirmed using flow cytometry. As compared to LPS-untreated macrophages, cells treated with Hb displayed an increase in the mean fluorescence intensity (MFI), indicating an increased expression of TF on their surface (► Figure 1E, F).

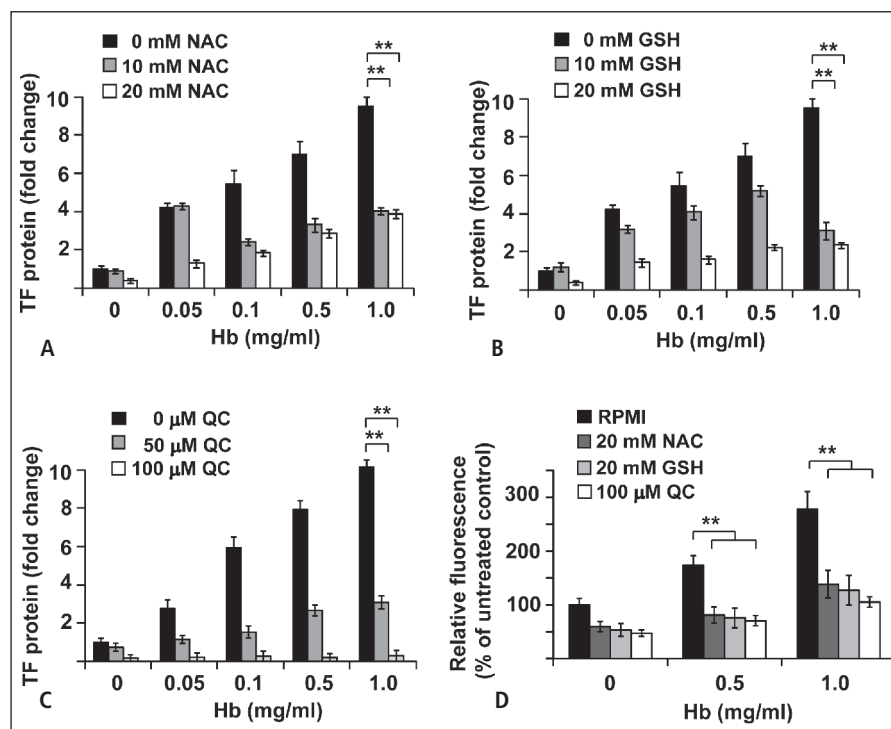
Because Hb is redox-active, we used the antioxidants, N-acetylcysteine (NAC), glutathione (GSH) and quercetin (QC) to probe whether the Hb-induced TF expression was redox regulated. Treatment with NAC,



**Figure 1: Hb induces TF expression in macrophages.** A) Macrophages were treated with the indicated concentrations of Hb (Hb was either left untreated or pre-treated with PMB for 30 min) for 2 h. As a positive control, cells were treated with LPS (10 ng/ml). Following incubation, total RNA was isolated and analysed for TF mRNA by qRT-PCR. TF mRNA levels were normalised using B2M. Results are expressed as fold change relative to untreated control using the  $\Delta\Delta C_t$  method, with B2M as an internal control. B) Macrophages were treated with Hb (with or without pre-treatment with PMB). After 6 h of treatment with Hb, total cellular proteins were extracted. Cell lysates were assayed for TF protein levels using ELISA. Results are expressed as fold change with respect to the untreated control. C) Macrophages were treated with Hb for 6 h, and TF activity on the cell surface and in total cell lysates was measured. Results are expressed as fold change with respect to the untreated control. D) Surface TF activity is expressed as a percentage of the total TF activity for each dose of Hb. B-D) Data are mean  $\pm$  SD from a representative of three independent biological replicates. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . E, F) Macrophages were treated with Hb for 6 h, and TF expression on cell surface was measured using flow cytometry. TF levels were assessed by staining with goat anti-TF antibody and Alexa Fluor 488-conjugated secondary antibody. E) Representative histograms are shown for Hb-treated and -untreated cells. The grey curve represents the background signal from the antibody isotype control. F) Surface TF expression levels are expressed as the mean fluorescence intensity (MFI), normalised with respect to the control. Data are mean  $\pm$  SD of three independent experiments. \*\*,  $p < 0.01$ .



**Figure 2: Hb-mediated TF upregulation is redox-dependent.** A-C) Macrophages were treated with (A) NAC, (B) GSH, (C) QC for 1 h, followed by Hb for 6 h. Total cellular proteins were extracted, and cell lysates were assayed for TF protein levels using ELISA. Results are expressed as fold change with respect to the untreated control. Data are mean  $\pm$  SD from a representative of three independent biological replicates. \*\*,  $p < 0.01$ . D) Macrophages were treated with the indicated concentrations of antioxidants for 1 h, followed by Hb for 6 h. Cells were treated with a cell-permeable ROS sensitive probe, DCFH-DA (100  $\mu$ M) for 30 min. Fluorescence was measured, with excitation at 485 nm and emission at 530 nm, using a microplate reader. Levels of intracellular ROS are expressed as a percentage of the untreated control. Data are mean  $\pm$  SD of three independent experiments. \*\*,  $p < 0.01$ .



GSH or QC caused a significant decrease in the upregulation of TF by Hb (► Figure 2A-C). To confirm that these anti-oxidants exerted an effect on the redox status of the cells, the levels of intracellular ROS were measured in cells treated with Hb in the presence or absence of these antioxidants. It was seen that while the addition of Hb resulted in a drastic increase in the intracellular ROS levels (► Figure 2D, black bars), the addition of antioxidants rescued the cells from the oxidative stress (► Figure 2D). This suggests that Hb-mediated TF expression was regulated by the redox status. Despite the presence of antioxidants, there was some dose-dependent increase in the TF protein levels, suggesting that Hb-induced TF expression was only partially mediated by redox effects.

