

# C1-inhibitor efficiently inhibits *Escherichia coli*-induced tissue factor mRNA up-regulation, monocyte tissue factor expression and coagulation activation in human whole blood

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Accepted for publication 27 February 2013  
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## Introduction

Sepsis is a serious condition with high lethality [1]. The invading microbes can overactivate the host inflammatory response harmfully through several cascade systems, such as the complement, kinin and coagulation systems [2,3]. In some cases, this overactivation leads to multi-organ failure and death.

Tissue factor (TF) is the most important activator of coagulation [4]. *In-vitro* stimulation with lipopolysaccharide (LPS) and bacteria up-regulate TF expression on the surface of monocytes [4]. Several cytokines can also induce

## Summary

Both the complement system and tissue factor (TF), a key initiating component of coagulation, are activated in sepsis, and cross-talk occurs between the complement and coagulation systems. C1-inhibitor (C1-INH) can act as a regulator in both systems. Our aim in this study was to examine this cross-talk by investigating the effects of C1-INH on *Escherichia coli*-induced haemostasis and inflammation. Fresh human whole blood collected in lepirudin was incubated with *E. coli* or ultrapurified *E. coli* lipopolysaccharide (LPS) in the absence or presence of C1-INH or protease-inactivated C1-INH. C3 activation was blocked by compstatin, a specific C3 convertase inhibitor. TF mRNA was measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and TF surface expression was measured by flow cytometry. In plasma, the terminal complement complex, prothrombin F1-2 (PTF1-2) and long pentraxin 3 (PTX3) were measured by enzyme-linked immunosorbent assay (ELISA). Cytokines were analysed using a multiplex kit. C1-INH (1.25–5 mg/ml) reduced both LPS- and *E. coli*-induced coagulation, measured as a reduction of PTF1-2 in plasma, efficiently and dose-dependently ( $P < 0.05$ ). Both LPS and *E. coli* induced marked up-regulation of TF mRNA levels and surface expression on whole blood monocytes. This up-regulation was reduced efficiently by treatment with C1-INH ( $P < 0.05$ ). C1-INH reduced the release of PTX3 ( $P < 0.05$ ) and virtually all cytokines measured ( $P < 0.05$ ). Complement activation was inhibited more efficiently with compstatin than with C1-INH. C1-INH inhibited most of the other readouts more efficiently, consistent with additional non-complement-dependent effects. These results indicate that complement plays a role in activating coagulation during sepsis and that C1-INH is a broad-spectrum attenuator of the inflammatory and haemostatic responses.

**Keywords:** C1-inhibitor, coagulation, complement, tissue factor, whole blood

TF surface expression on monocytes [5]. TF is expressed on extravascular cells and is involved in normal haemostasis [5]. TF is a transmembrane glycoprotein, and acts as a co-factor for FVIIa, which is formed from FVII upon autocatalysis and activation by other coagulation enzymes. The TF/FVIIa complex catalyses further the generation of limited amounts of FXa and FIXa during the initiation phase of coagulation [4]. Subsequently, the small amounts of thrombin generated by TF-bearing cells can activate platelets and FV and dissociate FVIII from von Willebrand factor, leading to the amplification phase and the activation of FXI.

Napoleone *et al.* reported that long pentraxin 3 (PTX3) increased LPS-induced TF mRNA up-regulation [6]. PTX3 is produced by several cells, including monocytes, and does not bind to any classical pentraxin ligands except for the complement component C1q [7].

The complement system is activated efficiently by whole Gram-negative bacteria, whereas pure LPS can activate the complement system only at high concentrations [8]. *Escherichia coli* (*E. coli*) activates mainly the classical and alternative pathways [9]. Inhibition of the complement component C3 has shown promising results in a baboon model of *E. coli* sepsis [10]. Complement activation leads to the release of the anaphylatoxin C5a, which can lead to a number of detrimental effects during sepsis [11]. C5a is also involved in the *Neisseria meningitidis*-induced TF surface expression on monocytes [12]. Interestingly, thrombin can also split C5 and participate in C5a generation [13].

C1-INH is a protease inhibitor in the serpin family [14]. C1-INH inhibits complement activation through both the classical pathway, by blocking C1r and C1s activation [15], and the lectin pathway, by inhibiting mannan-binding lectin-associated serine protease-1 (MASP-1) and MASP-2 [16,17]. In addition, C1-INH has an important regulatory effect on the kinin system [18]. C1-INH is used to treat angioedema in patients with hereditary C1-INH deficiency [19]. In addition, C1-INH has anti-inflammatory effects, and several of these effects are not dependent upon the protease inhibitory activity of C1-INH [20]. C1-INH treatment reduced *E. coli*-induced complement activation and cytokine release efficiently in baboons [21]. Treatment with C1-INH has been reported recently to reduce lethality significantly during human sepsis [22].

Several recent reports have indicated that cross-talk occurs between the coagulation and complement systems [23]. Because C1-INH participates as a regulator of both systems, in this study we investigated the role of C1-INH on *E. coli*- and LPS-induced TF up-regulation and inflammatory reactions. The selective C3 convertase inhibitor compstatin was also used to evaluate the role of complement activation in TF up-regulation.

## Materials and methods

### Inhibitors

Purified human C1-INH (Berinert® P C1 esterase inhibitor) was obtained from ZLB Behring GmbH (Marburg, Germany). Inactivated C1-INH (iC1-INH) was produced as described previously [24] and dissolved in LAL reagent water (Cambrex Bio Science, Inc., Walkersville, MD, USA). Endotoxin-free human serum albumin (HSA) was obtained from Octopharma AG (Lachen, Switzerland). An improved analogue of the C3 convertase inhibitor compstatin AMY-90, comprised of the following sequence Ac-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-mIle-NH<sub>2</sub>,

and a corresponding control peptide were synthesized as described previously [25] and provided by the co-author, J. D. Lambris. The concentration of compstatin used (20 µM) was chosen from initial dose–response experiments showing a maximal inhibitory effect (data not shown).

### Whole blood experiments

Experiments were performed with blood from 12 individual healthy donors (both female and male) using fresh human whole blood, as described previously [9]. The study was approved by the regional ethics committee in the Northern Norway Regional Health Authority. Whole blood samples were collected in 4.5 ml polypropylene tubes (Nunc, Roskilde, Denmark), using lepirudin (Refludan®, 50 mg/l; Celgene, Uxbridge, UK) as an anti-coagulant. Aliquots of fresh whole blood (5 parts) were distributed immediately into polypropylene tubes containing inhibitors or control protein in Dulbecco's phosphate-buffered saline (PBS, 2 parts). PBS with or without calcium chloride and magnesium chloride was purchased from Sigma-Aldrich (St Louis, MO, USA). All equipment, such as tubes, tips and solutions, were endotoxin-free. After 8 min of pre-incubation at 37°C, PBS, *E. coli* ( $1 \times 10^7$ /ml) or ultra-purified LPS (100 ng/ml) from *E. coli* 0111 (LPS-EB Ultrapure; InvivoGen, Eugene, OR, USA) was added to the blood. The LPS and *E. coli* concentrations used were selected after initial time–course and dose–response experiments on TF up-regulation and cytokine release. The *E. coli* strain LE392 (ATCC 33572) was obtained from the American Type Culture Collection (Manassas, VA, USA). The bacteria were grown and heat-inactivated as described previously [9]. The time zero sample (T<sub>0</sub>) was processed immediately, and the other samples were incubated on a rotator at 37°C for 120 min. Immediately following incubation, the blood was distributed into three different tubes. Citrate was added prior to flow cytometric analysis. No additive was used in the tubes designated for TF function analysis. For analysis of the complement activation products and mRNA levels, ethylenediamine tetraacetic (EDTA, 10 mM) was added to stop further complement activation. All tubes were centrifuged for 15 min at 3220 g at 4°C. The plasma was stored at –80°C. The pelleted cell fraction in the EDTA tubes was used for mRNA extraction. Cells were lysed using ×1 nucleic acid purification lysis solution (Applied Biosystems, Warrington, UK), and the lysates were frozen at –80°C until analysis.

