**Staphylococcus aureus**-induced complement activation promotes tissue factor-mediated coagulation

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**Essentials**

- Complement, Toll-like receptors and coagulation cross-talk in the process of thromboinflammation.
- This is explored in a unique human whole-blood model of *S. aureus* bacteremia.
- Coagulation is here shown as a downstream event of C5a-induced tissue factor (TF) production.
- Combined inhibition of C5 and CD14 efficiently attenuated TF and coagulation.

**Summary.** Background: There is extensive cross-talk between the complement system, the Toll-like receptors (TLRs), and hemostasis. Consumptive coagulopathy is a hallmark of sepsis, and is often mediated through increased tissue factor (TF) expression. Objectives: To study the relative roles of complement, TLRs and TF in *Staphylococcus aureus*-induced coagulation. Methods: Lepirudin-anticoagulated human whole blood was incubated with the three *S. aureus* strains Cowan, Wood, and Newman. C3 was inhibited with compstatin, C5 with eculizumab, C5a receptor 1 (C5aR1) and activated factor XII with peptide inhibitors, CD14, TLR2 and TF with neutralizing antibodies, and TLR4 with eritoran. Complement activation was measured by ELISA. Coagulation was measured according to prothrombin fragment 1 + 2 (PTF1 + 2) determined with ELISA, and TF mRNA, monocyte surface expression and functional activity were measured with quantitative PCR, flow cytometry, and ELISA, respectively. Results: All three strains generated substantial and statistically significant amounts of C5a, terminal complement complex, PTF1 + 2, and TF mRNA, and showed substantial TF surface expression on monocytes and TF functional activity. Inhibition of C5 cleavage most efficiently and significantly inhibited all six markers in strains Cowan and Wood, and five markers in Newman. The effect of complement inhibition was shown to be completely dependent on C5aR1. The C5 blocking effect was equally potentiated when combined with blocking of CD14 or TLR2, but not TLR4. TF blocking significantly reduced PTF1 + 2 levels to baseline levels. Conclusions: *S. aureus*-induced coagulation in human whole blood was mainly attributable to C5a-induced mRNA upregulation, monocyte TF expression, and plasma TF activity, thus underscoring complement as a key player in *S. aureus*-induced coagulation.

**Keywords:** bacteremia; blood coagulation; complement system proteins; *Staphylococcus aureus*; tissue factor; Toll-like receptor.

**Introduction**

Sepsis is a critical and potentially lethal syndrome with an increasing incidence [1]. No specific therapy has been developed, despite vigorous attempts to do so. Supportive care has improved survival over the past decades, but mortality and morbidity remain high [2]. With the discovery of persistent critical illness among sepsis survivors, new challenges regarding the syndrome have also emerged, implying that specific treatments are still greatly in demand [3]. However, the complex pathophysiology of...
sepsis makes this difficult [4]. Sepsis is now regarded as a failure of homeostasis at all levels, from intracellular signaling to overall organ function, characterized by a loss of specialized cell function and barrier function [5,6]. In this sense, it is no longer a uniquely immunemediated disease.

However, the innate immune system is crucial for initial pathogen recognition and response, leading to systemic inflammation that disrupts homeostasis. The complement system and the Toll-like receptors (TLRs) with its coreceptor CD14 plays pivotal roles in sepsis as key, upstream molecules of innate immunity recognizing danger [7–9]. The coagulation system is also closely linked to the complement system, and consumptive coagulopathy is a hallmark of severe sepsis [10–12]. Indeed, coincident coagulation and inflammation – immunothrombosis – has been postulated to be a physiological phenomenon of the microvasculature in response to danger [12]. Ekdahl et al. recently coined the term thromboinflammation, designating the collective activation of innate immunity and coagulation [13]. In sepsis, tissue factor (TF) is the main initiator of coagulation, and dysfunctional anticoagulation and impaired fibrinolysis further potentiate coagulation, resulting in consumptive coagulopathy [6,14]. Therefore, it is important to note that complement activation is known to induce TF expression [15–17].