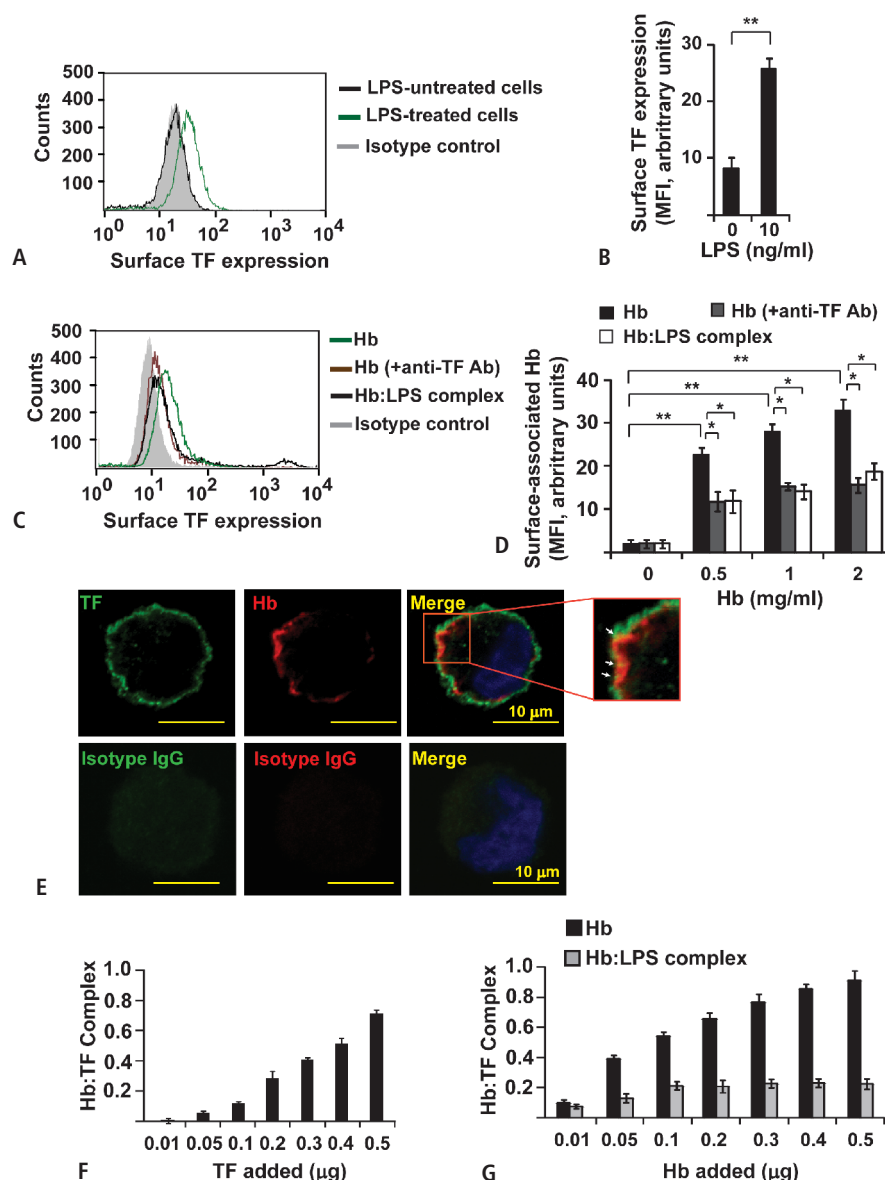
Treatment with various doses of Hb, LPS and antioxidants did not have any effect on the cell viability, as was measured using CellTiter Blue assay (Suppl. Figure 4, available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

### Hb binds TF and binding is diminished in the presence of LPS

Next, we examined whether TF expressed on the surface of macrophages interacts with extracellular Hb. Flow cytometry analysis showed that macrophages that had been treated with LPS displayed an increase in the expression of TF on their surface as compared to LPS-untreated macrophages (► Figure 3A, B). Addition of Hb to the cells resulted in a dose-dependent increase in the MFI, indicating binding of Hb to the macrophage surface (► Figure 3C, D, black bars). The specificity of the interaction between Hb and TF was examined by treating the cells with anti-TF antibody (R&D Systems, Indianapolis, IN, USA) before the addition of

Hb. TF<sup>+</sup> macrophages that were pre-treated with anti-TF antibody showed significant drop in Hb binding (► Figure 3C, D, grey bars), thus corroborating the association of Hb with TF expressed on the macrophage surface. However, pre-treatment of cells with anti-TF antibody to mask TF from Hb did not completely abolish the binding of Hb to the macrophage surface. This may be attributable to the binding of Hb to other cell-surface proteins such as SR-B1 (35). The well-known Hb scavenger receptor, CD163, that is found on macrophages is absent from THP-1 macrophages (36), and hence it is irrelevant here. Prior incubation of Hb with LPS led to a decline in the binding of Hb to TF<sup>+</sup> macrophages (► Figure 3D, white bars), implying that LPS competed with TF for binding Hb. Immunofluorescence staining coupled with confocal microscopy further confirmed the co-localisation of Hb with TF on the macrophage surface (► Figure 3E).