### Reverse transcription–quantitative polymerase chain reaction (RT–qPCR) of TF mRNA expression

Total RNA was isolated using reagents from Applied Biosystems (Foster City, CA, USA) total RNA chemistry and the AB6100 nucleic acid prep station (Applied Biosystems). The concentrations of RNA were measured using a Nanodrop

2000c (ThermoFisher Scientific, Wilmington, DE, USA). Reverse transcription was performed on 50 ng of total RNA using a high-capacity cDNA reverse transcription kit and a 2720 thermal cycler (Applied Biosystems). The thermal cycle conditions were 25°C for 10 min and 37°C for 120 min. Samples of cDNA were frozen at -80°C. TF mRNA was measured using the 7500 fast real-time PCR system (Applied Biosystems), *TaqMan* fast universal PCR master mix and predeveloped *TaqMan*<sup>®</sup> gene expression assays. The target gene TF (Hs00175225\_m1) and the stably expressed reference gene human beta-2-microglobulin ( $\beta$ 2M, assay ID 4326319E; Applied Biosystems) were analysed in a multiplex reaction using cycle conditions according to the manufacturer's instructions. RT-qPCR was performed using 3  $\mu$ l of cDNA (triplicate samples) in a final volume of 20  $\mu$ l. The comparative delta Ct-method was used to determine the relative concentration of TF mRNA. The values in the PBS controls after 120 min were set to 1 and served as the calibrator for the assays.

#### Flow cytometric analysis of TF surface expression

Monocyte TF expression was analysed using 12.5  $\mu$ l of whole blood stained with a fluorescein isothiocyanate (FITC)-conjugated anti-human TF antibody (product no. 4508CJ, clone VD8; American Diagnostica, Inc., Stamford, CT, USA) and a phycoerythrin (PE)-conjugated anti-CD14 antibody (Becton Dickinson, San Jose, CA, USA). Immunoglobulin (Ig)G1 FITC (BD 345815) was used as the isotype-matched control. After 15 min of incubation at room temperature, while protected from light, the blood was incubated with Easy Lyse (S2364; Dako Cytomation, Glostrup, Denmark) for 15 min at room temperature to lyse red cells. The leukocytes were then washed (PBS with 0.1% bovine serum albumin) and resuspended in PBS. The leukocytes were then acquired on a BD LSR II flow cytometer (Becton Dickinson) with the threshold on forward-scatter (FSC). Monocytes and granulocytes were gated in a PE/side-scatter (SSC) dot-plot, and the results were recorded as median fluorescence intensity (MFI).

#### Enzyme-linked immunosorbent assay (ELISA)

Coagulation cascade activation was measured as the concentration of prothrombin fragment 1.2 (PTF1.2) in plasma using an Enzygnost<sup>®</sup> F1+2 (monoclonal) kit from Dade Behring GmbH (Marburg, Germany). Human long PTX3 was analysed using a kit for detecting human PTX3/TSG-14 (from R&D Systems, Minneapolis, MN, USA). Activation of the terminal C5-C9 pathway was measured using the monoclonal antibody aE11, which is specific for a C9 neopeptide exposed in the sC5b-9 complex (TCC), as described previously [26]. Optical density was measured using an MRX microplate reader (Dynex Technologies, Denkendorf, Germany).

#### Cytokine assays

The Bio-Plex multiplex cytokine assay, human cytokine 27-plex panel from Bio-Rad Laboratories (Hercules, CA, USA) was used to measure cytokines including interleukin (IL)-1ra, IL-6, IL-8, IL-17, platelet-derived growth factor (PDGF) bb and tumour necrosis factor (TNF). The analysis was performed using the Bio-Plex 200 System (Bio-Rad).

#### TF functional activity in plasma microparticles

Whole blood samples were centrifuged as described above. The TF functional activity in plasma microparticles was analysed according to Engstad *et al.* [27]. In brief, microparticles were isolated by ultracentrifugation at 40 000 *g* for 90 min at 4°C from the previously isolated platelet-poor plasma. The microparticles were then resuspended in 200  $\mu$ l of 0.15 M NaCl and frozen at -70°C. Samples were then thawed and tested for TF activity using a two-stage amidolytic assay. The method is based on the ability of TF to accelerate the activation of FX by FVIIa, followed by the FXa-mediated conversion of prothrombin to thrombin in the presence of activated FV [27]. Crude rabbit brain extract was used as a standard for TF activity, with an undiluted activity assigned at 1000 mU/ml.

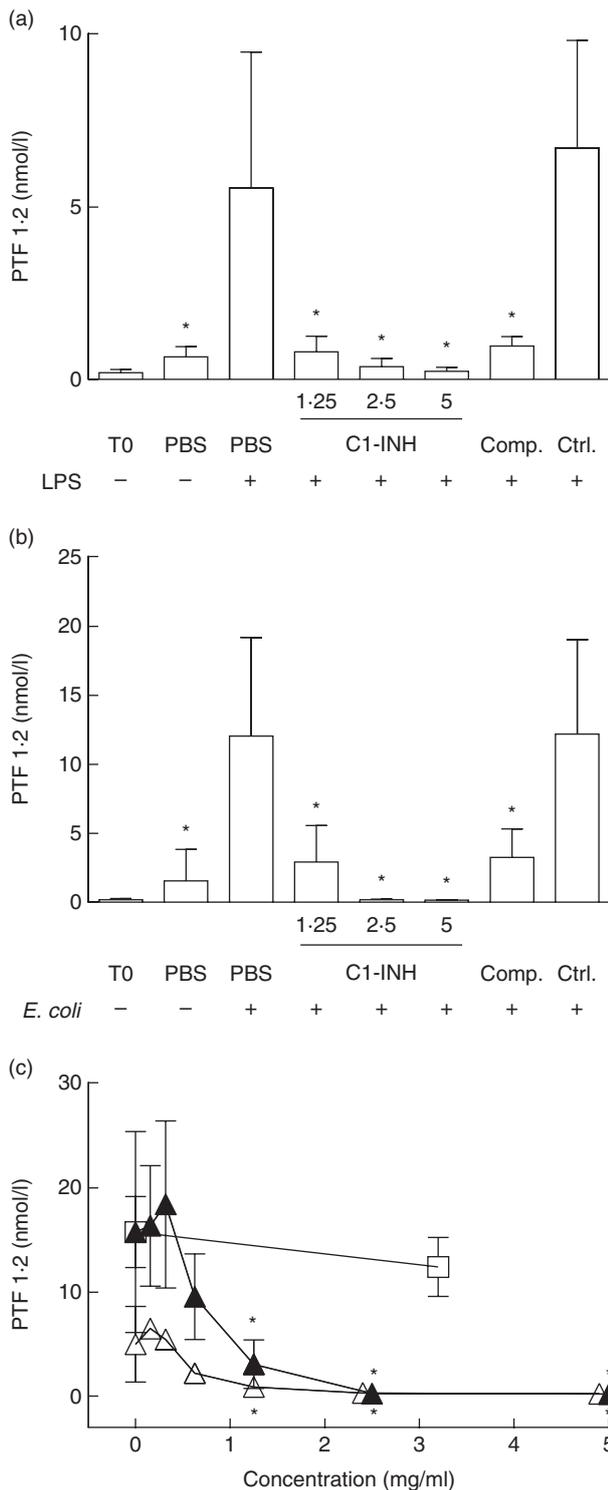
#### Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 from GraphPad Software (San Diego, CA, USA) using a one-way, repeated-measurements analysis of variance (ANOVA) and multiple comparisons. The results were analysed using *E. coli* or LPS as the control. The data were transformed logarithmically if the normality test failed.  $P < 0.05$  was considered the threshold for statistical significance.

