

# PAPER III

# Complement component 5 does not interfere with physiological hemostasis but is essential for *Escherichia coli*-induced coagulation accompanied by Toll-like receptor 4

A. Landsem <sup>\*,†</sup>, H. Fure,<sup>\*</sup>  
J. Krey Ludviksen,<sup>\*</sup> D. Christiansen,<sup>\*</sup>  
C. Lau,<sup>\*</sup> M. Mathisen,<sup>\*</sup> G. Bergseth,<sup>\*</sup>  
S. Nymo,<sup>\*,‡</sup> K. T. Lappégard,<sup>†‡</sup>  
T. M. Woodruff,<sup>\*\*</sup> T. Espevik,<sup>††</sup>  
T. E. Mollnes<sup>\*,†§,††</sup> and O.-L. Brekke<sup>\*†</sup>  
<sup>\*</sup>Research Laboratory and Department of  
Laboratory Medicine, Nordland Hospital  
Trust, Bodø, <sup>†</sup>Department of Clinical  
Medicine, UiT – The Arctic University of  
Norway, Tromsø, <sup>‡</sup>Division of  
Medicine, Nordland Hospital Trust, Bodø,  
<sup>§</sup>K. G. Jebsen TREC, UiT – The Arctic  
University of Norway, Tromsø, <sup>¶</sup>Department  
of Immunology, Oslo University Hospital  
Rikshospitalet and University of Oslo, Norway,  
<sup>\*\*</sup>School of Biomedical Sciences, Faculty of  
Medicine, The University of Queensland,  
Brisbane, Australia, and <sup>††</sup>Centre of  
Molecular Inflammation Research, and  
Department of Cancer Research and  
Molecular Medicine, Norwegian University of  
Science and Technology, Trondheim, Norway

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Correspondence: A. Landsem, Research  
Laboratory and Department of Laboratory  
Medicine, Nordland Hospital Trust, mailbox  
1480, N-8092 Bodø, Norway.

E-mail: anne.landsem@nordlandssykehuset.no

## Introduction

Sepsis is still a major threat to human health, and the incidence is rising [1]. It is commonly associated with coagulation disturbances ranging from coagulation activation to disseminated intravascular coagulation [2]. Tissue factor (TF) is suggested to be a major initiator of bacteria-induced coagulation in human sepsis [3].

TF initiates the extrinsic coagulation pathway and results in thrombin activation and clot formation. We have previously demonstrated that the *Escherichia coli*-induced

## Summary

There is a close cross-talk between complement, Toll-like receptors (TLRs) and coagulation. The role of the central complement component 5 (C5) in physiological and pathophysiological hemostasis has not, however, been fully elucidated. This study examined the effects of C5 in normal hemostasis and in *Escherichia coli*-induced coagulation and tissue factor (TF) up-regulation. Fresh whole blood obtained from six healthy donors and one C5-deficient individual (C5D) was anti-coagulated with the thrombin inhibitor lepirudin. Blood was incubated with or without *E. coli* in the presence of the C5 inhibitor eculizumab, a blocking anti-CD14 monoclonal antibody (anti-CD14) or the TLR-4 inhibitor eritoran. C5D blood was reconstituted with purified human C5. TF mRNA was measured by quantitative polymerase chain reaction (qPCR) and monocyte TF and CD11b surface expression by flow cytometry. Prothrombin fragment 1+2 (PTF1-2) in plasma and microparticles exposing TF (TF-MP) was measured by enzyme-linked immunosorbent assay (ELISA). Coagulation kinetics were analyzed by rotational thromboelastometry and platelet function by PFA-200. Normal blood with eculizumab as well as C5D blood with or without reconstitution with C5 displayed completely normal biochemical hemostatic patterns. In contrast, *E. coli*-induced TF mRNA and TF-MP were significantly reduced by C5 inhibition. C5 inhibition combined with anti-CD14 or eritoran completely inhibited the *E. coli*-induced monocyte TF, TF-MP and plasma PTF1-2. Addition of C5a alone did not induce TF expression on monocytes. In conclusion, C5 showed no impact on physiological hemostasis, but substantially contributed to *E. coli*-induced procoagulant events, which were abolished by the combined inhibition of C5 and CD14 or TLR-4.

**Keywords:** CD14, complement C5, *Escherichia coli*, tissue factor, Toll-like receptor 4

coagulation in human whole blood measured by prothrombin fragment 1+2 (PTF1-2) is TF-dependent, using a blocking antibody against TF [4]. The *E. coli*-induced monocyte TF surface expression was efficiently inhibited by the combined inhibition of complement C3 and CD14 [4]. Furthermore, the *Neisseria meningitidis*-induced monocyte TF surface expression was largely complement component 5 (C5)-dependent [5].

The complement system is a complex plasma cascade and an important component of innate immunity. The system is activated via three routes, the classical,

alternative and lectin pathways, all leading to the activation of C5 and the terminal pathway [6]. The terminal complement C5b-9 complex (TCC) can either be deposited on a surface as the membrane attack complex (MAC) or remain in the fluid phase as soluble TCC (sC5b-9) [6]. Complement opsonizes bacteria, enhances phagocytosis and may induce MAC-mediated lysis of some complement-sensitive bacteria, in particular *Neisseria* species [7]. Activation of C5 also induces formation of C5a, which is an important proinflammatory mediator with a wide range of effects on host cells, particularly leukocytes [8].

There are many links between the complement and coagulation systems [9], but the role of C5 in normal coagulation kinetics and hemostasis is only partially investigated. One reason for this is that genetic deficiency of C5 is extremely rare [10]. However, clinical use of the anti-C5 antibody eculizumab (Soliris®) is considerably increasing the patient population with a 'functional' C5 deficiency and, thus, increasing the relevance of studies on complement in hemostasis. Muhlfelder *et al.* showed that C5 increased the TF activity on leukocytes [11], due most probably to activation of C5a receptor type 1 (C5aR1) [5,12].

Earlier studies have suggested that C6-deficient rabbits had an increased tendency to bleed, as prolonged clotting time and reduced prothrombin consumption were observed, and reconstitution with purified C6 corrected this condition [13]. Furthermore, C6-deficient mice and rats also had prolonged bleeding [14]. In contrast, humans with complement C6-deficiency [15,16] and a C7-deficient individual [17] were shown to have normal hemostasis. Zymosan failed to aggregate platelets in both C3, C5, C6 and C7-deficient plasmas, suggesting a role of C5 in platelet aggregation [18].

Combined inhibition of C3 and CD14 significantly inhibited the *E. coli*-induced cytokine release, phagocytosis and oxidative burst in human whole blood [19]. CD14 is the co-receptor of several Toll-like receptors (TLRs), including TLR-4 and TLR-2 [20], indicating that TLRs may be involved in the *E. coli*-induced TF up-regulation.

The aim of this study was to investigate how C5 affects normal hemostasis and coagulation kinetics, and the roles of C5 as well as CD14 and TLR-4 in bacteria-induced coagulation in fresh human whole blood from healthy controls or a C5-deficient individual (C5D).

## Materials and methods

### Blood sampling for hemostasis investigations

Venous blood from the male C5D individual, who was diagnosed due to repeated meningococcal sepsis [10], and

healthy controls (one male and five females) for hemostasis investigation were collected in tubes with 3.2% sodium citrate (Vacuette; Greiner Bio-One GmbH, Kremensmünster, Austria). The tubes were centrifuged at 2500 g for 10 min at room temperature. The study was approved by the regional ethics committee of the Northern Norway Regional Health Authority. The donors provided written informed consent.