We further investigated the interaction between Hb and TF in a cell-free system. ELISA showed a dose-dependent binding of TF to Hb immobilised on Maxisorp™ plates (► Figure 3F), indicating that the interaction between Hb and TF does not require the presence of any cellular factors. Upon reversal of the order of binding, by immobilisation of TF and addition of Hb in the fluid phase, the amount of Hb-TF complex increased, suggesting that a solid surface-associated TF interacts better with soluble Hb (► Figure 3G, black bars). Moreover, binding of Hb to TF was suppressed when Hb was pre-complexed with LPS, confirming that there is competition between LPS and TF for binding to Hb (► Figure 3G, grey bars). To examine whether TF was able to bind LPS, ELISA was performed by immobilising LPS and adding increasing concentrations of TF. However, no complex formation was observed between TF and LPS, thus eliminating the possibility that Hb-LPS



**Figure 3: TF competes with LPS for binding Hb.** A-B) TF expression on macrophage surface after LPS stimulation was measured using flow cytometry. Macrophages were either untreated or stimulated with 10 ng/ml LPS for 6 h. TF levels were assessed by staining with goat anti-TF antibody and Alexa Fluor 488-conjugated secondary antibody. A) Representative histograms are shown for LPS-treated and -untreated cells. The grey curve represents the background signal from the antibody isotype control. B) Surface TF expression levels are expressed as MFI, normalised with respect to the control. Data are mean  $\pm$  SD of three independent experiments. \*\*,  $p < 0.01$ . C-D) Binding of Hb or Hb:LPS complex to TF<sup>+</sup> macrophages was measured using flow cytometry. TF<sup>+</sup> macrophages, with or without pre-treatment with anti-TF antibody, were treated with increasing doses of Hb for 30 min. Hb bound to the cell surface was detected by staining with mouse anti-Hb antibody and Alexa Fluor 488-conjugated secondary antibody. C) Representative histograms of binding of Hb (2 mg/ml) to cells. The grey curve represents the background signal from the isotype control. D) Levels of surface-associated Hb are expressed as mean fluorescence intensity (MFI), normalised with respect to the control. Data represent the mean  $\pm$  SD of three independent experi-

ments. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ . E) Confocal fluorescence microscopy confirmed the co-localisation of TF (green) with Hb (red), as shown (with white arrow) in merged panel. TF-expressing macrophages were treated with 0.5 mg/ml Hb for 30 min. Proteins were stained using primary goat anti-TF and mouse anti-Hb antibodies, followed by secondary Alexa Fluor 488-conjugated donkey anti-goat and Alexa Fluor 546-conjugated donkey anti-mouse antibodies. Isotype antibodies were used as negative controls. Scale bar, 10  $\mu$ m. F) ELISA was performed with 0.2  $\mu$ g of Hb immobilised on a Maxisorp™ plate. The unbound sites were blocked with 2% BSA, and TF was then added. Bound protein was detected with primary anti-TF antibodies and HRP-conjugated secondary antibody. OD<sub>405</sub> was read to indicate the extent of complex formation. Data are mean  $\pm$  SD of three independent experiments. G) ELISA was performed by immobilising 0.2  $\mu$ g of TF. Hb (with or without pre-treatment with a 4-fold excess of LPS) was added, and bound protein was detected using primary anti-Hb antibody and HRP-conjugated secondary antibody. OD<sub>405</sub> was read to indicate the extent of complex formation. Data are mean  $\pm$  SD of three independent experiments.

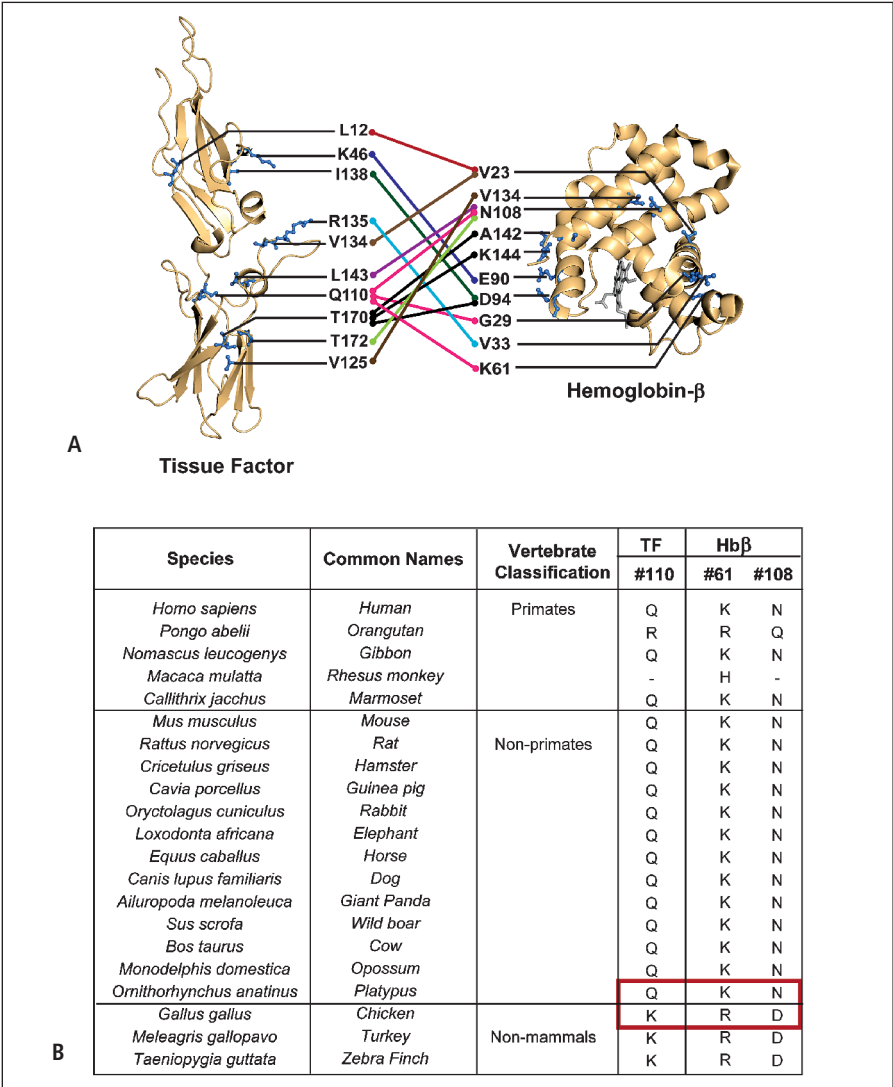
complexes bound to TF via LPS (Suppl. Figure 5, available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)).