#### Results

##### C1-INH inhibited LPS- and *E. coli*-induced coagulation cascade activation in human whole blood

The effect of C1-INH on LPS and *E. coli*-induced coagulation cascade activation was examined by analysing PTF1.2 levels in the plasma. The PTF1.2 levels increased only slightly after 120 min of incubation with PBS, from 0.19 nmol/l in the baseline sample T0 to 0.65 nmol/l in the spontaneous control (Fig. 1a). Incubating whole blood with LPS (100 ng/ml) enhanced the PTF1.2 levels 8.5-fold, from 0.65 nmol/l at baseline to 5.6 nmol/l after 120 min (Fig. 1a). C1-INH reduced the LPS-induced coagulation cascade activation dose-dependently and significantly ( $P < 0.05$ ) to baseline levels (i.e. T0). The C3 convertase inhibitor compstatin also inhibited the LPS-induced coagulation cascade



**Fig. 1.** C1-inhibitor (C1-INH) reduced efficiently lipopolysaccharide (LPS)- and *Escherichia coli* (*E. coli*)-induced coagulation activation measured as prothrombin factor 1.2 (PTF1.2) in human whole blood. (a) LPS (final concentration 100 ng/ml) and (b) *E. coli* (final concentration  $1 \times 10^7$ /ml) were added to whole blood and incubated for 120 min. Prior to incubation, phosphate-buffered saline (PBS), C1-INH (1.25, 2.5 and 5.0 mg/ml), the complement C3 inhibitor compstatin (Comp.) (20  $\mu$ M) or a control peptide (Ctrl.) (20  $\mu$ M) were added. PTF1.2 was analysed in plasma by enzyme-linked immunosorbent assay (ELISA) and expressed as nmol/l. T0 is the baseline sample at time zero. Values in (a) and (b) are means  $\pm$  standard deviation (s.d.) ( $n = 6$ ) of separate experiments with different donors. (c) Dose-response effect of C1-INH on *E. coli* (closed triangles) and LPS (open triangles) induced PTF1.2 levels after 120 min incubation ( $n = 2-8$ ). The effect of albumin (at the same molar concentration corresponding to C1-INH: 5 mg/ml) on *E. coli*-induced PTF1.2 is also indicated (open square). \* $P < 0.05$  compared to LPS or *E. coli* plus PBS.

and significantly ( $P < 0.05$ ) to baseline levels. Similarly, compstatin reduced coagulation cascade activation significantly ( $P < 0.05$ ) to almost the level of the spontaneous PBS control, consistent with the LPS data described above. C1-INH reduced LPS- and *E. coli*-induced coagulation activation dose-dependently (Fig. 1c), while the addition of HSA at the same molar concentration as C1-INH (5 mg/ml) had no effect.

### C1-INH inhibited LPS- and *E. coli*-induced TF mRNA up-regulation

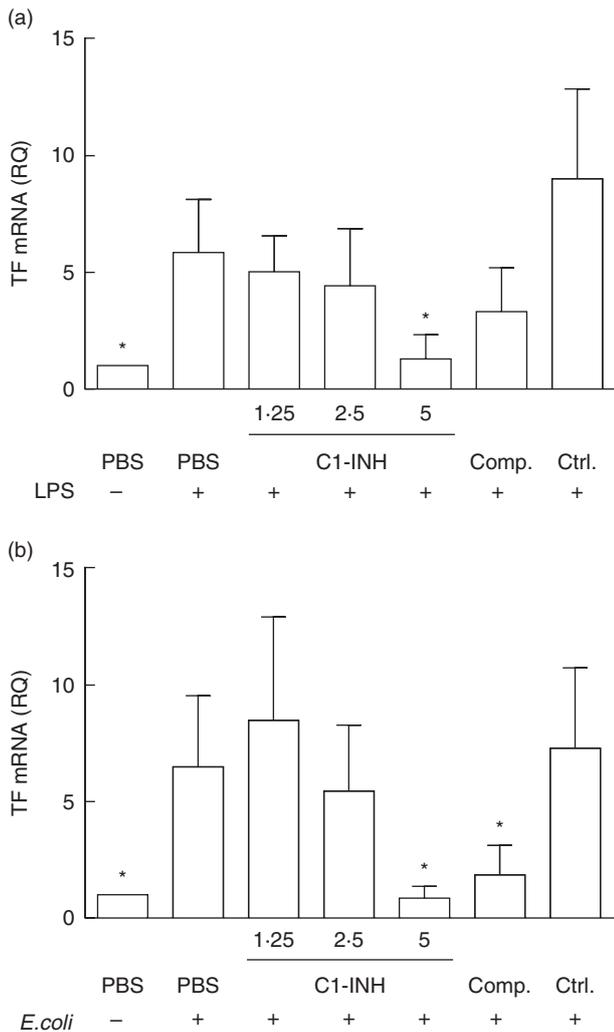
The regulation of TF at the mRNA level was examined using RT-qPCR. The TF mRNA levels were expressed as a relative quantity (RQ) in comparison to the TF mRNA level in the PBS controls after 120 min incubation, which were set to 1. Incubation with LPS (100 ng/ml) for 120 min enhanced TF mRNA levels from 1 RQ to 5.9 RQ (Fig. 2a). Incubation with *E. coli* ( $1 \times 10^7$ /ml) for 120 min enhanced TF mRNA levels from 1 RQ to 6.5 RQ (Fig. 2b). C1-INH reduced LPS- and *E. coli*-induced TF mRNA up-regulation dose-dependently and significantly ( $P < 0.05$ ) to the level of the spontaneous PBS control after 120 min of incubation (Fig. 2a,b). In comparison, compstatin reduced LPS-induced TF up-regulation non-significantly, while the effect on *E. coli*-induced TF mRNA up-regulation was significant.

### C1-INH inhibits LPS- and *E. coli*-induced TF cell surface expression on monocytes in whole blood and TF functional activity in plasma microparticles

The incubation time (120 min) was chosen from initial time-course studies on LPS- and *E. coli*-induced TF cell surface expression on monocytes (data not shown). The surface expression of TF on monocytes increased only slightly in the spontaneous control, increasing from an MFI of approximately 43 in the baseline sample T0 to an MFI of

activation extensively and significantly ( $P < 0.05$ ) to the level of the spontaneous PBS control.

Incubation with *E. coli* ( $1 \times 10^7$ /ml) enhanced PTF1.2 levels significantly by 8.1-fold, from 1.5 nmol/l in the spontaneous PBS control to 12.1 nmol/l after treatment for 120 min (Fig. 1b). Treatment with C1-INH reduced *E. coli*-induced coagulation cascade activation dose-dependently



**Fig. 2.** C1-inhibitor (C1-INH) reduced efficiently lipopolysaccharide (LPS)- and *Escherichia coli* (*E. coli*)-induced tissue factor (TF) mRNA up-regulation. (a) LPS (100 ng/ml) and (b) *E. coli* ( $1 \times 10^7$ /ml) were added to human whole blood and incubated for 120 min. TF mRNA was analysed using real-time reverse transcription–quantitative polymerase chain reaction (RT–qPCR) of whole blood total RNA. The TF mRNA level is expressed as relative quantity (RQ) to the TF mRNA level in the phosphate-buffered saline (PBS) sample, which was set to 1. The concentrations of inhibitors and controls are indicated in the legend to Fig. 1. Values are given as means  $\pm$  standard deviation (s.d.) ( $n = 6$ ) from separate experiments using different blood donors. \* $P < 0.05$  compared to LPS or *E. coli* plus PBS.

88 after 120 min of incubation with PBS (Fig. 3a). Incubation with LPS (100 ng/ml) for 120 min enhanced the monocyte cell surface expression of TF significantly ( $P < 0.05$ ), from 88 MFI to 275 MFI (Fig. 3a). C1-INH (2.5 and 5.0 mg/ml) reduced LPS-induced monocyte surface TF expression significantly ( $P < 0.05$ ) to almost baseline levels (Fig. 3a). Incubation with *E. coli* ( $1 \times 10^7$ /ml) for 120 min enhanced ( $P < 0.05$ ) cell surface expression of TF significantly on monocytes, from 200 MFI in the spontaneous

control to 1152 MFI (Fig. 3b). Furthermore, C1-INH reduced *E. coli*-induced cell surface expression of TF on monocytes dose-dependently and significantly ( $P < 0.05$ ) to spontaneous levels (Fig. 3b). Compstatin reduced both LPS- and *E. coli*-induced cell surface expression of TF significantly ( $P < 0.05$ ), but to a lesser degree than C1-INH.