We have previously shown that early, upstream inhibition of innate immunity efficiently reduces the levels of key inflammatory mediators whose levels are otherwise increased in response to, for example, Escherichia coli and Staphylococcus aureus [18–20].

We here hypothesize that TF expression is a downstream event of complement and TLR activation, and that combined inhibition of these systems will reduce coagulation in response to S. aureus. This has been documented in our whole blood model with E. coli, in which TF expression was largely complement-dependent [21]. The present study focuses on Gram-positive species, namely three pathogenic strains of S. aureus (strains Cowan, Wood, and Newman), which is one of the most commonly isolated bacteria in sepsis. S. aureus is also particularly prone to inducing coagulation and aggregation in blood [22,23]. We recently showed that the staphylococci trigger the release of several important cytokines related to TF expression and activation of coagulation, such as interleukin (IL)-1β, IL-8, and tumor necrosis factor, when added to human whole blood [18,24,25]. The aims of this study were therefore as follows: first, to investigate whether S. aureus induces coagulation in human whole blood through a complement-dependent and/or a TLR-dependent increase in TF expression; and second, to determine whether this induction could be abrogated through inhibition of complement and TLRs as a rationale for a future therapy.

Materials and methods

Whole blood model of bacteremia

Whole blood experiments were performed with whole blood from eight different donors and carried out as previously described [26]. In brief, blood drawn by venipuncture was immediately distributed into polystyrene tubes containing Dulbecco’s phosphate-buffered saline (PBS), inhibitors, or controls. The samples were preincubated for 9 min at 37 °C as described in the following. After preincubation, PBS with CaCl₂ and MgCl₂ (Sigma-Aldrich, St Louis, MO, USA), heat-inactivated S. aureus strains Cowan 1 (American Type Culture Collection [ATCC] 12598), Wood (ATCC 10382) or Newman (ATCC 25904) were added to a final whole blood concentration of 1 × 10⁸ mL⁻¹. In the dose–response experiments, 1 × 10⁷ mL⁻¹, 3 × 10⁷ mL⁻¹ and 1 × 10⁸ mL⁻¹ were used. The bacteria were purchased from the ATCC (Manassas, VA, USA).

The time zero (T0) sample was processed immediately after blood sampling. After 60, 120 and 180 min of incubation at 37 °C, blood was distributed into three different sets of tubes. Two sets contained 3.2% citrate solution (1 : 9 v/v), and the blood was used for flow cytometry and TF functional analysis in plasma microparticles (MPs). The last set contained EDTA (10 mM), and the plasma was used for analysis by ELISA, and the cells of the whole blood were used for quantitative PCR (qPCR).

The regional ethics committee of the Northern Norway Regional Health Authority approved the study. All equipment, tips and working solutions were endotoxin-free. Lepirudin (Refludan; Celgene, Uxbridge, UK; 50 mg L⁻¹) was used as the anticoagulant in polystyrene tubes (4.5 mL; Nunc, Roskilde, Denmark).