### Whole blood experiments and inhibitors

Whole blood experiments were performed according to the previously described whole blood model [21]. Blood was collected in 4.5 ml Nunc polypropylene tubes (Roskilde, Denmark) containing a 50 mg/l final concentration of lepirudin (Refludan®; Celgene, Uxbridge, UK). All equipment and reagents were endotoxin-free. Blood (975 µl) was preincubated for 5 min with 195 µl of phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO, USA) or inhibitor. Anti-human C5 IgG2/4 eculizumab (Alexion Pharma GmbH, New Haven, CT, USA) was used at a final concentration of 77 mg/l, an immunoglobulin (Ig) G2/4 isotype control antibody (NHDL, produced in our laboratory) at 77 mg/l, anti-human CD14 IgG2/4 r18D11 (anti-CD14 [22]) at 15 mg/l and the TLR-4 inhibitor eritoran (E5564, kindly provided from Eisai, Andover, MA, USA) at 1 µM. PMX53 [AcF-(OPdChaWR)] was synthesized as described previously [23], purified by reverse-phase high-performance liquid chromatography, and used at a final concentration of 10 µM. In experiments with blood from the C5D, purified human C5 at a final concentration of 50 mg/l (Complement Technology, Tyler, TX, USA) or 17.5 mg/l human serum albumin (HSA) (Octapharma, Lachen, Switzerland) were added. After preincubation, 195 µl of PBS, heat-inactivated *E. coli* (final concentration  $1 \times 10^7$ /ml; strain LE392, ATCC 33572; American Type Culture Collection, Manassas, VA, USA) [21] or ultrapure lipopolysaccharide (LPS) (final concentration 100 ng/ml; LPS-EB Ultrapure from *E. coli* strain 0111; InvivoGen, Eugene, OR, USA) were added. The time zero sample (T0) was processed immediately after blood sampling. Samples for flow cytometric analysis of CD11b surface expression were incubated at 37°C for 10 min. All other samples were incubated at 37°C and tilted up and down 10 times per min on a Rock-n-Roller (Labinco, Breda, the Netherlands) for 120 min before further processing. For flow cytometric analysis of TF, 45 µl of whole blood was added to 5 µl citrate. For analysis of PTF1-2, soluble TCC, cytokines and TF mRNA, ethylenediamine-tetraacetic acid (EDTA, 10 mM final concentration) was added to stop further coagulation and complement activation. All tubes were centrifuged at 3000 g at 4°C for 20 min, and plasma was stored at -80°C until analysis, except for quantitative PCR (qPCR) analysis. Here, the volume of plasma removed above the

blood cells was replaced with PBS without  $\text{CaCl}_2$  and  $\text{MgCl}_2$  and Tempus<sup>TM</sup> blood RNA solution (Applied Biosystems, Foster City, CA, USA) was added at twice the blood volume. The tubes were stored at  $-20^\circ\text{C}$  until analysis. For analysis of TF function in plasma micro-particles (TF-MP), 25  $\mu\text{l}$  citrate solution was added to 225  $\mu\text{l}$  blood. The tubes were centrifuged twice, first at 1500  $g$  at room temperature for 15 min to remove cells. The plasma was centrifuged a second time at 13 000  $g$  at room temperature for 2 min and the supernatant was stored at  $-80^\circ\text{C}$  until analysis.

### Coagulation analyses

Routine coagulation analyses were performed in citrated plasma using a STA-R Evolution<sup>®</sup> instrument and reagents from Diagnostica Stago (Asnières, France). STA<sup>®</sup> SPA+ reagent was used for prothrombin time-international normalized ratio (PT-INR), and STA<sup>®</sup>-PPT for activated partial thromboplastin time (APTT; Diagnostica Stago) and clot detection methods. The colorimetric kits STACHROM<sup>®</sup> protein C and STACHROM<sup>®</sup> ATIII (Diagnostica Stago) were used for analysis of protein C and anti-thrombin III, respectively. The clot-based test kit STACLOT<sup>®</sup> protein S (Diagnostica Stago) and the COATEST<sup>®</sup> APC<sup>TM</sup> Resistance V kit from Chromogenix (Bedford, MA, USA) were used for analysis of protein S and activated protein C (APC) resistance, respectively. FVIII, von Willebrand factor antigen and activity, lupus anti-coagulant, anti-cardiolipin IgG and IgM and anti-beta2-glycoprotein I IgG and IgM were estimated in blood samples from the C5D individual by routine analyses at the Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway.

### Thromboelastometry on whole blood

Clot development in fresh citrated blood was analyzed using ROTEM<sup>®</sup> delta (Tem Innovations GmbH, Munich, Germany) following the manufacturer's instructions. The non-activated thromboelastometry (NATEM) analyses were performed as previously described [24,25]. Star-tem reagent (Tem Innovations GmbH) containing  $\text{CaCl}_2$  was used to recalcify the samples.

### Platelet function analysis in whole blood

Platelet function in whole blood was analyzed using the INNOVANCE<sup>®</sup> PFA200 instrument from Siemens Healthcare (Marburg, Germany) and Dade PFA collagen/ADP test cartridges (Siemens Healthcare). The test mimics venous damage and platelet adhesion and aggregation repair of a vessel injury. Closure time is the time before the hole in the cuvette is closed by platelets, and the results are given in seconds.

### Real-time quantitative polymerase chain reaction (qPCR) of TF mRNA levels

The MagMax<sup>TM</sup> for stabilized Blood Tubes RNA Isolation kit from Thermo Fisher Scientific (Vilnius, Lithuania) was used to isolate the RNA. The concentration of RNA was measured using a Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE, USA). The TaqMan<sup>®</sup> RNA-CT<sup>TM</sup> 1-step kit (Applied Biosystems) was used to analyze the TF and control mRNA levels from 2 ng of total RNA per sample. Hs00175225\_m1 (Applied Biosystems) was used to amplify the TF mRNA. Human beta-2-microglobulin (Hu B2 M; Applied Biosystems) was used as an endogenous control, as it is stably expressed in the whole blood model [26]. The samples were analyzed in triplicate using QuantStudio 6 Flex from Applied Biosystems. The delta-delta Ct-method was used to calculate the relative concentration of TF mRNA. The results are given as relative quantitation (RQ), using the PBS control sample incubated for 120 min at  $37^\circ\text{C}$  as a calibrator and set to 1.

### Flow cytometric analysis of TF surface expression

The monocyte TF surface expression in whole blood (12.5  $\mu\text{l}$ ) was analyzed by adding fluorescein isothiocyanate (FITC)-labeled anti-human TF antibody (2.5  $\mu\text{l}$ ) (product no. 4508CJ, clone VD8; American Diagnostica, Inc., Stamford, CT, USA) or a FITC-labeled mouse IgG1 isotype control antibody (2.5  $\mu\text{l}$ ) (Becton Dickinson, San Jose, CA, USA). To gate the monocytes and leukocytes, phycoerythrin (PE) (Becton Dickinson)-labeled anti-CD14 antibody (2.5  $\mu\text{l}$ ) and peridinin-chlorophyll (PerCP)-labeled anti-CD45 antibody (2.5  $\mu\text{l}$ ) (Becton Dickinson) were added to the blood samples in a 96-well microtiter plate (Nunc) and incubated in the dark for 15 min at room temperature. The samples were lysed by adding EasyLyse<sup>™</sup> (250  $\mu\text{l}$ ) (S2364; Dako Cytomation, Glostrup, Denmark) and incubated for 15 min at room temperature protected from light. The samples were centrifuged at 300  $g$  for 5 min and washed with PBS containing 0.1% (w/v) bovine serum albumin (BSA), and the cells were resuspended in PBS. The samples were analyzed using a BD LSR II flow cytometer (Becton Dickinson) and results expressed as median fluorescence intensity (MFI). The MFI results are given as the differences between the MFI with anti-TF antibody and the isotype control antibody.