Co-evolutionary analysis reveals correlated mutations between Hbβ and TF

Previous work in bioinformatics has shown that when proteins bind each other to achieve a significant functional effect, their sequences often show extensive co-evolution, meaning statistical co-variation in their amino acid mutations during the course of evolution. To test whether the evolutionary histories of TF and Hb show significant co-variation (which would serve as an indicator of the functional significance of their binding) we performed co-evolutionary analysis using ComplexCorr, which employs ELSC and OMES algorithms to calculate scores of co-evolutionary strength (30). To convert ELSC and OMES scores into predictions, Gershoni et al. determined the specificity and sensitivity of different ELSC and OMES thresholds, based on high-confidence interactions (i.e. protein pairs that had been crystallised in a bound

state). They found an ELSC score threshold of 15, or an OMES score threshold of 3.5 would provide 80% specificity, at the cost of lower sensitivity (30). We used these pre-determined thresholds to decide whether our observed level of statistical co-variation would be predictive of binding. The results from ComplexCorr suggested no interaction for TF and Hbα as the scores were below the thresholds (ELSC score: 11.95, OMES score: 2.74), but the scores for co-variation of TF and Hbβ were above the thresholds (ELSC score: 16.37, OMES score: 3.53). The fourteen pairs of residues with strongest co-variation are shown in ►Figure 4A. The residues at these positions switched to another amino acid at a similar time during evolution. For example, position 110 of TF showed co-evolution with residues at positions 61 and 108 of Hbβ (►Figure 4B). In sum, the co-evolution between TF and Hbα was weak, while the co-evolution between TF and Hbβ was significant enough to predict, on purely statistical grounds, that they are likely to bind. Thus it is conceivable that the interaction observed empirically between TF and Hb may play a physiologically important role.

**Figure 4: TF has co-evolved with Hbβ.** A) Co-evolving residue pairs between TF and Hbβ were predicted by ComplexCorr, using ELSC and OMES algorithms. The top scoring residue pairs identified by both the algorithms are mapped onto the structures of TF (PDB id: 1BOY) and Hbβ (PDB id: 1HGB). The figure was generated using PyMOL (v1.0, DeLano Scientific). B) Two highly co-evolving residue pairs between Hbβ and TF are shown. Position 110 of TF displayed strong co-evolution with residues 61 and 108 of Hbβ. Red box indicates simultaneous change in the amino acids during vertebrate evolution from non-mammals to mammals.