Inhibitors and agonists

Anti-CD14 Fab̄₂ (lipopolysaccharide [LPS] concentration of < 3.9 EU mL⁻¹) was obtained from Diatec Monoclonals (Oslo, Norway), and added to a final concentration of 10 μg mL⁻¹. The C3 convertase inhibitor compstatin (lot CP20) and its corresponding control peptide, synthesized as previously described [27], were kind gifts from J. Lambris. Compstatin was used at a final concentration of 20 μM. The C5 mAb eculizumab (Soliris) was obtained from Alexion Pharmaceuticals (Zürich, Switzerland), and added to a final concentration of 100 μg mL⁻¹. The cyclic hexapeptide PMX53 (sequence Ace-Phe-[Orn-Pro-dCha-Trp-Arg]), which blocks C5a receptor 1 (C5aR1), was synthesized and purified as previously described [28], and used at final concentration.
of 10 μM. The mouse anti-TF monoclonal blocking antibody (IgG1; Sekisui 4509) was obtained from American Diagnostica (Pfungstadt, Germany), and the isotype-matched control anti-HIV-1 gp120 (clone G3-519) was a kind gift from M. Fung. Both were used at a final concentration of 5 μg mL\(^{-1}\). Activated factor XII (FXIIa)-blocking recombinant human albumin-infestin-4 (infestin) was kindly provided by R. Spirig, CLS Behring (Marburg, Germany), and its inhibitory effect was confirmed by incubating lepirudin plasma in glass tubes for 60 min. Uninhibited plasma generated 20 898 ± 13 669 pmol L\(^{-1}\) prothrombin fragment 1 + 2 (PTF1 + 2), whereas coincubation with infestin reduced PTF1 + 2 levels to 975 ± 464 pmol L\(^{-1}\). The T2.5 monoclonal anti-TLR2 antibody was obtained from Hycult Biotech (Uden, the Netherlands) and used at 10 μg mL\(^{-1}\), whereas the TLR4/MD2-blocking lipid A analog eritoran (E5664) was obtained from Eisai (Andover, MA, USA) and used at 1 μM. The specific blocking of TLR2 and TLR4 with T2.5 and eritoran, respectively, was confirmed by incubating whole blood with the respective agonists FSL-1 (50 ng mL\(^{-1}\)) and \(E.\) coli (S2364; Invivogen, San Diego, CA, USA) and the plasma tested for TNF (data not shown).

**ELISAs**

PTF1 + 2 plasma levels were measured with the Enzymost PTF1 + 2 (monoclonal) kit from Dade Behring (Marburg, Germany). Human pentraxin 3 was analyzed with an ELISA kit from R&D Systems (Minneapolis, MN, USA). TF activity in plasma MPs was assayed with the Zymuphen MP TF kit from Aniara (West Chester, OH, USA), according to the kit insertions. Blood samples were citrated and centrifuged at 1500 \(\times\) g for 15 min at ambient temperature. Plasma was centrifuged at 13 000 \(\times\) g for 2 min, and supernatants were frozen at \(-80^\circ\)C. In this assay, a mAb coated on the wells of the microplate captures human TF without interfering with the active site. Plasma is removed in a washing step, and activated FXII and FX are introduced. Finally, an activated FX-specific substrate is added, producing a yellow color. Soluble terminal complement complex (TCC) levels (sC5b-9) were measured by use of a mAb against a C9 neoepitope specific for TCC, as previously described [29]. An MRX microplate reader (Dyax Technologies, Denkendorf, Germany) was used to measure ODs.

**qPCR of TF mRNA levels**

Total RNA was isolated with a MagMAX for Stabilized Blood Tubes RNA Isolation kit. The RNA concentrations were analyzed with a NanoDrop 2000c from Thermo Fisher Scientific (Wilmington, DE, USA). The TF mRNA levels were measured with the QuantStudio 6 Flex Real-Time PCR System from Life Technologies (Carlsbad, CA, USA). The TaqMan RNA-to-Ct 1 step kit (PN 4392938) was used. Predeveloped TaqMan gene expression assays were used for the target gene, i.e. the TF gene (Hs00175225_m1), and the reference gene was the human \(\beta_2\)-microglobulin gene ( assay ID 4326319E). The template input was 4 ng of RNA, and the samples were analyzed in triplicate. Plates (MicroAmp Fast 96-Well Reaction Plate) were sealed with optical adhesive film (MicroAmp Optical Adhesive film) by use of an applicator. The relative TF mRNA levels were measured with the comparative \(\Delta\Delta\) Ct method by the use of QuanTStudio 6 FLEX software. The TF mRNA levels in the samples after 120 min of incubation with PBS only were set to 1 and used to calibrate the results. All reagents and consumables were from Thermo Fisher Scientific.