### Flow cytometric analysis of CD11b surface expression

Increasing amounts of human recombinant C5a (Hycult Biotechnology, Uden, the Netherlands) were added to blood from healthy donors. After a 10-min incubation at  $37^\circ\text{C}$ , 25  $\mu\text{l}$  whole blood was added to 25  $\mu\text{l}$  0.5% (v/v) paraformaldehyde (PFA; Sigma Aldrich, Oslo, Norway), and cells were fixed at  $37^\circ\text{C}$  for 4 min. The

fixed blood cells were stained with PE-labeled anti-CD11b (Becton Dickinson) or PE-labeled mouse IgG2a (Becton Dickinson) in the dark for 15 min at room temperature. In addition, PerCP-labeled CD45 (Becton Dickinson) and FITC-labeled anti-CD14 (Becton Dickinson; clone MP9) were used to gate leukocytes and monocytes. The samples were mixed with 1 ml PBS and analyzed using NovoCyte flow cytometer (ACEA Biosciences, Inc., San Diego, CA, USA) and results recorded as the median fluorescence intensity (MFI).

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

Soluble TCC levels were measured in plasma using an antibody against a specific C9 neoepitope in the TCC complex, as described previously [27]. PTF1-2 in plasma was measured using an Enzygnost<sup>®</sup>F1+2 (monoclonal) kit from Siemens Healthcare (Marburg, Germany). The PTF1-2 results in Fig. 2b,d were normalized to the sample incubated for 120 min at 37°C with *E. coli*, which was set to 100%. TF-MP was measured in plasma using the Zymuphen MP-TF kit (Aniara Diagnostica, West Chester, OH, USA). Optical density was measured using an MRX microplate reader (Dynex Technologies, Denckendorf, Germany).

#### Statistical analysis

Analysis of normality distribution was performed using the Shapiro–Wilk test in IBM SPSS Statistics for Windows version 22.0 (IBM Corp., Armonk, NY, USA). If the normality test failed, the results were log-transformed. The results that were still skewed were analyzed using non-parametric tests. Data were analyzed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). The results from experiments with stimuli were analyzed with a paired Student's *t*-test comparing the results with and without *E. coli* or LPS and the exact *P*-value is reported. Wilcoxon's matched-pairs signed-rank test was used to analyze non-parametric data. Furthermore, a one-way repeated-measures analysis of variance (ANOVA) and Dunnett's multiple comparisons test using PBS as a control were used to compare the effects of various additives. Non-parametric data were analyzed using Friedman's test and Dunn's multiple comparisons test. The Pearson correlation in GraphPad Prism was used to analyze the correlation between coagulation measured as PTF1-2 and the monocyte TF surface expression or TF-MP in plasma. Two-way repeated-measures ANOVA and Sidak's multiple comparison tests were used to compare results in the presence or absence of the C5a receptor 1 (C5aR1) antagonist PMX53 at different C5a concentrations. A *P*-value below 0.05 was considered statistically significant.

## Results

### No impact of C5 on hemostasis or coagulation kinetics under physiological conditions

Samples from the healthy blood donors were within the reference ranges for the selected routine coagulation analysis (Table 1). Plasma from the C5D individual displayed normal coagulation measured by PT-INR, APTT, protein C, protein S and anti-thrombin levels, APC resistance, von Willebrand factor activity, lupus anti-coagulant and D-dimer (Table 1).

Analyses of coagulation kinetics in fresh blood from both the healthy donors and the C5D using thromboelastometry and platelet function analyzer showed that C5 had no apparent impact on clot formation and platelet aggregation under physiological conditions (Table 2). A statistical comparison of the ROTEM results in the C5D and the six healthy donors after adding eculizumab was not possible due to data from only one C5D individual.

### C5 inhibition and absence of C5 (C5D) reduced the *E. coli*-induced TF whole blood mRNA levels and monocyte TF surface expression

Incubation for 120 min with *E. coli* significantly ( $P = 0.0056$ ) increased the TF mRNA level in blood from healthy donors compared with control samples incubated with PBS (Fig. 1a). Eculizumab significantly ( $P < 0.05$ ) reduced the *E. coli*-induced TF mRNA level from 6.4 to 1.9 RQ compared to PBS, whereas the control antibody had no effect. In blood from healthy donors, monocyte TF surface expression was significantly ( $P = 0.0002$ ) increased from MFI = 88 in the PBS control to MFI = 851 in the presence of *E. coli* (Fig. 1b). Eculizumab significantly ( $P < 0.05$ ) reduced the *E. coli*-induced TF surface expression to MFI = 327, whereas the control antibody had no effect. Eculizumab completely blocked the *E. coli*-induced complement activation measured as formation of sC5b-9 in plasma (data not shown).

In blood from the C5D individual, *E. coli* increased the TF mRNA level from 1.0 to 7.5 RQ (Fig. 1c). After reconstitution with purified C5, the *E. coli*-induced TF mRNA level further increased to 23.4 RQ. Monocyte TF surface expression increased in the presence of *E. coli* to MFI = 232 compared to MFI = 59 in the PBS control (Fig. 1d). Reconstitution with purified C5 further increased the *E. coli*-induced TF surface expression to MFI = 470. The HSA control had no effect on either *E. coli*-induced TF mRNA or monocyte TF surface expression.

### C5 inhibition and absence of C5 (C5D) reduced the *E. coli*-induced TF-MP and PTF1-2 levels

Incubation of whole blood from healthy donors with *E. coli* for 120 min significantly ( $P = 0.0002$ ) increased

the levels of TF-MP from 0.84 to 8.8 pg/ml (Fig. 2a). Eculizumab significantly ( $P < 0.05$ ) reduced the *E. coli*-induced TF-MP levels to 4.9 pg/ml, whereas the control antibody had no effect. The impact of C5 inhibition on coagulation was evaluated by analyzing the PTF1-2 levels in plasma (Fig. 2b). *E. coli* significantly ( $P < 0.05$ ) increased the PTF1-2 levels from 4.4% in the PBS control to 100% after 120 min incubation with *E. coli* (set to 100%). Eculizumab significantly ( $P < 0.05$ ) reduced the *E. coli*-induced PTF1-2 level to 44% of the PBS control. The control antibody had no effect on the *E. coli*-induced PTF1-2 level; however, the results displayed high biological variation.

In blood from the C5D, *E. coli* increased the TF-MP levels from 0.36 to 2.9 pg/ml (Fig. 2c). After reconstitution with purified C5, *E. coli* increased the TF-MP levels from 2.9 to 5.7 pg/ml and the PTF1-2 level from 100% in the absence of recombinant C5 to 471% in C5 reconstituted blood (Fig. 2d). In the absence of *E. coli* and C5, PTF1-2 were 12% of the sample incubated with *E. coli*. The HSA control had no effect on either the *E. coli*-induced TF-MP or *E. coli*-induced PTF1-2 levels.

#### ***E. coli*-induced PTF1-2 levels correlated with procoagulant activity of TF-MP**

We then tested if the functional measure of coagulation activation, reflected by plasma PTF1-2, correlated significantly with TF surface expression on monocytes and TF-MP. Data for PTF1-2, TF-MP and monocyte TF surface expression from one experiment with each blood donor were included in the correlation analysis ( $n = 6$ ). PTF1-2 did not correlate with TF surface expression on monocytes ( $r = 0.49$ ,  $P > 0.05$ , Fig. 3a), but with TF-MP levels ( $r = 0.85$ ,  $P = 0.0319$ , Fig. 3b).