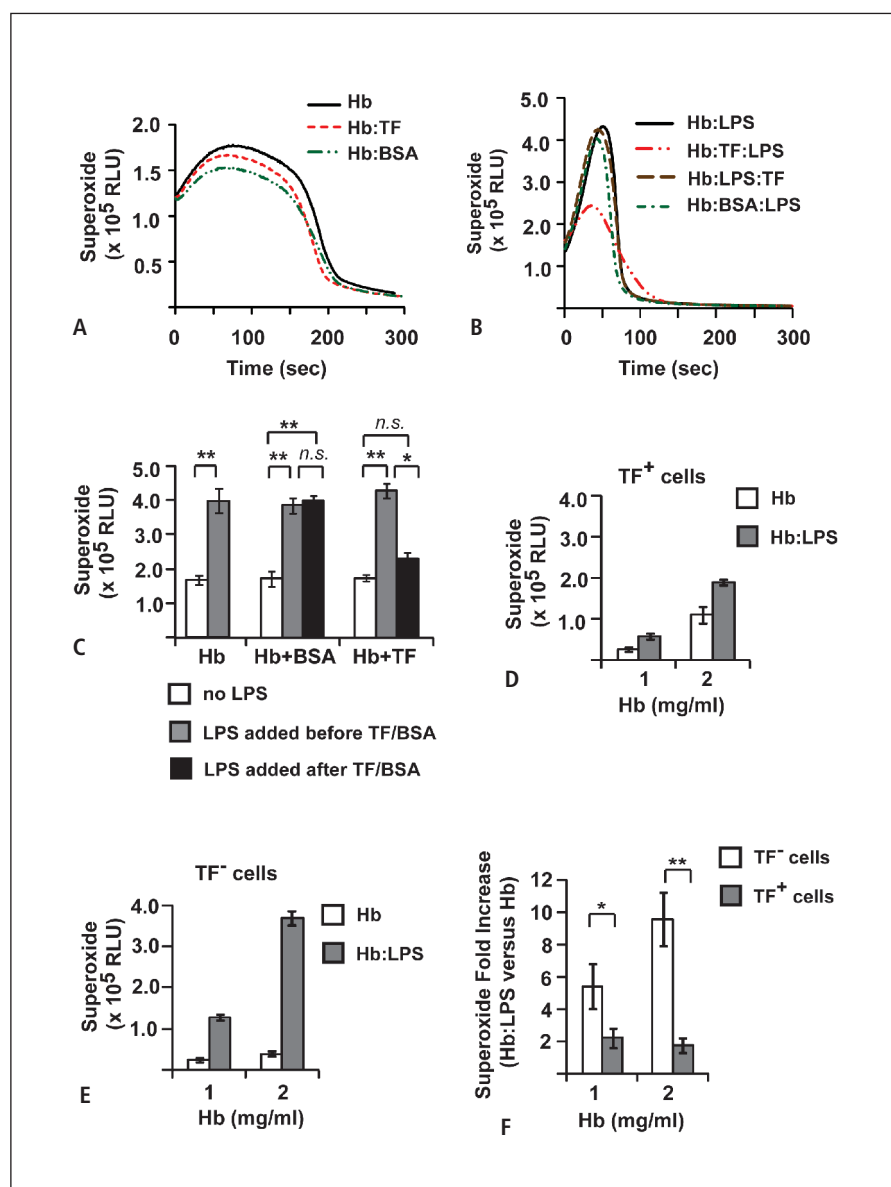


## TF inhibits the LPS-induced redox activity of Hb

We investigated whether the redox activity of Hb was influenced by its interaction with TF. The pseudoperoxidase activity of Hb catalytically converts peroxide into superoxide, which can be measured using a chemiluminescence assay. In cell-free experiments, the concentration (10 µg/ml) of Hb used was governed by the detection limit imposed by the assay. Previous work has indicated a binding ratio of Hb:LPS to be 1:3–5 (37), suggesting one LPS molecule per Hb subunit. Hence, LPS was added at a molar ratio of 1:4 of Hb:LPS. TF was maintained equimolar to Hb, assuming a one-to-one interaction between Hb and TF. Hb pre-treated with TF produced the same level of superoxide as Hb alone (► Figure 5A, C), indicating that TF had no effect on the redox behaviour of Hb. In accordance with previous reports (5), we found that the redox activity of Hb was doubled in the presence of LPS

(► Figure 5C, grey bars). To test whether the LPS-induced redox behaviour of Hb could be regulated by TF, Hb was incubated with TF, either before or after its treatment with LPS. We found that addition of TF to Hb prior to LPS led to an inhibition of the LPS-induced increase in superoxide production by Hb (► Figure 5B, C). In contrast, when TF was added after LPS, it did not inhibit the increase in the superoxide production by Hb. Moreover, addition of bovine serum albumin to Hb, prior to LPS, did not affect the redox activity of Hb, thus indicating the specificity of TF-mediated suppression of superoxide production.

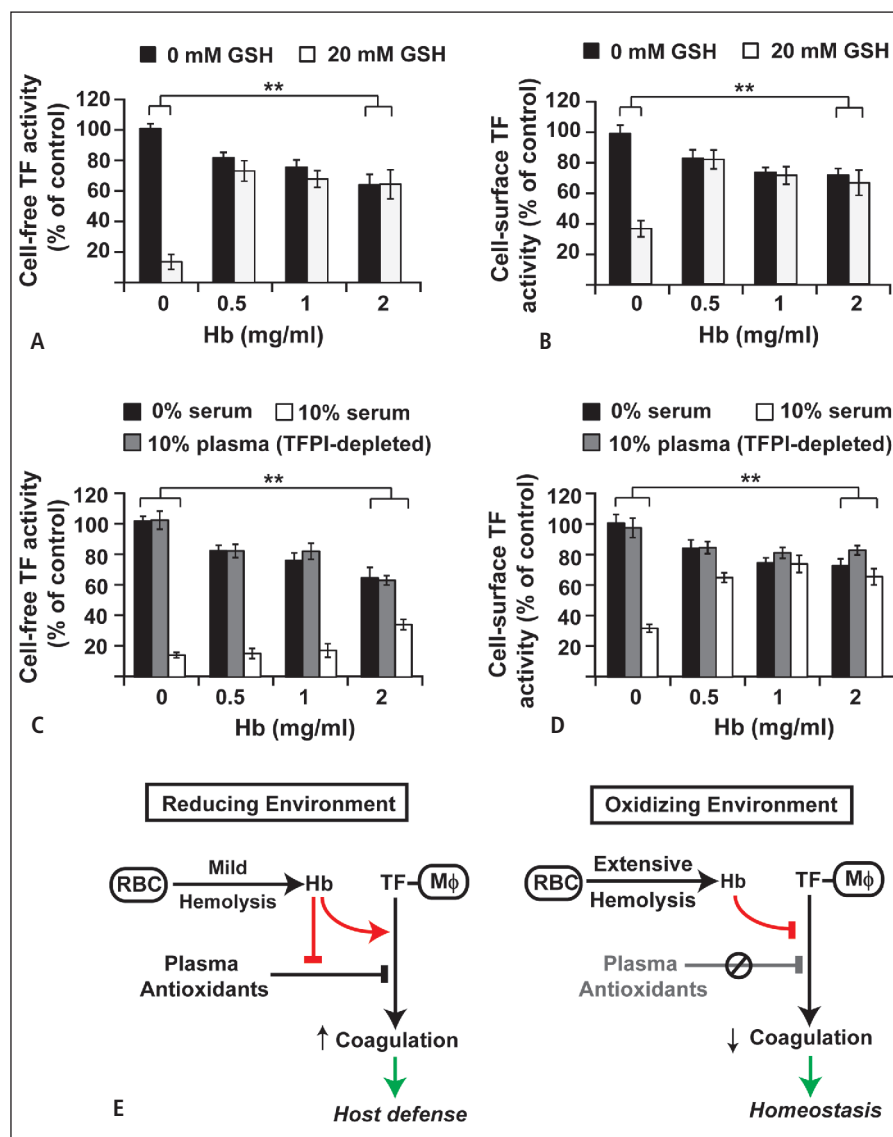
We next tested whether a similar dampening of the LPS-mediated redox activity of Hb occurred upon binding of Hb to TF anchored on the macrophage surface. There was a dose-dependent increase in the superoxide production by Hb bound to TF<sup>+</sup> macrophages (► Figure 5D, white bars). As seen from the *in vitro* assay, TF alone did not suppress the redox activity of Hb. Hence, Hb