**Flow cytometric analysis of TF surface expression**

Monocyte TF surface expression was quantified with a BD LSR II flow cytometer (Becton Dickinson, San Jose, CA, USA). Whole blood (12.5 μL) was stained with fluorescein isothiocyanate (FITC)-conjugated anti-human TF (product no. 4508CJ, clone VD8; American Diagnostica) and phycoerythrin (PE)-conjugated anti-CD14 (Becton Dickinson) antibodies. IgG1 FITC (BD 345815) was used as an isotype-matched control mAb. The blood was incubated for 15 min at room temperature in the dark. Easy lyse (S2364; Dako Cytomation, Glostrup, Denmark) was added to the blood, which was incubated for another 15 min at room temperature for erythrocyte lysis. The leukocytes were washed with PBS with 0.1% (w/v) bovine serum albumin, and finally resuspended in PBS. Monocytes were gated in a CD14 PE/side-scatter dot-plot, and the results are reported as the median fluorescence intensity.

**Colony-forming units (CFUs)**

Blood was serially diluted in Dulbecco’s modified Eagle’s medium/F-12 (Thermo Fisher), and 100 μL was evenly distributed on a blood agar dish. The number of colonies were counted on the following day, and the numbers were corrected for the dilution.

**Statistics and data presentation**

The data were analyzed with GraphPad Prism 6.h (GraphPad Software, San Diego, CA, USA). Normality was tested for with the Kolmogorov–Smirnov test, and any non-normal or skewed data were log-transformed before testing by one-way repeated measures ANOVA with Dunnett’s post hoc test comparing all columns with that of activated, uninhibited whole blood (the positive control), and comparing all columns with each other by the use of Tukey’s multiple comparisons test. Sidak’s post hoc test was used for the comparison between live and dead bacteria. We assumed equal variability of variances, thus
factoring out interindividual variations, for greater power. Dose–response experiments were analyzed by use of a two-way repeated measure ANOVA with Tukey’s multiple comparisons test, and correlation was determined by computing Pearson’s coefficient, r. Definitive outliers (Q = 1%) were identified by ROUT (robust regression and outlier removal), and one donor’s PTF$_{1+2}$ levels in response to the Wood strain were excluded. P-values of < 0.05 were considered to be significant, and all graphs show means and 95% confidence intervals as well as all individual points.

**Results**

*S. aureus*-induced TF generation and coagulation are both time-dependent and dose-dependent

TF mRNA levels and TF expression on monocytes (which can be viewed as molecular assays of coagulation) and the activity of TF in MPs (MP-TF) and the levels of PTF$_{1+2}$ (which can be viewed as functional assays of coagulation) were all increased in response to experimental bacteremia (Fig. 1). They correlated with both increasing doses of *S. aureus* Cowan strain (final concentrations of $1 \times 10^7$, $3 \times 10^7$ and $1 \times 10^8$ mL$^{-1}$) and incubation times (60, 120 and 180 min). The TF mRNA levels were elevated at all time points as compared with the PBS control, and peaked at 120 min (Fig. 1, upper left panel).

TF surface expression increased steadily to 180 min, and was greater than that in non-activated samples at all time points (Fig. 1, upper right panel). The bacterial concentration of $1 \times 10^8$ mL$^{-1}$ induced higher levels than $1 \times 10^7$ mL$^{-1}$ at 60 min and 120 min, but not than $3 \times 10^7$ mL$^{-1}$ at 120 min and 180 min. MP-TF and PTF$_{1+2}$ levels were increased at 120 min and continued to increase to 180 min in response to all three concentrations of bacteria, although the differences between the three concentrations were not statistically significant (Fig. 1, lower right panel). On the basis of these findings, we decided to use a final concentration of $1 \times 10^8$ staphylococci per milliliter of whole blood for an incubation time of 120 min in the following experiments.