#### **Recombinant human C5a increased the CD11b surface expression but not monocyte TF surface expression in whole blood**

We next examined if recombinant human C5a alone enhanced the TF surface expression on monocytes. The effect of increasing doses of C5a on TF and CD11b surface expression on monocytes in human whole blood was examined using flow cytometry. CD11b surface expression was used as a control readout, as C5a was reported to enhance CD11b surface expression [28]. Human C5a increased CD11b surface expression from MFI = 3187 at baseline to MFI = 9161 after a 10-min incubation with the highest C5a concentration in a dose-dependent manner (Fig. 4). The C5aR1 antagonist PMX53 significantly ( $P < 0.05$ ) reduced C5a-induced CD11b expression on monocytes. In contrast, TF surface expression was unchanged after a 120-min incubation with C5a and PMX53 had no effect (Fig. 4).

#### **The combination of TLR-4- and C5-inhibition reduced *E. coli*-induced monocyte TF surface expression, TF-MP and plasma PTF1-2**

We then examined the possible additional role for C5 and TLR-4 and its co-receptor CD14 on the *E. coli*-induced monocyte TF surface expression using the TLR-4/MD2-specific inhibitor eritoran and a blocking anti-CD14 mAb (Fig. 5). As a positive control, the effect of TLR-4 inhibition on ultrapure *E. coli* LPS-induced monocyte TF surface expression, TF-MP levels and PTF1-2 was also examined.

Eculizumab alone and anti-CD14 alone did not significantly reduce the *E. coli*-induced monocyte TF surface expression, whereas the combination significantly ( $P < 0.05$ ) reduced the monocyte TF surface expression from MFI = 1066 to MFI = 176 (Fig. 5a). Eritoran alone did not significantly reduce the *E. coli*-induced monocyte TF surface expression, whereas combined with eculizumab the reduction was significant ( $P < 0.05$ ) to MFI = 227.

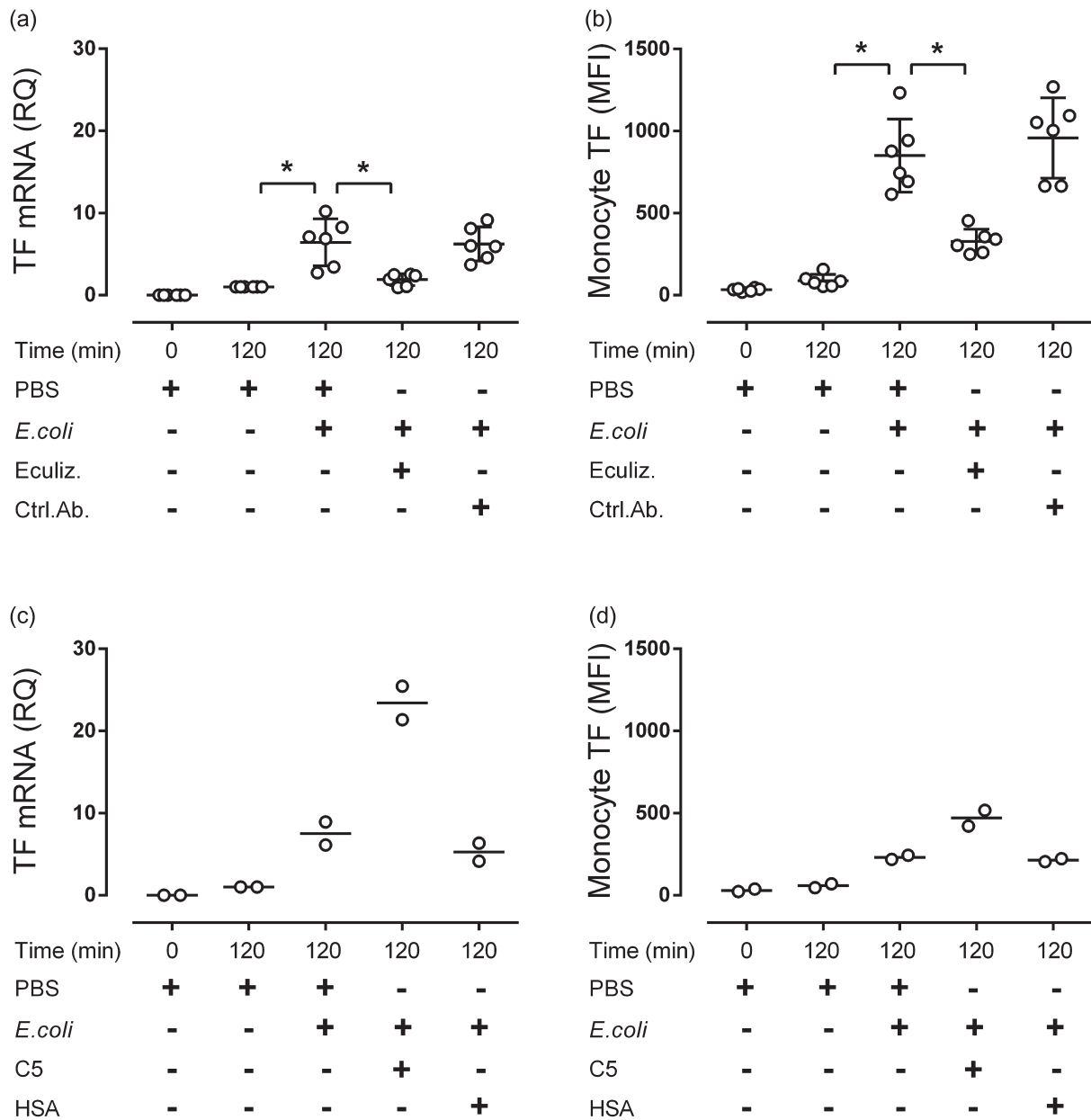
Eculizumab alone significantly ( $P < 0.05$ ) reduced the *E. coli*-induced TF-MP from 9.0 to 6.4 pg/ml (Fig. 5b). Anti-CD14 alone did not significantly reduce TF-MP, whereas combined with eculizumab the reduction from 9.0 to 3.4 pg/ml was significant ( $P < 0.05$ ). Eritoran alone did not significantly reduce TF-MP, whereas combined with eculizumab the reduction from 9.0 to 3.8 pg/ml was significant ( $P < 0.05$ ). The control antibody unexpectedly and significantly increased ( $P < 0.05$ ) the *E. coli*-induced TF-MP.

Eculizumab alone and anti-CD14 alone did not significantly reduce the *E. coli*-induced PTF1-2, whereas the combination significantly ( $P < 0.05$ ) reduced the PTF1-2 from 18.8 to 2.7 nmol/ml analyzed by ANOVA (Fig. 5c). However, a paired Student's *t*-test showed that eculizumab alone significantly ( $P = 0.0356$ ) reduced the *E. coli*-induced PTF1-2 (Fig. 5c). Eritoran alone did not significantly reduce the PTF1-2, whereas combined with eculizumab the reduction from 18.8 to 4.0 nmol/ml was significant ( $P < 0.05$ ).

Eritoran significantly ( $P < 0.05$ ) reduced LPS-induced monocyte TF surface expression (Fig. 5a), LPS-induced TF-MP (Fig. 5b) and LPS-induced PTF1-2 (Fig. 5c). Data on LPS and *E. coli*-induced monocyte TF surface expression were displayed in representative histograms for one donor (Fig. 6).