TF functional activity in plasma microparticles increased only slightly following 120 min of incubation with PBS, from 0.04 mU/ml in the baseline sample T0 to 0.06 mU/ml in the spontaneous control (Fig. 3c). Incubation with LPS (100 ng/ml) for 120 min enhanced the TF functional activity significantly ( $P < 0.05$ ) in plasma microparticles, from 0.06 at baseline to 0.47 mU/ml (Fig. 3c). C1-INH reduced LPS-induced TF activity in plasma microparticles dose-dependently and significantly ( $P < 0.05$ ) to the level of the spontaneous control (Fig. 3c). Incubation with *E. coli* ( $1 \times 10^7$ /ml) for 120 min increased TF functional activity significantly ( $P < 0.05$ ), from approximately 0.1 in the spontaneous control to 0.81 mU/ml (Fig. 3d). C1-INH at 5 mg/ml also reduced *E. coli*-induced TF activity significantly ( $P < 0.05$ ) to the level of the spontaneous control (Fig. 3d). In comparison, compstatin reduced the TF functional activity in plasma microparticles to 0.30 mU/ml, but the results were not significant.

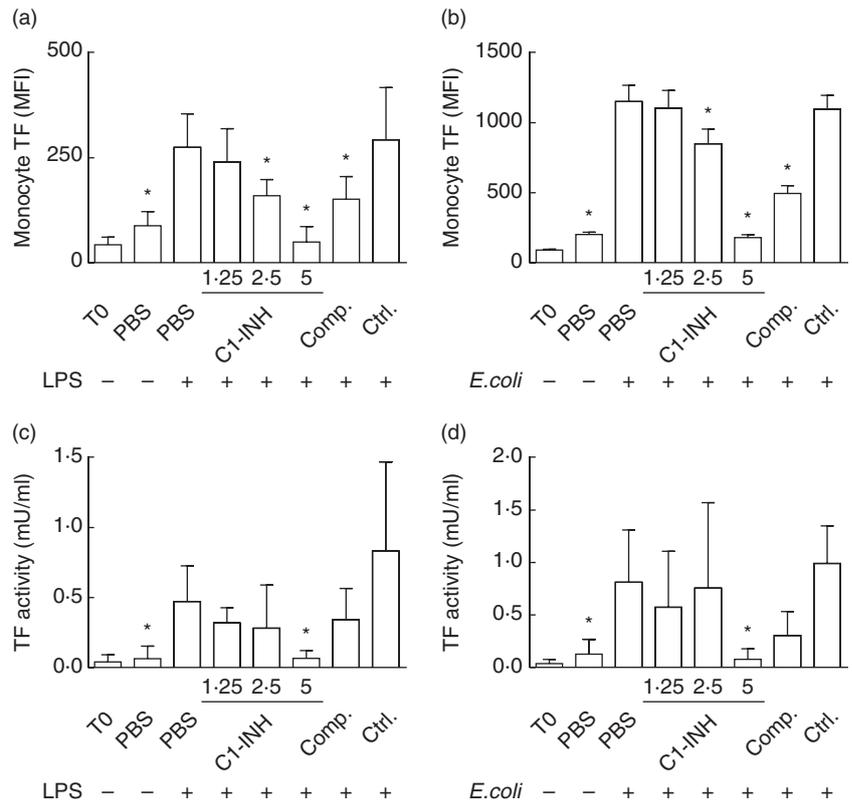
#### C1-INH reduced LPS- and *E. coli*-induced PTX3 release

PTX3 levels increased spontaneously from approximately 0.3 ng/ml in the baseline sample T0 to 3–4 ng/ml in the PBS control after 120 min of incubation (Fig. 4a,b). Incubation with LPS (100 ng/ml) for 120 min increased the PTX3 levels further to 7.5 ng/ml (Fig. 4a). Incubation with *E. coli* ( $1 \times 10^7$ /ml) increased the PTX3 levels to 6.8 ng/ml (Fig. 4b). C1-INH reduced LPS- and *E. coli*-induced PTX3 release dose-dependently and significantly ( $P < 0.05$ ). The highest concentration of C1-INH (5.0 mg/ml) reduced the PTX3 concentrations to below the spontaneous PBS control and almost down to the baseline sample T0. In comparison, compstatin reduced LPS- and *E. coli*-induced PTX3 release significantly ( $P < 0.05$ ) to the level of the spontaneous control.

#### C1-INH reduced the LPS- and *E. coli*-induced release of cytokines

The effect of C1-INH on LPS- and *E. coli*-induced cytokine release was studied. LPS enhanced IL-6 and IL-8 release from 21 to 1997 and 229 to 1890 pg/ml, respectively, after 120 min (Fig. 5a,c). LPS enhanced the TNF release from 216 to 6807 pg/ml. In comparison, *E. coli* enhanced IL-6 and IL-8 release from 844 to 3490 and 348 to 4488 pg/ml, respectively, after 120 min (Fig. 5b,d). *E. coli* enhanced the release of TNF from 137 to 17 956 pg/ml (Fig. 5f). C1-INH reduced the LPS- and *E. coli*-induced IL-6, IL-8 and TNF release significantly. In comparison, compstatin reduced

**Fig. 3.** C1-inhibitor (C1-INH) reduced efficiently lipopolysaccharide (LPS)- and *Escherichia coli* (*E. coli*)-induced tissue factor (TF) surface expression on whole blood monocytes. (a,c) LPS (100 ng/ml) or (b,d) *E. coli* ( $1 \times 10^7$ /ml) was added to human whole blood and incubated for 120 min. (a,b) TF expression on monocytes was analysed using flow cytometry and expressed as median fluorescence intensity (MFI). (c,d) TF functional activity was analysed in plasma microparticles using a chromogenic method and expressed as mU/ml. The concentrations of inhibitors and controls are indicated in the legend to Fig. 1. Values are given as means  $\pm$  standard deviation (s.d.) ( $n = 6$ ) from single experiments with different blood donors. \* $P < 0.05$  compared to LPS or *E. coli* plus phosphate-buffered saline (PBS).



only IL-8 release. The control protein HSA (3.2 mg/ml) had no effect (data not shown). IL-17 levels increased spontaneously from 16 pg/ml in the baseline sample T0 to 34 pg/ml in the PBS control after 120 min of incubation (Fig. 6b). *E. coli* enhanced the release of the proinflammatory cytokine IL-17 to 74 pg/ml (Fig. 6b). C1-INH reduced *E. coli*-induced IL-17 dose-dependently, and the effect at 5 mg/ml was statistically significant ( $P < 0.05$ ). In comparison, treatment with compstatin reduced the IL-17 levels slightly and non-significantly. LPS did not enhance IL-17 levels significantly (Fig. 6a).