![Fig. 1. Effects of *Staphylococcus aureus* Cowan on tissue factor (TF) upregulation and coagulation in human whole blood. TF mRNA levels, TF surface expression on monocytes, the activity of TF in microparticles (MP-TF) and the levels of prothrombin fragment 1 + 2 (PTF$_{1+2}$) were all increased in response to *S. aureus* Cowan, and correlated with both increasing doses of bacteria (final concentrations $1 \times 10^7$, $3 \times 10^7$ and $1 \times 10^8$ mL$^{-1}$) and incubation times (60, 120 and 180 min). TF surface expression on monocytes was analyzed by flow cytometry, MP-TF with an amidolytic assay, and PTF$_{1+2}$ levels with ELISA. Data were obtained from six consecutive and independent experiments on blood from six different donors, and are represented as means with 95% confidence intervals. *P < 0.05, when analyzed with repeated measures, two-way ANOVA with Tukey’s multiple comparisons post hoc test, comparing means at 60, 120 and 180 min with each other. FI, fluorescence intensity; PBS, phosphate-buffered saline; RQ, relative quantification.](https://www.wiley.com/en-us)
S. aureus induces high levels of complement and coagulation activation products in human whole blood

Incubating three different strains of staphylococci in human whole blood increased the levels of C5a, TCC, TF mRNA, TF surface expression, MP-TF, and PTF1+2 (Table 1). The levels of the complement activation products C5a and TCC increased substantially (P < 0.01) in response to the Cowan, Wood and Newman strains. Total TF mRNA levels, TF surface expression on monocytes, MP-TF levels and PTF1+2 levels also increased in response to the Cowan, Wood and Newman strains. This rank order was also seen for the ratios of MP-TF and PTF1+2 (Fig. 2, left panel). However, the different parameters increased five-fold to 55-fold, but the ratios of the functional assays (MP-TF and PTF1+2) of coagulation to the molecular assays (TF mRNA and TF surface expression) were different between strains (Table 1). The MP-TF/TF mRNA ratios were 2.6 for the Wood strain, 4.0 for the Cowan strain, and 11.0 for the Newman strain. Likewise, the ratios of PTF1+2 levels to TF mRNA levels and to TF surface expression, respectively, were 1.1 and 0.9 for the Wood strain, 1.8 and 1.1 for the Cowan strain, and 7.4 and 4.1 for the Newman strain. This rank order was also seen for the ratios of MP-TF and PTF1+2 levels to C5a and TCC levels, where the Newman strain induced both the highest levels of complement activation products and the highest levels of MP-TF and PTF1+2 (Table 1). In extension of this, C5a, TCC and TF surface expression were all positively correlated with MP-TF and PTF1+2 (Fig. 2, left panel). However, little correlation was seen between TF mRNA levels and MP-TF or PTF1+2 levels at 120 min.

Effect of inhibiting complement, CD14 or both on coagulation in response to S. aureus

Whole blood was preincubated with the complement inhibitors compstatin (C3) or eculizumab (C5), and a neutralizing anti-CD14 antibody alone or in combination with the complement inhibitors, before a further 120 min of incubation with the Cowan strain (Fig. 3, upper two panels). Compstatin activation as measured according to C5a and TCC was completely abolished by compstatin and eculizumab, indicating efficient complement inhibition, whereas anti-CD14 had no effect on complement activation (Fig. 3, upper panels).

Eculizumab alone and both complement inhibitors combined with anti-CD14 inhibited TF mRNA generation, whereas anti-CD14 and compstatin alone only tended to do so (Fig. 3, middle left panel).

Both complement inhibitors inhibited TF expression, and combining them with anti-CD14 enhanced this effect, as combined inhibition with compstatin and anti-CD14 had a more pronounced effect on TF surface expression than that obtained with either inhibitor alone (Fig. 3, middle right panel). Notably, TF expression was the only assay of coagulation that was inhibited to a statistically significant extent by anti-CD14 alone.

Both complement inhibitors when used alone or in combination with anti-CD14 inhibited MP-TF, whereas anti-CD14 alone had no effect. The inhibitory effect of eculizumab was stronger than that of compstatin.

Compstatin and eculizumab when used alone or in combination with anti-CD14 reduced PTF1+2 levels. The effect of eculizumab was more pronounced than that of compstatin alone, and the effect of the combination of eculizumab and anti-CD14 was more pronounced than that of compstatin and anti-CD14 (Fig. 3, lower right panel). Anti-CD14 did not affect PTF1+2 levels.