#### **Discussion**

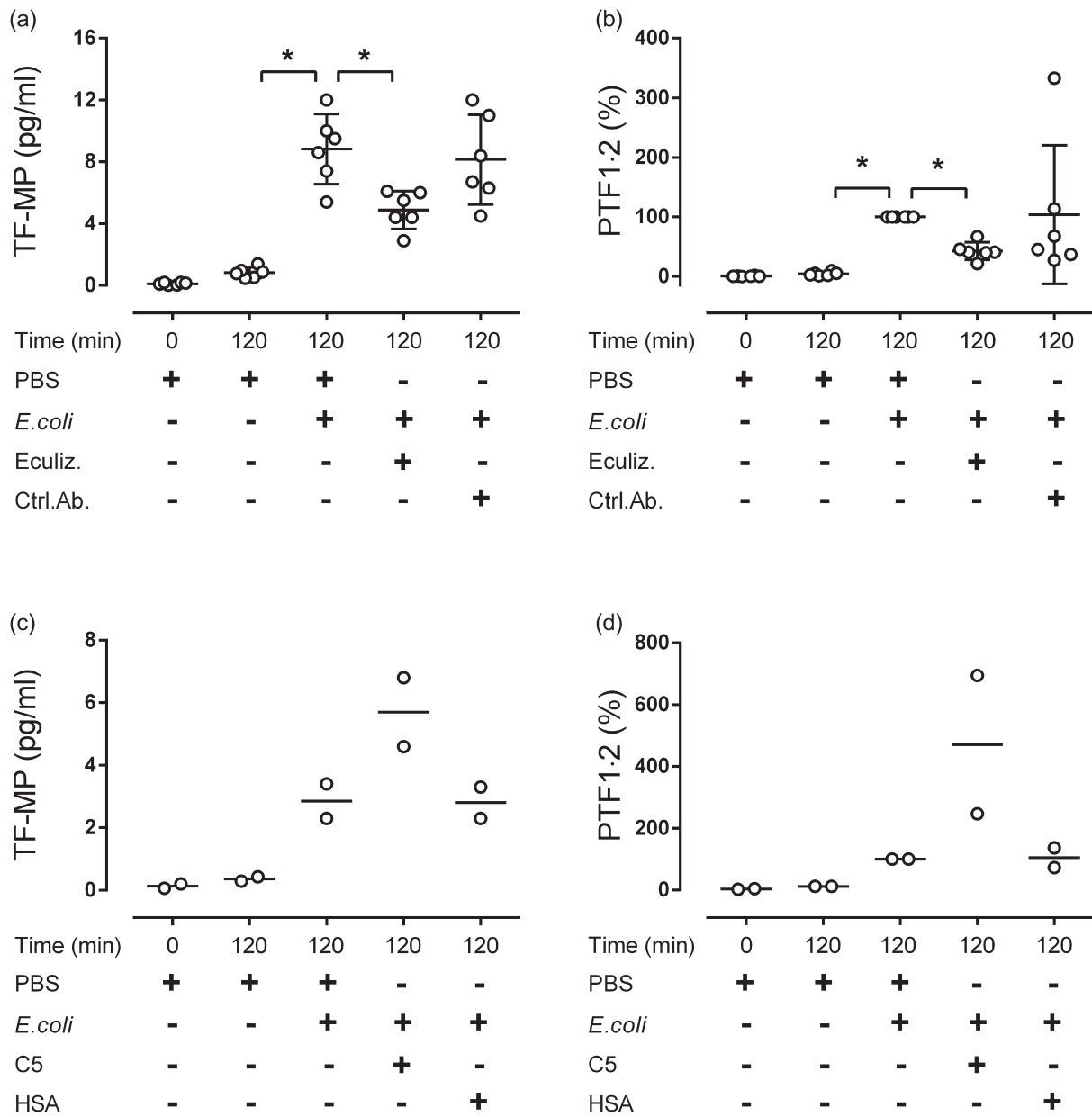
The present data demonstrate that blood from a C5-deficient (C5D) individual displayed completely normal concentrations of hemostatic proteins and functional hemostatic analyses as normal individuals. Consistently,



**Fig. 1.** Effect of the anti-human C5 antibody eculizumab and C5 deficiency on *Escherichia coli* (*E. coli*)-induced tissue factor (TF) mRNA levels and monocyte TF surface expression on whole blood monocytes. Whole blood from healthy donors was preincubated with phosphate-buffered saline (PBS), eculizumab (Eculiz.) or control antibody (Ctrl.Ab.). The blood samples were either processed immediately (time zero samples) or incubated for 120 min at 37°C with PBS (control) or  $1 \times 10^7$ /ml *E. coli*. TF mRNA expression (a,c) was examined using real-time quantitative PCR (qPCR) and expressed as the relative quantity (RQ) compared to the PBS control (set to 1). Monocyte TF surface expression (b,d) was examined using flow cytometry and given as median flow intensity (MFI). Whole blood from the C5-deficient individual was incubated with PBS, purified complement component C5 or human serum albumin (HSA) prior to incubation with *E. coli* (c,d). The results are given as means  $\pm$  standard deviation (s.d.) for normal blood experiments ( $n = 6$ ), and as mean (line) and scatterplot of two experiments performed on two different days with C5D blood. \* $P < 0.05$ .

C5 had no impact on the physiological coagulation kinetics *in vitro* using whole blood samples from healthy controls inhibited with eculizumab. In contrast, C5 was crucially important in the *E. coli*-induced hemostatic

disturbance, reflected by increased TF mRNA generation, monocyte TF surface expression, plasma TF-MP and PTF1-2 formation. The effect of C5 was most pronounced at the mRNA level. Finally, combined inhibition of C5



**Fig. 2.** Effect of the anti-C5 antibody eculizumab and C5 deficiency on *Escherichia coli* (*E. coli*)-induced tissue factor function in plasma microparticles (TF-MP) and on coagulation (PTF1.2). Whole blood from healthy donors was preincubated with phosphate-buffered saline (PBS), eculizumab (Eculiz.) or control antibody (Ctrl.Ab.) prior to incubation with  $1 \times 10^7$ /ml *E. coli* (a,b). Whole blood from the C5-deficient individual was incubated with PBS, purified C5 or human serum albumin (HSA) prior to the incubation (c,d). *E. coli*-induced coagulation was measured by prothrombin fragment 1+2 (PTF1.2) in plasma using enzyme-linked immunosorbent assay (ELISA) (b,d), and expressed as a percentage of the positive control sample incubated with *E. coli* (set to 100%). TF-MP (a,c) are given in pg/ml. The results are expressed as means  $\pm$  standard deviation (s.d.) for normal blood experiments ( $n = 6$ ), and as means (line) and scatterplot of two experiments performed on two different days with C5-deficient blood. \* $P < 0.05$ .

and CD14 or TLR-4 most efficiently blocked the *E. coli*-induced coagulation, with only minor effects of each inhibitor alone.

Inhibition of complement with eculizumab emerges as an important strategy for treatment of an increasing

number of diseases involving complement activation, and is Food and Drug Administration (FDA)-approved for clinical use with paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome and myasthenia gravis [29,30]. Here, we show that the



**Table 1.** Results from routine coagulation analysis of the C5D individual and healthy donors

Analyses	Results		
	Healthy donors	C5D <sup>a</sup>	Reference ranges
P-PT-INR <sup>b</sup>	1.0 ± 0.1	1.1	<1.2
P-APTT <sup>c</sup> (s)	34 ± 1.6	38	30–42
P-D-dimer (mg/l)	n.d. <sup>d</sup>	0.3	<0.5
P-fibrinogen (g/l)	n.d.	2.0	1.5–4.0
P-anti-thrombin (%)	102 ± 5.0	92	80–120
P-protein C (%)	99 ± 15	76	>70
P-protein S (%)	88 ± 12	97	>65
P-n-APC <sup>e</sup> -sensitivity ratio	1.0 ± 0.02	1.03	>0.80
P-von Willebrand antigen (%)	n.d.	68	50–200
P-von Willebrand activity (%)	n.d.	66	50–200
P-factor VIII (%)	n.d.	68	50–150
P-lupus anti-coagulant SCT	n.d.	1.07	<1.25
P-anti-cardiolipin-antibody IgG (GPL) <sup>f</sup>	n.d.	3.9	<40
P-anti-cardiolipin-antibody IgM (MPL) <sup>g</sup>	n.d.	6.1	<40
P-anti-beta2-GPI IgG (SGU) <sup>h</sup>	n.d.	5.0	<20
P-anti-beta2-GPI IgM (SMU) <sup>i</sup>	n.d.	5.5	<20