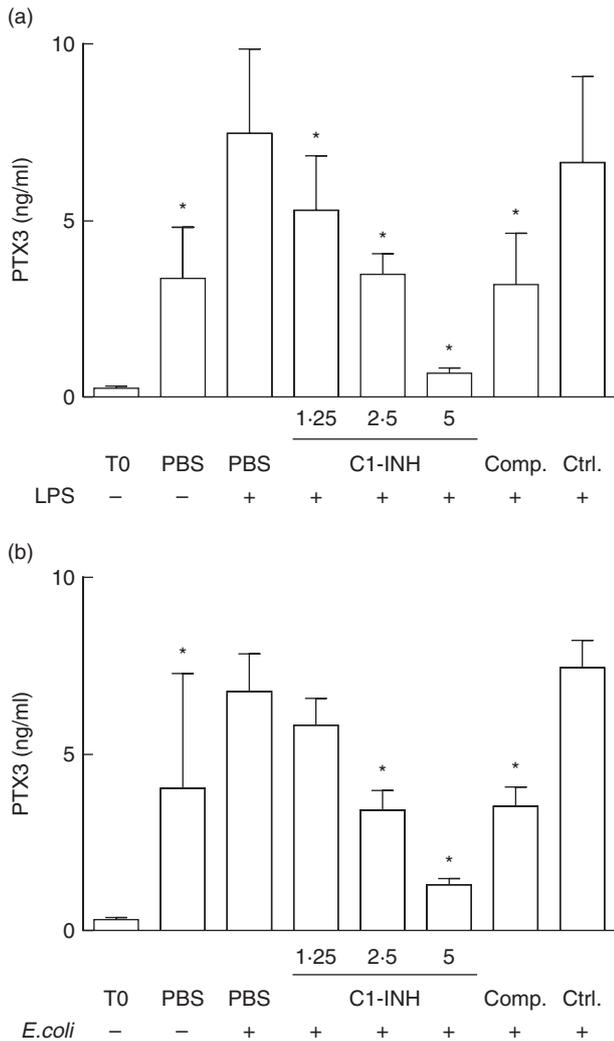
The levels of the anti-inflammatory cytokine IL-1ra increased from 59 pg/ml in the baseline sample T0 to 232 pg/ml in the PBS control after 120 min of incubation (Fig. 6c). LPS and *E. coli* enhanced the IL-1ra levels to 706 and 670 pg/ml, respectively (Fig. 6c,d). Incubation with C1-INH at 5 mg/ml reduced both LPS- and *E. coli*-induced IL-1ra release significantly ( $P < 0.05$ ), while compstatin had no effect.

PDGF bb is released from  $\alpha$ -granules in activated platelets. PDGF bb levels increased from 308 pg/ml in the baseline sample T0 to 1717 pg/ml in the PBS control after 120 min of incubation (Fig. 6f). Incubation with *E. coli* enhanced PDGF bb release to 2880 pg/ml (Fig. 6f). Treatment with C1-INH at concentrations of 2.5 and 5.0 mg/ml reduced *E. coli*-induced PDGF bb release dose-dependently and significantly ( $P < 0.05$ ), whereas compstatin did not reduce PDGF bb release.

### The effects of inactivated C1-INH on LPS- and *E. coli*-induced PTF1-2, TF mRNA, TF expression on monocytes and IL-8 release

We then examined whether inactivated C1-INH (iC1-INH) could influence LPS (100 ng/ml)- and *E. coli* ( $1 \times 10^7$ /ml)-induced coagulation cascade activation, measured through PTF1-2 levels. iC1-INH reduced LPS-induced PTF1-2 levels markedly from 20.5 to 3.4 nmol/l, similar to C1-INH, although this result was not statistically significant (Fig. 7a). iC1-INH reduced *E. coli*-induced PTF1-2 levels significantly (Fig. 7b). In contrast, iC1-INH enhanced LPS- and *E. coli*-induced TF mRNA levels (Fig. 7c,d). Similarly, iC1-INH treatment increased LPS-induced TF surface expression, while *E. coli*-induced surface expression was unaffected (Fig. 7e,f). In contrast, C1-INH reduced LPS- and *E. coli*-induced TF mRNA up-regulation and TF surface expression levels significantly, consistent with the previous data (Fig. 7c-f).

LPS (100 ng/ml) enhanced IL-8 release, from 309 pg/ml in the spontaneous PBS control to 1933 pg/ml after 120 min of incubation (Fig. 7g). Similarly, *E. coli* enhanced IL-8 release to 3784 pg/ml after 120 min of incubation (Fig. 7h). iC1-INH enhanced LPS-induced IL-8 release significantly ( $P < 0.05$ ), while no effect was found for the *E. coli* treatment. C1-INH reduced significantly ( $P < 0.05$ ) both LPS- and *E. coli*-induced IL-8 release to 290 and 942 pg/ml, respectively (Fig. 7g,h). However, iC1-INH (5 mg/ml)



**Fig. 4.** C1-inhibitor (C1-INH) reduced efficiently lipopolysaccharide (LPS)- and *Escherichia coli* (*E. coli*)-induced long pentraxin 3 (PTX3) release. (a) LPS (100 ng/ml) and (b) *E. coli* ( $1 \times 10^7$ /ml) were added to human whole blood and incubated for 120 min. The concentrations of inhibitors and controls are indicated in the legend to Fig. 1. PTX3 in plasma was analysed using enzyme-linked immunosorbent assay (ELISA), and the results are expressed as ng/ml. Results are given as means  $\pm$  standard deviation (s.d.) ( $n = 6$ ) from separate experiments with each blood donor. \* $P < 0.05$  compared to LPS or *E. coli* plus phosphate-buffered saline (PBS).

reduced the LPS- and *E. coli*-induced release of several of the other cytokines analysed non-significantly (data not shown). In these experiments, we used PBS as control (bar to the left Fig. 7a–h), as we did not have access to a buffer control treated in the same way as when C1-INH was used to make iC1-INH.

#### C1-INH reduced *E. coli*-induced complement cascade activation

Finally, we examined *E. coli*-induced complement cascade activation in the fluid phase. TCC levels increased from

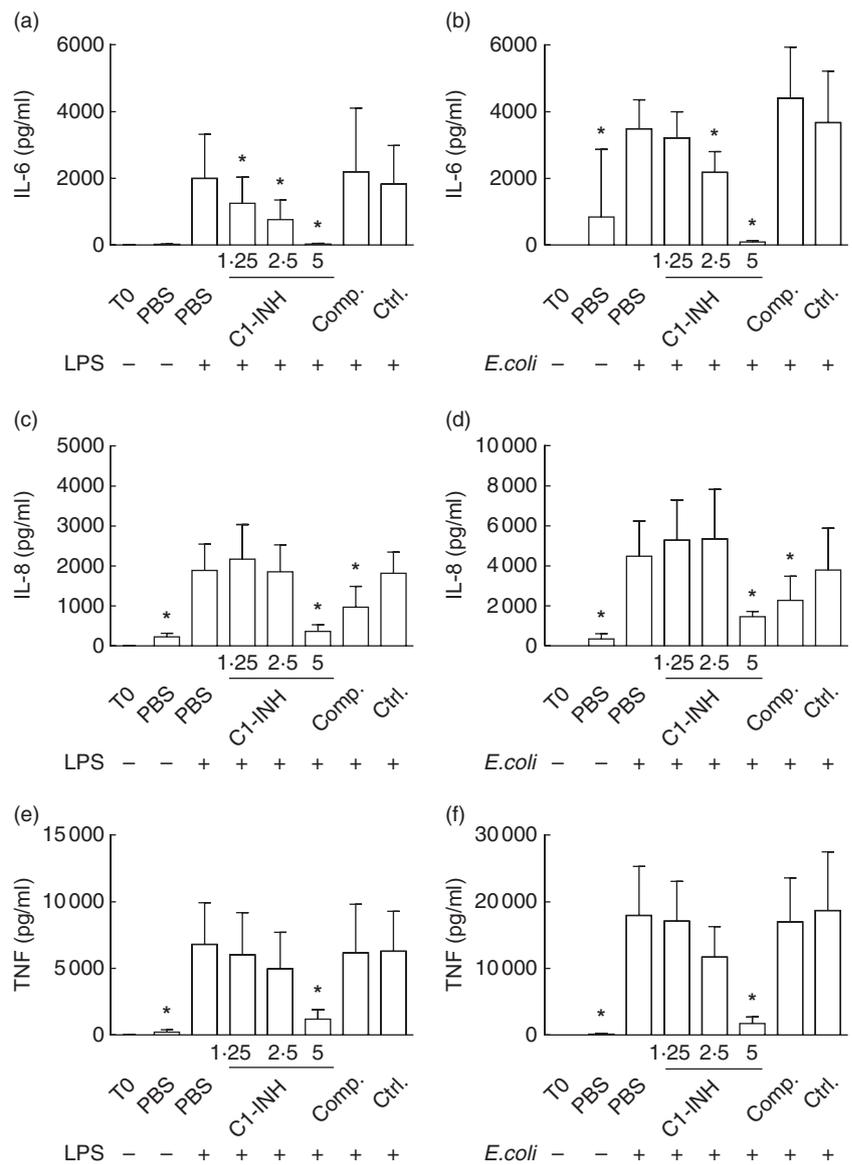
0.3 AU/ml in the baseline sample T0 to 6.3 AU/ml in the PBS control after 120 min of incubation (Fig. 8). *E. coli* ( $1 \times 10^7$ /ml) enhanced complement cascade activation significantly ( $P < 0.05$ ), measured as TCC levels, to 17 AU/ml (Fig. 8). C1-INH reduced *E. coli*-induced TCC levels significantly ( $P < 0.05$ ) and dose-dependently to 4.5 AU/ml, approximately the same levels as in the spontaneous PBS control. As expected, the complement inhibitor compstatin abolished TCC formation ( $P < 0.05$ ) efficiently, confirming the efficient effect of compstatin to inhibit complement compared to C1-INH. In comparison, iC1-INH (5 mg/ml) enhanced the *E. coli*-induced TCC levels to 34 AU/ml.