**Figure 5: TF suppresses the LPS-induced redox activity of Hb.** A) Hb was incubated with equimolar TF, or with BSA as a control. H<sub>2</sub>O<sub>2</sub> and CLA were added, and the kinetics of superoxide production by Hb was measured as the chemiluminescence emitted by CLA. The extent of superoxide formed is expressed as relative luminescence units (RLU). Data are representative of three independent experiments. B) Hb was treated with TF and LPS in varying orders of addition, and the kinetics of superoxide production was measured. Hb:TF:LPS – LPS was added before TF, Hb:LPS:TF – LPS was added after BSA. C) The peak chemiluminescence intensity for the various combination treatments, obtained from the superoxide production curves was plotted. Data are mean ± SD of three independent experiments. \*, p < 0.05, \*\*, p < 0.01, ns – not significant. D) TF<sup>+</sup> macrophages were treated with Hb (1 and 2 mg/ml). Cells were washed and left untreated or treated with LPS, followed by addition of H<sub>2</sub>O<sub>2</sub> and CLA. The superoxide production, measured as the chemiluminescence emitted by CLA, was quantified by the peak chemiluminescence intensity and is expressed as relative luminescence units (RLU). E) TF<sup>+</sup> macrophages obtained by blocking TF<sup>+</sup> macrophages with anti-TF antibody were treated as described in (A), with Hb+/-LPS and the superoxide production was measured after addition of H<sub>2</sub>O<sub>2</sub> and CLA. F) The fold-change in the superoxide formed by the cells treated with Hb and LPS, versus those treated with Hb alone was plotted. TF<sup>+</sup> indicates cells expressing TF, TF<sup>-</sup> indicates cells treated with anti-TF antibody to block TF. Data are mean ± SD of three independent experiments. \*, p < 0.05, \*\*, p < 0.01.



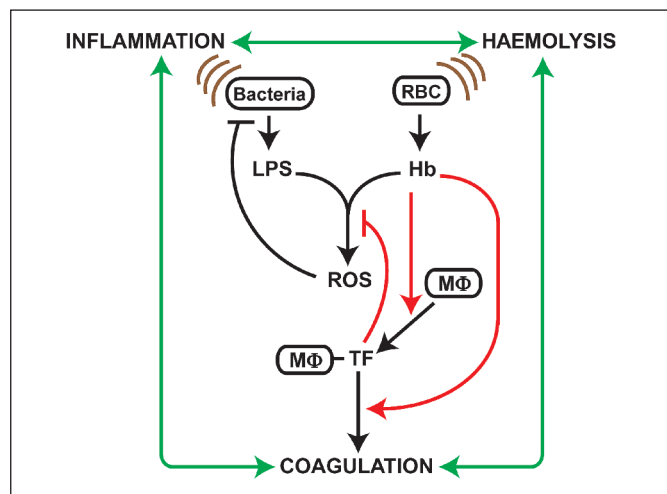
**Figure 6: Effect of Hb on the procoagulant activity of TF.** A, C) Hb, with or without pre-treatment with GSH or serum, was mixed with lipidated TF. The change in the activity of TF was measured using ACTICHROME® TF activity assay kit, and is plotted as a percentage of the Hb-untreated control. B, D) TF<sup>+</sup> macrophages were treated in a similar way with Hb, with or without pre-treatments with GSH or serum. Cells were washed, and TF activity on the cell surface was measured. Activity is expressed as a percentage of the untreated control samples. Data are mean  $\pm$  SD of three independent experiments. \*\*,  $p < 0.01$ . E) The dual effects of Hb on TF procoagulant activity are schematically depicted. Under mild haemolysis, plasma antioxidants are present at sufficient levels to inhibit TF procoagulant activity. Extracellular Hb binds to TF on macrophages to overcome the inhibitory effects of antioxidants, and may promote coagulation for maintaining host defense. In severe oxidative stress, plasma antioxidants are depleted and their control over TF procoagulant activity would be lost. Hb would then bind to TF to downregulate and stabilise TF activity, which may contribute to maintaining homeostasis. Thus, in both oxidising and reducing conditions, Hb would preserve the TF procoagulant activity to maintain the coagulation processes for host defense. M $\Phi$ : macrophage; red arrows: experimental findings; green arrows: proposed implications.



bound to TF<sup>+</sup> macrophages displayed redox activity in a concentration-dependent manner. Hb bound to TF<sup>-</sup> macrophages also displayed some redox activity, indicating that Hb was bound to these cells, albeit non-specifically (► Figure 5E, *white bars*). Addition of LPS to Hb bound to TF<sup>-</sup> macrophages led to a significant increase in its redox activity (► Figure 5E, *grey bars*), while only a marginal increase was observed with Hb bound to TF<sup>+</sup> cells. These results imply that while non-specific binding of Hb to the macrophage surface did not suppress its redox behaviour, binding to TF downregulated the LPS-induced redox activity of Hb. The redox activity of Hb was quantified by computing the fold change in superoxide produced by Hb in the presence of LPS versus Hb alone. Hb bound to TF<sup>-</sup> cells displayed a six- to 10-fold increase in its redox activity in the presence of LPS. However, upon binding to TF<sup>+</sup> cells, the redox activity was increased by only two-fold (► Figure 5F).