Results from C5-deficient individual ( $n = 1$ ) and healthy donors given in means ± standard deviation (s.d.) ( $n = 6$ ). C5D<sup>a</sup> = complement component 5-deficient individual; PT-INR<sup>b</sup> = prothrombin international normalized ratio; APTT<sup>c</sup> = activated partial thromboplastin time; n.d.<sup>d</sup> = not done; APC<sup>e</sup> = activated protein C; (GPL)<sup>f</sup> = immunoglobulin (Ig)G phospholipid units corresponding to 1 µg/ml of cardiolipin antibody; (MPL)<sup>g</sup> = IgM phospholipid units corresponding to 1 µg/ml of cardiolipin antibody; (SGU)<sup>h</sup> = standard IgG anti-beta-2-GPI unit; (SMU)<sup>i</sup> = standard IgM anti-beta-2-GPI unit.

presence of eculizumab in normal human blood did not affect platelet closure times or the coagulation kinetic readouts, implying that eculizumab does not interfere with normal hemostasis, and that C5 most probably does not play a role in hemostasis under physiological conditions. This is in agreement with the observation that C5-deficient individuals do not have a general bleeding tendency [18].

Keshari *et al.* recently observed that coagulation and hemostasis can be activated independently of significant complement activation, as infusion of FXa and phosphatidylcholine–phosphatidylserine in baboons did not activate complement [31]. Notably, in rodents and rabbits complement C6 and C7 deficiencies were accompanied by increased bleeding tendency or other

**Table 2.** Thromboelastometry and platelet function analyses of blood from healthy donors and the C5D individual

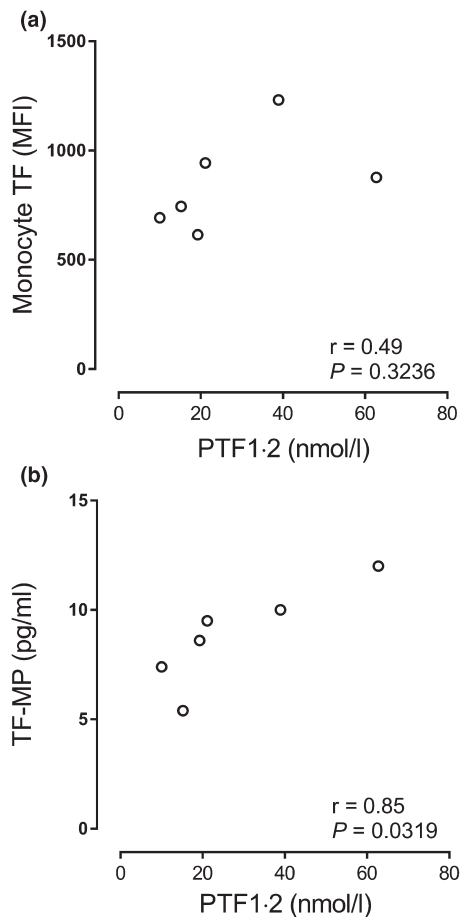
Test	Additions	Healthy donors	C5D <sup>a</sup>
CT <sup>b</sup> (s) <sup>c</sup>	Undiluted	824 ± 159	968 ± 109
	PBS <sup>d</sup>	1073 ± 143	1059 ± 370
	Eculizumab	1059 ± 83	n.d. <sup>e</sup>
	Control antibody <sup>f</sup>	934 ± 94	n.d.
Alpha-angle (°) <sup>g</sup>	Undiluted	47 ± 7.0	28 ± 1.0
	PBS	41 ± 6.9	32 ± 8.5
	Eculizumab	39 ± 4.6	n.d.
	Control antibody	39 ± 5.2	n.d.
CFT <sup>h</sup> (s)	Undiluted	259 ± 63	514 ± 22
	PBS	333 ± 76	481 ± 187
	Eculizumab	366 ± 72	n.d.
	Control antibody	351 ± 61	n.d.
MCF <sup>i</sup> (smm) <sup>j</sup>	Undiluted	48 ± 5.1	43 ± 2.3
	PBS	46 ± 5.0	43 ± 1.5
	Eculizumab	46 ± 3.7	n.d.
	Control antibody	46 ± 4.3	n.d.
PFA200 <sup>k</sup> (s)	Undiluted	80 ± 21	106 ± 4.9
	PBS	86 ± 24	121 ± 12
	Eculizumab	94 ± 20	n.d.
	Control antibody	103 ± 20	n.d.

Thromboelastometry was performed using non-activated thromboelastometry (NATEM) reagents. Results are expressed as seconds, degrees or millimeter and are given as mean ± standard deviations (s.d.) for healthy donors ( $n = 6$ ). Results for the C5D are from two analyses performed at two different days and are given as means ± s.d.

C5D<sup>a</sup> = complement component 5-deficient individual; CT<sup>b</sup> = clotting time; (s)<sup>c</sup> = second; PBS<sup>d</sup>, phosphate-buffered saline; n.d.<sup>e</sup> = not done; (°)<sup>g</sup> = degrees; CFT<sup>h</sup> = clot formation time; MCF<sup>i</sup> = maximum clot firmness; (mm)<sup>j</sup> = millimeter; PFA200<sup>k</sup>, Siemens Healthcare platelet function analyzer 200 closure time.

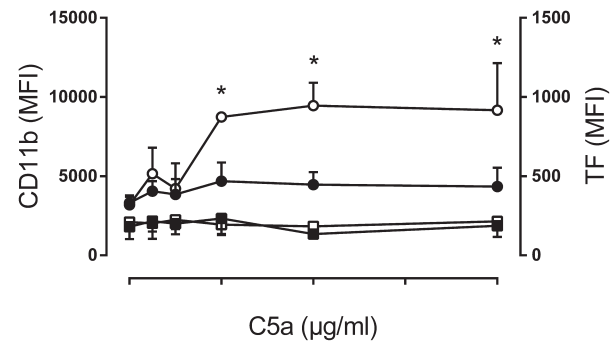
coagulation disorders [13,14]. However, previous studies with human complement deficiencies, including C6 and C7, could not find such effects [15–17]. In the present study, the results from thromboelastometry, platelet function and coagulation tests imply that human C5 deficiency neither causes hemostatic disorders nor pathological coagulation kinetics.

The relatively high biological variation of PTF1-2 levels is due most probably to different levels of TF in high and low TF responders [32]. Eculizumab significantly reduced the *E. coli*-induced PTF1-2 when three, but not seven, groups were compared by one-way repeated-measures ANOVA. This is due probably to weaker statistical power when many groups are compared. When *E. coli* with and without eculizumab were compared using a paired Student's *t*-test, the effect on PTF1-2 was statistically significant in all experiments. However, when the results after incubation with *E. coli* plus eculizumab were



**Fig. 3.** Correlations between coagulation activation measured as prothrombin fragment 1+2 (PTF1.2) in plasma and monocyte tissue factor (TF) surface expression (a) or tissue factor function in plasma microparticles (TF-MP) (b). The PTF1.2 level was analyzed by enzyme-linked immunosorbent assay (ELISA) and expressed in nmol/l. The TF surface expression on monocytes was analyzed by flow cytometry and expressed as median fluorescence intensity (MFI). The TF-MP levels were expressed in pg/ml. The Pearson's correlation analyses were performed using GraphPad Prism. Results from separate experiments with each blood donor are included ( $n = 6$ ).

compared with those of *E. coli* plus the control antibody, the results were significantly different in six of seven readouts. The significant inhibitory effect of eculizumab on the *E. coli*-induced coagulation in human whole blood indicated, however, an important role of C5 in bacteria-induced coagulation. C5 blockade by eculizumab may reduce the complement-mediated lysis of live *E. coli* bacteria and reduce the free LPS level in plasma [33]. It was not possible to examine the effect of eculizumab on bacterial survival in this study, as we used heat-inactivated *E. coli* bacteria. Patients treated with eculizumab may possibly have a reduced risk of *E. coli*-induced coagulation. As bacteria-induced thromboembolic events are significantly increased in PNH and catastrophic