#### Discussion

This study found that supraphysiological concentrations of C1-INH inhibited efficiently LPS and *E. coli*-induced TF mRNA up-regulation and surface expression on monocytes as well as the release of PTF1-2, PTX3 and several cytokines. Several similar effects of the C3 convertase inhibitor compstatin could imply that many of the effects of C1-INH could be explained by complement inhibition. Interestingly, however, activities of C1-INH independent of protease-binding were responsible for several of its effects in this and a previous study [24]. These findings are consistent with a number of recent studies highlighting C1-INH as a multifunctional protein with modulating effects also on, e.g. LPS and P-selectin [28]. Notably, following a challenge by LPS or *E. coli*, C1-INH is not a particularly efficient complement inhibitor compared to compstatin [24].

The dose–response data indicate that C1-INH reduced LPS- and *E. coli*-induced coagulation activation dose-dependently, and C1-INH at 1.25 mg/ml reduced LPS-induced PTF1-2 levels efficiently by 97%. We used PTF1-2 levels to evaluate coagulation activation. This is because the use of lepirudin as an anti-coagulant in the collected samples inhibits thrombin and the formation of thrombin–anti-thrombin complexes (TAT) selectively. The slightly increased PTF1-2 levels in the spontaneous control compared to the baseline sample (T0) after 120 min were due most probably to coagulation activation by the plastic surface *in vitro*. Activation of FXII and the contact and complement systems following contact between blood and polypropylene plastic in the test tube has been well documented [29]. In our experiments, the LPS- and *E. coli*-induced coagulation activation was due most probably to the up-regulation of TF. However, TF-induced coagulation activation can be modulated by post-translational modifications of TF [30] and phosphatidylserine expression [31], highlighting the complex regulation of TF-induced coagulation system activation.

Incubation with LPS and *E. coli* enhanced TF mRNA up-regulation and levels of TF surface expression rapidly on monocytes. TF expression is important for the development of disseminated intravascular coagulation (DIC) in sepsis.

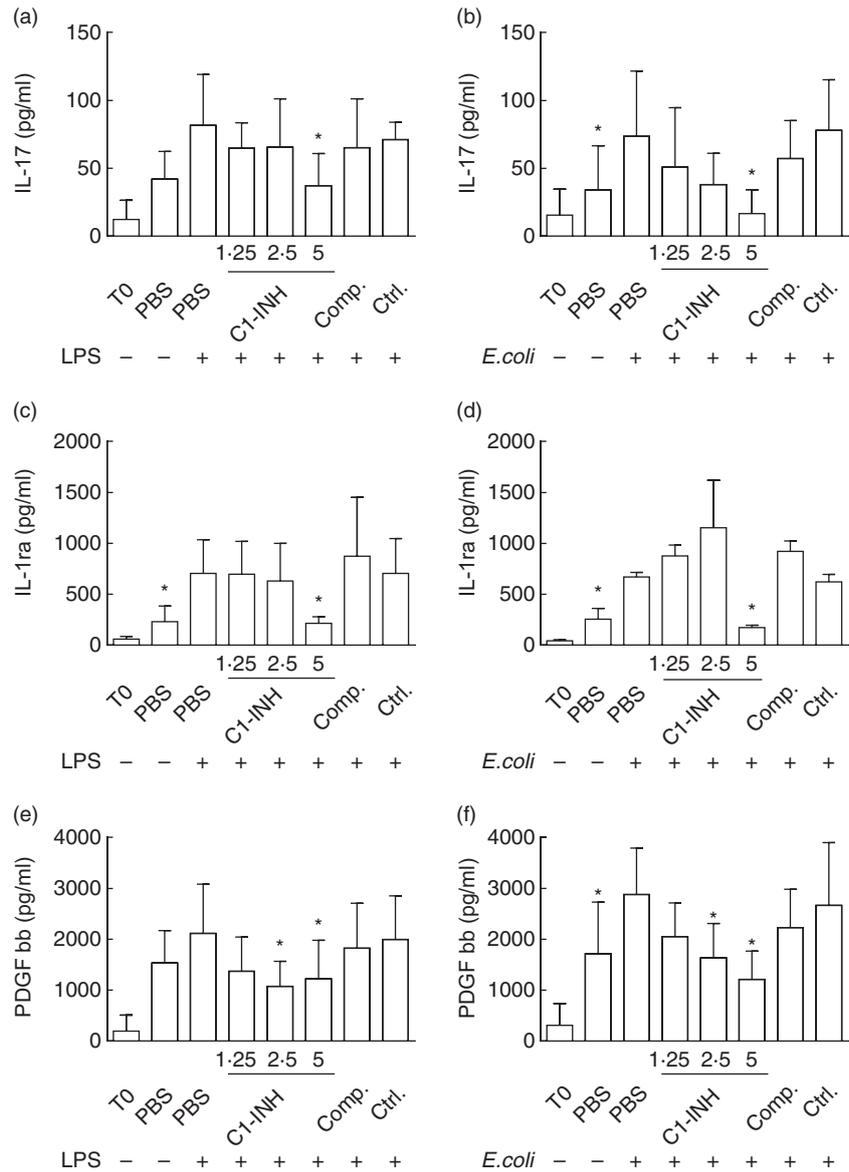


**Fig. 5.** C1-inhibitor (C1-INH) reduced efficiently lipopolysaccharide (LPS)- and *Escherichia coli* (*E. coli*)-induced interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF) release. (a,c,e) LPS (100 ng/ml) and (b,d,f) *E. coli* ( $1 \times 10^7$ /ml) were added to human whole blood and incubated for 120 min. The concentrations of inhibitors and controls are indicated in the legend to Fig. 1. The concentrations of IL-6, IL-8 and TNF in the samples were measured using a multiplex cytokine assay. The results are expressed as pg/ml. Results are given as means  $\pm$  standard deviation (s.d.) ( $n = 6$ ) from separate experiments with different blood donors. \* $P < 0.05$  compared to LPS or *E. coli* plus phosphate-buffered saline (PBS).

DIC increases tissue damage and may lead to organ failure and death. A treatment that reduces TF mRNA up-regulation could therefore be of great benefit in treating sepsis [32]. This study showed how the C3 convertase inhibitor compstatin and C1-INH reduced *E. coli*-induced TF mRNA up-regulation and TF surface expression on monocytes efficiently. Compstatin reduced LPS-induced TF mRNA up-regulation non-significantly, suggesting that the LPS-induced TF mRNA up-regulation was not complement-dependent. However, compstatin reduced *E. coli*-induced TF mRNA up-regulation efficiently after 120 min. We have no explanation for this difference, but we speculate that it may be related to inhibitory effects of compstatin on the time-dependent phagocytosis of *E. coli* bacteria. Thus, our data suggest that the *E. coli*-induced TF mRNA up-regulation is mainly complement-dependent. Surprisingly, our study showed that compstatin reduced

LPS-induced TF expression on monocytes. However, the LPS-induced complement cascade activation is very weak (data not shown). Both C1-INH and compstatin reduced monocyte TF expression significantly, suggesting that TF expression on monocytes is at least partly complement-dependent. However, C1-INH reduced TF expression on monocytes down to the level in the spontaneous control, suggesting that other mechanisms in addition to complement were also involved.