The results for the Wood strain (Fig. 4) were almost identical to those described for the Cowan strain above. Compstatin had a (statistically) stronger effect on TF mRNA levels in response to the Wood strain (Fig. 4), whereas the similar effect of anti-CD14 when used alone on TF surface expression was only statistically significant in response to the Cowan strain (Fig. 3).

The Newman strain (Fig. 5) induced greater responses than the Cowan and Wood strains, but the effects of the different inhibitors were similar with respect to complement activation, TF mRNA levels, TF surface expression, and PTF1+2 levels. The inhibitory effects of either single

Table 1 Percentage upregulation of inflammatory and coagulation markers in response to Staphylococcus aureus strains Cowan, Wood and Newman in human whole blood as compared with phosphate-buffered saline

| Biomarker               | Cowan Fold change | Wood Fold change | Newman Fold change |
|-------------------------|-------------------|------------------|-------------------|
|                         | P-value           | P-value          | P-value           |
| C5a (pg mL⁻¹)           | 19 ± 3*           | 0.0007           | 16 ± 2            | 0.0002           | 25 ± 3            | 0.0002           |
| TCC (AU mL⁻¹)           | 30 ± 4            | 0.0004           | 24 ± 5            | 0.0030           | 32 ± 5            | 0.0017           |
| TF mRNA (RQ)            | 6 ± 1             | 0.0106           | 7 ± 1             | 0.0038           | 5 ± 4             | 0.0009           |
| TF expression (MFI)     | 10 ± 1            | 0.0008           | 9 ± 1             | 0.0006           | 9 ± 1             | 0.0016           |
| MP-TF (pg mL⁻¹)         | 25 ± 6            | 0.0069           | 18 ± 3            | 0.0032           | 56 ± 17           | 0.0232           |
| PTF1+2 (pmol L⁻¹)       | 11 ± 3            | 0.0163           | 8 ± 2             | 0.0258           | 37 ± 14           | 0.0411           |

PTF1+2, prothrombin fragment 1 + 2; MFI, median fluorescence intensity; MP-TF, activity of tissue factor in microparticles; RQ, relative quantification; TCC, terminal complement complex; TF, tissue factor. *Data are from six consecutive experiments in whole blood from six different donors (n = 6), and are presented as mean and standard error of the mean.

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or combined inhibition on MP-TF were less pronounced and not statistically significant (Fig. 5).

In summary, for the experiments examining the activation of complement and coagulation after 120 min of whole blood incubation with all three staphylococcal strains, the combined inhibition of complement (either eculizumab or compstatin) and TLR (anti-CD14) reduced the levels of 17 of the 18 (six per strain) parameters measured.

*PTF*<sub>1 + 2</sub> was generated as a result of increased TF levels

To study whether coagulation, quantified as PTF<sub>1 + 2</sub>, was mediated by TF, we conducted a second set of experiments with a neutralizing anti-TF mAb. The anti-TF mAb effectively reduced the *S. aureus* Cowan-induced, Newman-induced and Wood-induced PTF<sub>1 + 2</sub> levels towards baseline values for all three strains (Fig. 6), consistent with the correlated levels of complement-activation products with increased MP-TF and PTF<sub>1 + 2</sub> levels. Importantly, PTF<sub>1 + 2</sub> generation was not completely abolished by blocking of TF. In a follow-up experiment conducted in lepirudin-anticoagulated plasma, recombinant TF induced PTF<sub>1 + 2</sub> generation in a similar fashion to the whole bacteria (Fig. 2, right panel). This PTF<sub>1 + 2</sub> generation was, in turn, completely abolished both in the presence of anti-TF and in the presence of the FXIIa inhibitor infestin.