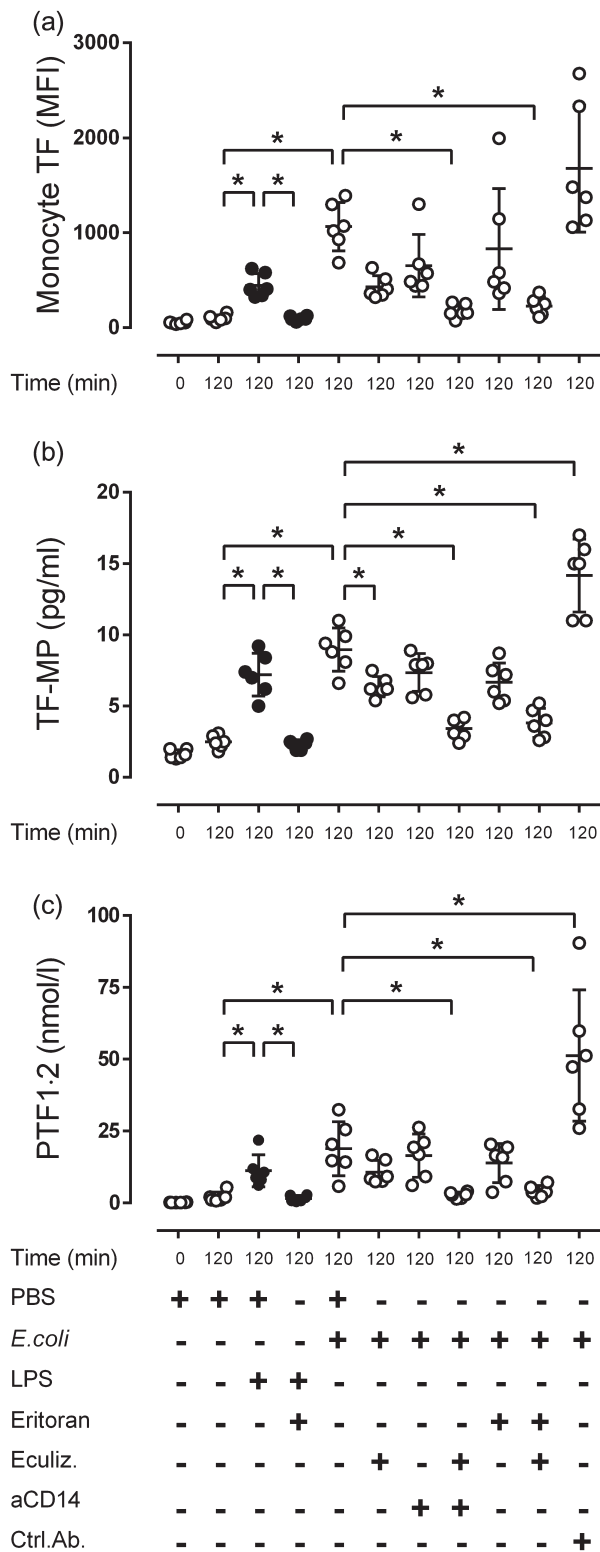


**Fig. 4.** Effect of recombinant C5a on monocyte CD11b and tissue factor (TF) surface expression. Whole blood from healthy donors was incubated with phosphate-buffered saline (PBS) or increasing concentrations of purified human C5a for 10 min at 37°C (CD11b analysis) or 120 min (TF analysis) followed by flow cytometry. The results from separate experiments using blood from healthy blood donors ( $n = 4$ ) are expressed as median fluorescence intensity (MFI)  $\pm$  standard deviation (s.d.). The left y-axis indicates monocyte CD11b surface expression; samples added C5a (open circles) and samples added C5a and PMX53 (closed circle). The right y-axis indicates monocyte TF surface expression samples added C5a (open square) and samples added C5a and PMX53 (closed square).

anti-phospholipid syndrome, two diseases indicative for eculizumab treatment, the putative protective effect of eculizumab on bacteria-induced thrombosis should be further examined [34].

This report indicates a significant role of C5 in the bacteria-induced TF up-regulation. We have previously reported that the *E. coli*-induced coagulation is predominantly TF-dependent [4]. In this study, we reveal that the *E. coli*-induced increase in TF mRNA levels in blood from healthy donors was virtually abolished by the addition of eculizumab, and was significantly enhanced in the C5D individual after reconstitution with purified C5. In reconstituted C5D blood, C5 reached a final concentration of 50 mg/l, which is within the normal physiological range [35].

TF gene transcription can be induced rapidly by various stimuli. In fact, Gregory *et al.* showed that monocyte TF mRNA expression is mainly regulated on the transcriptional level, and that the TF mRNA half-life of approximately 1.5 h is somewhat short [36]. In accordance, monocytes were previously reported to have nearly undetectable amounts of TF stored in their cytoplasm in resting state [32]. Like *N. meningitidis* [5], *E. coli* induced TF surface expression on monocytes in a highly C5-dependent manner. We assumed that nearly all monocyte TF surface expression originated from newly produced TF mRNA due to the significant role of C5 in the *E. coli*-induced TF mRNA up-regulation. However, the *E. coli*-induced monocyte TF surface expression was only partially blocked by C5 inhibition using eculizumab. Both