C1-INH reduced LPS- and *E. coli*-induced TF functional activity significantly in plasma microparticles, while the effects of compstatin were not statistically significant. The plasma microparticles are derived most probably from activated platelets or apoptotic monocytes [33]. These results indicate that LPS- and *E. coli*-induced TF functional activity in plasma microparticles is not complement-dependent. PDGF bb is stored in the  $\alpha$ -granules of platelets. The obser-



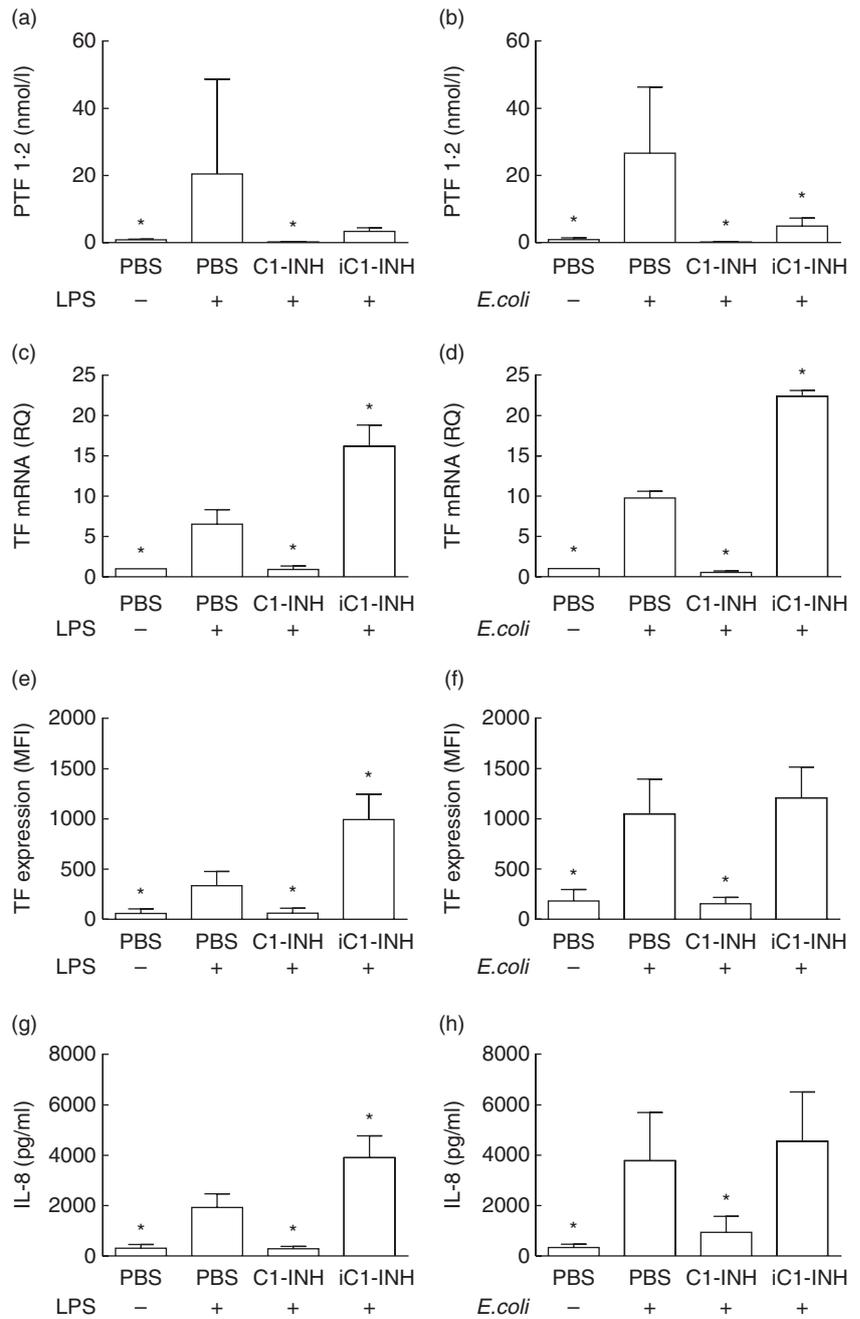
**Fig. 6.** Effect of C1-inhibitor (C1-INH) on lipopolysaccharide (LPS)- and *Escherichia coli* (*E. coli*)-induced interleukin (IL)-17, IL-1ra and platelet-derived growth factor (PDGF) bb release. (a,c,e) LPS (100 ng/ml) and (b, d, f) *E. coli* ( $1 \times 10^7$ /ml) were added to human whole blood and incubated for 120 min. The concentrations of inhibitors and controls are indicated in the legend to Fig. 1. The concentrations of cytokines in the samples were measured using a multiplex cytokine assay measuring 27 cytokines. The results are expressed as pg/ml. Results are given as means  $\pm$  standard deviation (s.d.) ( $n = 6$ ) from separate experiments with each blood donors. \* $P < 0.05$  compared to LPS or *E. coli* plus phosphate-buffered saline (PBS).

vation that C1-INH inhibited *E. coli*-induced PDGF bb levels suggests that C1-INH also affected the activation of platelets. The effect of C1-INH on TF functional activity in plasma microparticles may be due in part to inhibitory effects on platelet activation [34], but this hypothesis remains to be tested.

PTX3 has been reported previously to enhance LPS-induced TF mRNA up-regulation in monocytes [6]. Our data indicated that both LPS and *E. coli* stimulation increased PTX3 release. C1-INH and compstatin reduced LPS- and *E. coli*-induced PTX3 release. Mannose-binding lectin (MBL) binds to pathogen-associated molecular patterns, including LPS. Thereafter, PTX3 may bind to MBL and enhance complement activation if C1q is present [35]. Compstatin reduced both LPS- and *E. coli*-induced PTX3 release to the levels of the spontaneous control, indicating

that the PTX3 release is complement-dependent. The spontaneous increase in PTX3 and IL-7 release after 120 min is due probably to plastic-induced complement activation and activation of immune competent cells due to contact with the plastic surface in the tubes, implicating a limitation in our model. C1-INH reduced the PTX3 release further down to the levels of the baseline sample, indicating that its effects may be due in part to a significant reduction of LPS- and *E. coli*-induced cytokines, such as IL-1 $\beta$  and TNF, both of which stimulate PTX3 release [36].

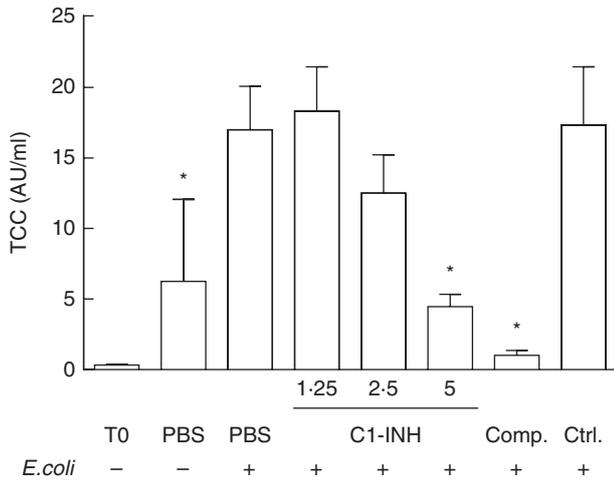
Cytokines are important mediators of inflammation and TF up-regulation [5]. IL-17 is an important component of the proinflammatory response in sepsis through its induction of cytokines and recruiting of neutrophils to the infection site [37]. IL-17 may also play a role in monocyte TF production [38]. This report confirmed that *E. coli* stimu-