## Hb stabilises the procoagulant activity of TF

We asked whether Hb exerted a reciprocal regulatory effect on the procoagulant activity of TF. The procoagulant activity of TF was measured in the presence of Hb, both in a cell-free system and *in situ* in macrophages, with and without antioxidants. In a cell-free system without antioxidants, Hb caused a dose-dependent decline in TF activity (► Figure 6A). At a concentration of 2 mg/ml of Hb, there was a 40% inhibition of TF activity (► Figure 6A, *black bars*). Previous studies have found TF activity to be strongly suppressed by antioxidants (38, 39). In accordance with these reports, we too observed a dramatic inhibition of TF activity by GSH (► Figure 6A, *white bars*). The combination of Hb with GSH showed a neutralisation effect – in the presence of Hb, TF activity was unaffected by GSH (► Figure 6A, *white bars*). Serum contains an assortment of physiological antioxidants, and serum at 1:10 dilution (without Hb) drastically attenuated TF ac-



**Figure 7: A model to illustrate the multi-functional interplay between Hb and TF – implications on inflammation, haemolysis and coagulation.** Infection elicits crosstalk between inflammation, haemolysis and coagulation. Infection-inflammation condition triggers haemolysis, elevating extracellular Hb, which is redox-active. LPS binds extracellular Hb and activates it to generate ROS, resulting in microbial killing. Our findings (red arrows) reveal a novel link between hemolysis and coagulation. Hb upregulates TF expression on macrophages, and further binds to cell-anchored TF, and maintains the coagulant activity of TF at steady levels. Binding to TF inhibits the LPS-induced redox activity of Hb. MΦ: macrophage, red arrows: experimental findings.

tivity (► Figure 6C, *white bars*). However, a high dose of Hb (2 mg/ml) rendered the activity of TF insensitive to serum-mediated inhibition. Serum also contains tissue factor pathway inhibitor (TFPI), which could contribute to the impact of serum. TF activity in presence of TFPI-depleted plasma (1:10 dilution) was similar to the buffer controls (► Figure 6C, *grey bars*), suggesting that the effects of serum are more complex than the antioxidant effects alone. Hb led to a decrease in the TF activity, stabilising it at ~60% of the control. In sum, Hb increased the activity of TF in the presence of glutathione, even though it had the opposite effect without the antioxidant. In TF<sup>+</sup> macrophages, we observed similar trends (► Figure 6B, D). Hb stabilised the activity of TF at ~80% of untreated control, regardless of the presence of GSH or serum. No TF activity was detected upon treatment of TF with anti-TF antibody, in both cell-free and macrophage experiments, thus suggesting that binding of antibody to TF abrogates its procoagulant activity.

## Discussion

Many pathological conditions such as sickle cell disease (SCD), thalassemia, paroxysmal nocturnal haemoglobinuria (PNH) and infection are characterised by extensive intravascular haemolysis. Plasma Hb levels have been reported to be 1–2.5 mg/ml in sepsis (7, 8), 0.5–2 mg/ml in PNH (40), and up to 0.4 mg/ml in patients with SCD (41). Hb levels may also reach up to 10 mg/ml during

episodes of severe haemolysis in PNH. An increased incidence of thrombosis has also been reported under these conditions (42, 43). Haemolysis is known to promote coagulation via several mechanisms (21), including exposure of phosphatidyl serine on the surface of red blood cells, scavenging of nitric oxide by Hb, endothelial dysfunction, and increased expression of TF. Intravascular coagulation results in fibrin deposition that causes haemolysis by mechanical damage to erythrocytes. Here, we have identified a functional interplay between Hb and TF that provides a new link between haemolysis and coagulation during infection.

Previous studies showed that while Hb enhanced the LPS-induced production of TF in endothelial cells, Hb by itself failed to induce TF expression (13). Heme, however, has been shown to induce TF expression in endothelial cells (22). Here, we found that Hb levels ranging from 0.05 mg/ml to 1.0 mg/ml significantly induced expression of functional TF in macrophages. Whether heme or apoglobin (Hb without heme) exerts an influence on TF expression may be further tested to dissect the molecular mechanism of the Hb-mediated TF expression. It is known that many pathological conditions with intravascular haemolysis are characterised by an elevated TF expression (43). Therefore, the presence of Hb-mediated TF induction in macrophages, and not in endothelial cells, suggests an intriguing parallel with published instances where haemolytic coagulopathies were caused by non-endothelial sources of excessive TF (25, 27).

Hb, with its intrinsic redox activity, is a potent source of free radicals. The role of oxygen free radicals in inducing the expression of TF by monocytes has been demonstrated previously, as treatment with hydrogen peroxide led to an increase in TF expression, which was abrogated upon treatment with NAC (44). LPS-induced TF expression by monocytes and endothelial cells has been shown to be inhibited upon treatment with NAC (45). It has been suggested that antioxidants suppress TF expression at the post-transcriptional level via inhibition of translation or degradation of the translated protein (45). Thus, we propose that attenuation of the Hb-induced TF expression by NAC treatment may be attributed to a similar mechanism.

TF purified from human brain tissue has been found to contain a heterodimer of TF linked to Hb alpha chain through a disulfide (46). This disulfide was interpreted as resulting from the cell lysis procedure, because the amount of heterodimer was dependent on the duration of the procedure in the lab, and because the disulfide involved a cysteine from the cytoplasmic tail of TF. Nonetheless, the abundance of this specific disulfide pairing, rather than a non-specific effect with mixed partners, is consistent with TF and Hb being in proximity, prior to oxidation. In melanoma, Hb has been shown to form a complex with melanoma cell-derived TF (47). Here, we demonstrated the ability of Hb to form a complex with TF expressed on the macrophage surface. Flow cytometry and ELISA showed that Hb pre-treated with LPS was unable to bind TF effectively, suggesting that TF and LPS compete for binding Hb.