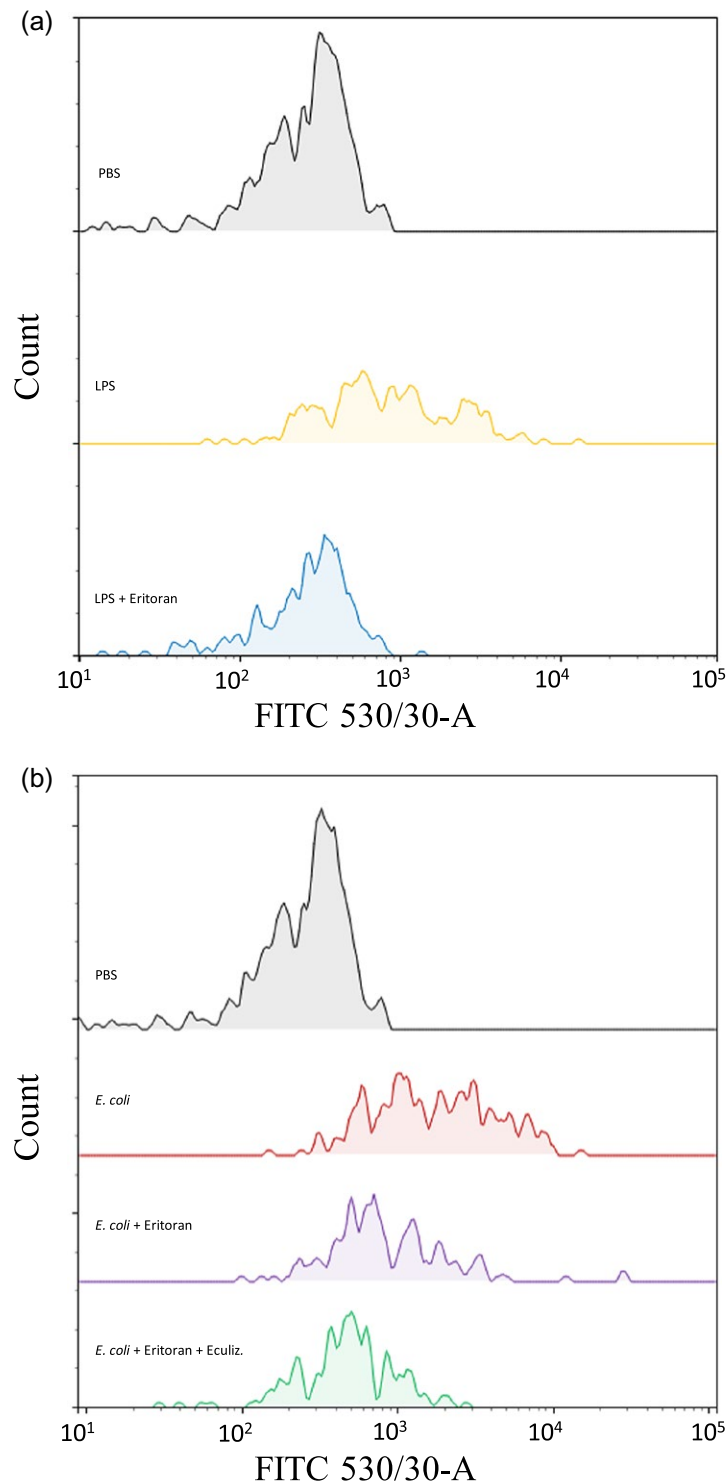


**Fig. 5.** Effect of eculizumab, anti-CD14 or eritoran on *Escherichia coli* (*E. coli*)-induced monocyte tissue factor (TF) surface expression (a), tissue factor function in plasma microparticles (TF-MP) (b) and prothrombin fragment 1+2 (PTF1-2) levels in plasma (c). The blood samples from healthy donors were preincubated with eritoran, eculizumab (Eculiz.), anti-CD14 blocking monoclonal antibody (mAb) (aCD14), control antibody (Ctrl.Ab.) or a combination, as indicated, before they were either processed immediately (time zero samples) or incubated for 120 min at 37°C with phosphate-buffered saline (PBS) (control), 100 ng/ml ultrapure *E. coli* lipopolysaccharide (LPS) (closed circles) or  $1 \times 10^7$ /ml *E. coli* (open circles). Monocyte TF surface expression was measured using flow cytometry and given as median fluorescence intensity (MFI). TF-MP and PTF1-2 in plasma were analyzed using enzyme-linked immunosorbent assay (ELISA) and expressed as pg/ml and nmol/l, respectively. In the PTF1-2 analysis, one data point of the control antibody data set was an outlier and replaced by the mean of this data set, both in the figure and the statistical analysis. All results are given as means  $\pm$  standard deviation (s.d.) ( $n = 6$ ). \* $P < 0.05$ .

TF mRNA and monocyte surface TF were slightly increased in the absence of C5, indicating that other molecules and receptors are involved.

Egorina *et al.* previously showed that LPS is necessary to induce exocytosis of TF [32]. Also, there are several functional links between the LPS receptor CD14/TLR-4 and CD11b or complement receptor CR3 (CD11b/CD18) [37]. We found that the TLR-4 inhibitor eritoran completely inhibited LPS-induced, but did not significantly reduce the *E. coli*-induced, TF surface expression. Together, these results indicate that *E. coli*-induced monocyte TF surface expression is C5 and TLR-4-dependent. *E. coli* bacteria also activates TLR-2 [38]. CD14 blockade has a broader effect on several TLRs than inhibition of TLR-4 by eritoran as CD14 is a co-receptor of several TLRs, including TLR-2 and -4 [39]. This study indicates that combined inhibition of TLR-4 with eritoran and eculizumab or combined inhibition of several TLRs by anti-CD14 and eculizumab have similar effects on *E. coli*-induced TF, suggesting that TLR-4 is the main TLR involved.

In the present study, addition of human C5a alone to normal whole blood did not induce monocyte TF surface expression, despite its positive effect on CD11b. This is in contrast to a study by Janco *et al.*, which showed that C5a induced procoagulant activity in isolated monocytes [40]. We have no explanation for this discrepancy, but a possible LPS contamination in the previously used C5a preparation should be considered. Importantly, in blood, C5a is immediately cleaved by carboxypeptidase N to



**Fig. 6.** Representative histograms from the flow cytometric analysis of the monocyte tissue factor (TF) surface expression in blood from one of six healthy blood donors. The median fluorescence (MFI) signal derived from the fluorescein isothiocyanate (FITC)-labeled anti-TF antibody is depicted at the x-axis and the monocyte count at the y-axis. The isotype control antibody data were nearly identical for all samples and not included in this illustration. (a) Whole blood was incubated for 120 min at 37°C with phosphate-buffered saline (PBS) (gray) or ultrapure *E. coli* lipopolysaccharide (LPS) (100 ng/ml) in the absence (yellow) or presence of eritoran (blue). (b) Whole blood was incubated for 120 min with PBS (gray) or *E. coli* ( $1 \times 10^7$ /ml) in the absence (red) or presence of eritoran (purple) or a combination of eritoran and eculizumab (Eculiz.) (green).

inactive C5a des-Arg [41] which, however, has some affinity to C5aR and may induce CD11b up-regulation [42]. However, the selective C5aR1 antagonist PMX53 [43] ablated the C5a-induced CD11b expression on monocytes. This indicates that C5a-mediated induction of CD11b in human monocytes is wholly dependent upon C5aR1 signaling, similar to that described for human neutrophils [44].

We have demonstrated earlier that *E. coli*-induced TF expression on plasma microparticles is partly C5-dependent [45], and have now verified this observation using C5-deficient blood reconstituted with C5. Importantly, TF negative microparticles may also induce coagulation through a procoagulant negatively charged surface and FXI [46]. In addition, LPS-induced shedding of TF positive plasma microparticles has been observed in high responders [32]. However, the high correlation between TF-MP and PTF1-2 levels found in our model suggests that TF positive plasma microparticles play a significant role in the coagulation activation and PTF1-2 formation. The *E. coli*-induced PTF1-2 was not significantly correlated with the TF surface expression on monocytes, due possibly to the limited number of blood donors. We demonstrated that LPS induces TF-MP in a TLR-4-dependent manner in our model. *E. coli*-induced TF-MP levels, however, were significantly reduced only by the combined inhibition of CD14 or TLR-4 and C5, indicating a role for both TLR-4 and complement C5 for whole bacteria-induced TF-MP. We have no explanation for the unexpected increase in the *E. coli*-induced TF-MP and PTF1-2 levels by the control antibody. However, eculizumab did not have these effects, indicating that this is probably related to some unknown effects of the control antibody itself.

A limitation of this study is the inhibition of thrombin and the lack of endothelial cells as well as of blood flow in the whole blood model. This precludes us from studying the effects of these factors in the experimental system. However, the contribution of TF from endothelial cells in sepsis is unclear, as a study on the endothelial TF-gene knock-out mice did not affect coagulation in an endotoxin model [47]. The TF-positive microparticles seem to be an important contributor to TF's effect in sepsis [48]. Nevertheless, so far the current whole blood model is the best available model to study the role of complement *ex vivo* in interaction with other biological systems. Another limitation is that only one C5D individual was included in our study, as this deficiency is extremely rare. It is therefore possible that our observations from this C5D individual do not reflect the theoretical entirety of C5D individuals in general.

Taken together, this study revealed that C5 plays a critical role together with TLR-4 in *E. coli*-induced coagulation without affecting physiological hemostasis. Consequently,

C5-deficient and patients treated with eculizumab may have a reduced risk of *E. coli*-induced coagulation.

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## Disclosures

None.

## Author contributions

A. L., C. L., S. N., K. T. L., T. E., T. E. M. and O. L. B. planned the experiments. A. L., H. F., D. C., G. B., M. M. and J. K. L. performed the experiments. T. M. W. contributed key reagents and assisted in experimental design and data interpretation. All authors participated in writing of the manuscript and approved the final version.

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