**Fig. 7.** Effect of inactivated C1-inhibitor (iC1-INH) on lipopolysaccharide (LPS)- and *Escherichia coli* (*E. coli*)-induced prothrombin factor 1.2 (PTF1-2), tissue factor (TF) mRNA up-regulation, TF expression on monocytes and interleukin (IL)-8 release. (a,c,e,g) LPS (100 ng/ml) and (b,d,f,h) *E. coli* ( $1 \times 10^7$ /ml) were added to human whole blood and incubated for 120 min. C1-INH and iC1-INH were added at final concentrations of 5 mg/ml. (a,b) PTF1-2 in plasma was analysed using enzyme-linked immunosorbent assay (ELISA) and expressed as nmol/l. (c,d) TF mRNA up-regulation was analysed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and expressed as relative quantity (RQ) to the TF mRNA level in the phosphate-buffered saline (PBS) sample after 120 min, which was set to 1. (e,f) TF surface expression on monocytes was analysed by flow cytometry and given as median fluorescence intensity (MFI). (g,h) IL-8 in plasma was analysed using multiplex ELISA and given as pg/ml. Results are given as means  $\pm$  standard deviation (s.d.) ( $n = 3-7$ ) from separate experiments with each blood donor. \* $P < 0.05$  compared to LPS or *E. coli* plus PBS.

lated IL-17 release. However, our data indicated that the IL-17 release was reduced only by the highest C1-INH concentration, suggesting that the inhibitory effects of C1-INH on TF were explained only partly by its effects on IL-17 release. IL-17 release was not complement-dependent, as compstatin had no effect. This finding indicates that the effects of compstatin on TF are not mediated by reduced IL-17 release. Complement inhibition has been reported to increase the levels of the anti-inflammatory cytokine IL-1ra [12]. The inhibitory effect of C1-INH treatment on IL-1ra levels and the slightly enhanced IL-1ra levels found after incubation with compstatin confirmed that C1-INH and

compstatin affect cytokine release differentially. We have no explanation for this different effect on IL-1ra release. However, we speculate that the compstatin-induced IL-1ra release may be involved in the anti-inflammatory effect of compstatin. We analysed several other cytokines, including IL-6, IL-8 and TNF, which have been reported to increase monocyte TF surface expression [5,6]. C1-INH reduced almost all analysed cytokines, while compstatin reduced only LPS- and *E. coli*-induced IL-8 release, in line with previous findings [39]. The efficient reduction of cytokine release by C1-INH could, potentially, explain its more pronounced inhibition of TF surface expression on monocytes



**Fig. 8.** C1-inhibitor (C1-INH) reduced *Escherichia coli* (*E. coli*)-induced complement activation in human whole blood, but less efficiently than compstatin. *E. coli* ( $1 \times 10^7$ /ml) was added to human whole blood and incubated for 120 min. The concentrations of inhibitors and controls are indicated in the legend to Fig. 1. Complement activation measured as the terminal complement complex (TCC) in plasma was analysed by enzyme-linked immunosorbent assay (ELISA). The results are expressed as AU/ml. Values are given as means  $\pm$  standard deviation (s.d.) ( $n = 6$ ) from single experiments with different blood donors. \* $P < 0.05$  compared to *E. coli* plus phosphate-buffered saline (PBS).

when compared with compstatin. C1-INH reduced *E. coli*-induced cytokine release efficiently in human whole blood using live *E. coli* bacteria [24], in line with this study using heat-inactivated bacteria. The effect of C1-INH on heat-inactivated *E. coli*-induced cytokine release and other parameters analysed was somewhat more pronounced compared to live bacteria [24]. We have no explanation for these differences, but speculate that it may be due to the higher bacterial load used in the previous study, as live bacteria may multiply before the start of the experiment. However, iC1-INH at 5 mg/ml could not reduce LPS- or *E. coli*-induced IL-8 release, or that of any of the other analysed cytokines (data not shown). We speculate that the slight increase in IL-8 release was due to the competition of iC1-INH with the normal C1-INH present in plasma.

Compstatin, in contrast to C1-INH and iC1-INH, reduced *E. coli*-induced complement activation efficiently measured as TCC levels. We have shown previously that iC1-INH activates the classical pathway of complement and increased IL-8 release [24]. In several other experiments we have seen that IL-8 is largely complement-dependent. The IL-8 increase we observed when adding iC1-INH may therefore be generated via a classical pathway complement activation. We speculate that this enhancement of complement activation by iC1-INH may explain the increase in LPS- and *E. coli*-induced TF mRNA levels observed in this study. The reason for classical pathway activation by iC1-INH remains to be examined in detail. As suggested by Zic-

cardi, native C1-INH prevents C1r autoactivation [40]. C1-INH levels lower than approximately 25% allow C1r to autoactivate and to start activating C1s [40]. Ziccardi also believed that native C1-INH inhibited C1r-autoactivation without forming a covalent bond with C1r. Davis *et al.*, however, asked if native C1-INH rather controlled C1r autoactivation by forming a covalent complex with tiny amounts of spontaneously generated C1r [18], thus blocking an amplification loop. If so, we speculated that a large surplus of iC1-INH, containing 93% of the amino acids in native C1-INH, could displace the loosely associated native C1-INH from the C1 complex, allowing C1r to autoactivate.

The effect of C1-INH may, potentially, be explained by several mechanisms. First, the effect of C1-INH at high concentrations may be due to the non-protease inhibitory effects of C1-INH, as C1-INH can bind directly to Gram-negative bacteria [18]. In our study, iC1-INH could not reduce LPS- or *E. coli*-induced TF mRNA up-regulation or TF surface expression on monocytes, indicating that TF was inhibited via the protease inhibitory function of C1-INH. C1-INH and iC1-INH have been shown to directly bind LPS and inhibit LPS-induced Toll-like receptor (TLR)-4 activation on immune cells [41]. Because treatment with iC1-INH did not reduce LPS-induced TF mRNA levels, the almost complete inhibition of LPS-induced TF mRNA up-regulation by C1-INH that we observed was most probably via its anti-protease effects. LPS is known to bind to CD14, myeloid differentiation factor 2 (MD2) and TLR-4, the main receptor for LPS [42]. One possible mechanism could be that C1-INH inhibits the recently reported MASP-1-induced coagulation activation [43]. The inhibitory effect of C1-INH on lectin-pathway activation [16] may therefore reduce coagulation activation, and this could possibly explain why C1-INH was a more efficient inhibitor than compstatin.

Supraphysiological concentrations of C1-INH at or above 1.25 mg/ml can be achieved only in experimental models or after intravenous or intra-arterial injection of C1-INH [21,44,45]. In one study, the levels of C1-INH after intravenous infusion reached a level equivalent to three times the physiological concentration [46]. The C1-INH concentration of 1.25 mg/ml in our study is equivalent to approximately five times the physiological concentration. Regional intra-arterial administration of C1-INH directly into organs, however, could potentially reach much higher localized levels of C1-INH than intravenous administration and reach higher concentrations in the organs than those used in our study. Therefore, the high C1-INH doses that we used are clinically relevant. Interestingly, a recent report showed that intravenous injection of C1-INH increased the survival of sepsis patients significantly [22]. This finding indicates that C1-INH may be a potential therapeutic medication in sepsis patients.

In conclusion, our study showed for the first time, to our knowledge, that C1-INH reduced LPS- and *E. coli*-induced

TF mRNA up-regulation, TF surface expression, coagulation activation and PTX3 release. The effects were partially dependent upon complement inhibition. Our findings emphasize the broad-spectrum, modulating effects of C1-INH treatment on many of the harmful mechanisms that contribute to the development of sepsis.

### Acknowledgements

This study was supported by grants from the Northern Norway Regional Health Authority and The Odd Fellow Foundation. This work was supported by National Institutes of Health Grants GM-62134 and AI-068730 to J.D. Lambris. We thank Dr. Hongchang Qu for synthesizing the synthetic peptides used in this study.

### Disclosure

The co-author, Professor J.D. Lambris, is the holder of several patent applications on complement inhibitors. The other authors declare that they have no competing financial or other interest in relation to their work.

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