Computational analysis identified a strong pattern of co-evolution between TF and in Hbβ, indicating that they probably form a direct interaction. Proteins that bind each other to accom-

plish a beneficial function often have correlated mutations, because when one mutation occurs by chance and disrupts the binding, the host would gain an evolutionary advantage by adopting a compensatory mutation in the other protein to restore the interaction. The number and strength of co-evolving residues can be interpreted in bulk as statistical evidence for the existence of binding, but the individual pairs of amino-acids may not be structural evidence for the location of binding. Previous studies of interacting proteins have found that the majority of strongly co-evolving residue pairs were not located at the protein-protein binding interface (48, 49). Although our finding of co-evolution is only statistical in nature, it is a noteworthy complement to our *in vitro* experiments because it provides an entirely independent source of support for the binding of Hb to TF. Moreover, if there is a genuine evolutionary preference for the amino acids of Hb $\beta$  to co-vary with the amino acids of TF, then the Hb-TF relationship must have some importance *in vivo*, or else it could not affect the evolutionary fitness of the organism. Future experiments using animal models could validate the predicted *in vivo* significance of Hb-TF interaction.

Extracellular Hb is redox-active as it generates ROS via its pseudoperoxidase activity (10). The redox behaviour of extracellular Hb is augmented by partial unfolding of the protein structure, such as in the presence of LPS (11). Heightened generation of ROS then occurs in the vicinity of pathogens and serves as an anti-microbial defense mechanism (5). Here, we found that if Hb bound TF prior to binding LPS, TF abated the LPS-induced redox activity of Hb. This prompted us to reason that TF may be binding to Hb to induce a conformational change or a steric hindrance that blocks LPS from binding to Hb. Such an anti-oxidative mechanism may protect the host from cellular damage that may ensue if plasma Hb were to encounter LPS in proximity to a host cell during a Gram-negative bacterial infection.

We found that the antioxidant, GSH, inhibited TF activity, both in a cell-free system and on macrophages. Addition of serum also led to an attenuation of the TF activity. However, Hb reversed these effects and restored ~60-80% of TF activity independent of the redox environment. The Cys186-Cys209 disulfide of TF has been proposed to regulate the decryption of the procoagulant function (38, 39), but disulfide formation is not necessary for all cases of TF decryption (50). One possible interpretation of our results is that Hb-binding causes a change in the redox microenvironment of TF that restores its procoagulant activity. Perhaps more likely, Hb-binding induces a contextual or conformational change that alters the redox sensitivity of TF. Future work may test whether the Hb-TF mode of interaction relates to a more general switch in TF sensitivity to redox regulation. It is conceivable that in contexts that lack antioxidants, Hb would dampen the procoagulant activity of TF, which could be homeostatic in a highly pro-oxidative environment (► Figure 6E). In haemolytic contexts where antioxidants are not depleted, Hb would induce TF activity as well as promote TF expression, thus contributing to host defense (► Figure 6E). In both oxidising and reducing conditions, TF would decrease LPS-induced Hb redox activity on the surface of host cells and decrease oxidative damage to the host (► Figure

### What is known about this topic?

- Extracellular haemoglobin (Hb) produces reactive oxygen species (ROS), and this redox activity is enhanced when Hb binds bacterial lipopolysaccharide (LPS). The resulting ROS contributes to host defence by killing bacteria.
- Tissue factor (TF) is upregulated during infection to promote coagulation, but coagulation also causes haemolysis. Factors released during haemolysis can create a feedback loop leading to additional coagulation.

### What does this paper add?

- Extracellular Hb induces TF expression in macrophages and stabilises TF procoagulant activity by desensitising TF activity to the effects of antioxidants.
- TF exerts a regulatory influence on the redox activity of Hb by suppressing the LPS-induced activation of Hb redox activity during a Gram negative bacterial infection.
- TF and Hb appear to have co-evolved, and the functional effect of their binding bears a resemblance to the invertebrate interaction between haemocyanin (a respiratory factor) and Factor B (a coagulation protease).

7). Future experiments with primary macrophage cells may be performed to further substantiate our present findings with THP-1 cell line.

To summarise, our results conclusively show the existence of a multi-functional interplay between Hb and TF that regulates TF expression and activity, and Hb redox activity. Compared with the invertebrates, where regulation of phenol oxidase activity of haemocyanin is unknown, vertebrates have apparently evolved a significant safeguard against over-activation of the pseudoperoxidase activity of Hb. The interaction between Hb and TF helps limit oxidative damage to the host while stabilising the coagulation processes that contribute to host defense.

### Conflicts of interest

None declared.

### Abbreviations

B2M,  $\beta$ 2microglobulin; CHX, cycloheximide; CLA, cypridina-luciferin analog; DCFH-DA, dichlorofluorescein diacetate; ELSC, explicit likelihood of subset covariation; EU, endotoxin units; GSH, glutathione; Hb, haemoglobin; MFI, mean fluorescence intensity; NAC, N-acetylcysteine; NO, nitric oxide; OMES, observed minus expected squared; PMA, phorbol 12-myristate 13-acetate; PMB, polymyxin B; PNH, paroxysmal nocturnal haemoglobinuria; QC, quercetin; ROS, reactive oxygen species; SCD, sickle cell disease; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

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