**C5aR1 blockade and TLR2 inhibition reduced complement and coagulation comparably to combined inhibition of C5 and CD14 in response to *S. aureus***

Next, using *S. aureus* Cowan only, we explored whether the effects of CD14 inhibition could be attributed to TLR2 or TLR4, the two main CD14-dependent TLRs, and whether the effects of complement inhibition could be attributed to reduced activity in the C5a–C5aR1 axis. TLR2 was inhibited by a mAb, TLR4 by eritoran, and C5aR1 by PMX53.

TF mRNA levels, TF surface expression on monocytes and MP-TF and PTF<sub>1 + 2</sub> levels were measured. Following the 120-min incubation, all four assays were significantly (*P < 0.05) and equally inhibited by the C5aR1 antagonist and eculizumab (Fig. 7), indicating that the complement effects were mediated through C5aR1 activation.

Anti-TLR2 alone significantly reduced TF expression on monocytes but not the levels of the other three markers of coagulation, consistent with the results obtained with anti-CD14 (Fig. 7; also shown in Fig. 3). The addition of a complement inhibitor also enhanced the effect of CD14 or TLR2 inhibition on TF expression, but no additional effects of anti-CD14 or anti-TLR2 were seen on the other three markers.

**Staphylococcal loads were reduced during the whole blood experiments, and the effects of complement inhibition were identical to the responses induced by both live and dead bacteria**

First, we examined CFUs of live *S. aureus*. We observed a decrease in the number of *S. aureus* CFU from 0 min to 120 min (Fig. S1). This reduction was less pronounced when eculizumab was used than when the other inhibitors were used. Second, we compared live and dead *S. aureus*. Live *S. aureus* generated greater levels of PTF<sub>1 + 2</sub> than dead bacteria, whereas the levels of TF mRNA, TF surface expression and MP-TF increased comparably in
response to both live and dead bacteria (Fig. S2). Likewise, the effects of the different inhibitors on these responses were almost identical for both live and dead bacteria, except for a somewhat more pronounced effect of complement inhibition on TF surface expression in response to dead bacteria. Finally, inhibiting TF, but not FXIIa, inhibited the PTF$_1 + 2$ generation in response to both live and dead bacteria, whereas TF mRNA levels, surface expression of TF and MP-TF were unaffected by both TF and FXIIa blockade (Fig. S2).

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**Discussion**

In this study, we have documented time-dependent and dose-dependent coagulation in human whole blood in response to three different strains of *S. aureus*, isolated from human infections. We revealed that this coagulation occurs through early and upstream complement-dependent increases in TF expression and activity, subsequently leading to downstream coagulation with the release of PTF1 + 2. Complement inhibition at the level of C5,
confirmed to be mediated through C5aR1, efficiently attenuated coagulation, particularly when C5 inhibition was combined with CD14 and TLR2 inhibition. Although complement-dependent upregulation of TF has been documented before [15–17], we show that the complement-mediated coagulation via TF in response to S. aureus can be efficiently reduced by inhibiting complement, suggesting that it is an important mechanism with clinical relevance and therapeutic potential.

In line with our previous study of S. aureus-induced cytokine release in whole blood [18], TF expression was largely complement-dependent. Blockade of the TLR

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Fig. 5. Responses to Staphylococcus aureus Newman in human whole blood. Heat-inactivated S. aureus Newman (1 × 10^9 mL⁻¹) increased the levels of complement fragment C5a and terminal complement complex (TCC) in plasma, and tissue factor (TF) mRNA, monocyte surface expression of TF, TF activity in microparticles (MP-TF) and prothrombin fragment 1 + 2 (PTF1 + 2) levels in humans after 120 min of incubation as compared with the phosphate-buffered saline (PBS) control. All of these, except for MP-TF, were reduced towards baseline levels when whole blood was preincubated with the C5-inhibitory antibody eculizumab (Eculiz.). The C3-inhibitory peptide compstatin (Comp.) attenuated the levels of C5a, TCC and MP-TF only. The anti-CD14 antibody (aCD14) exerted no inhibitory effect on its own. The rightmost column (control) represents stimulated whole blood preincubated with PBS containing control peptide and control antibody. Data were from six consecutive and independent experiments using blood from six different donors, and are represented as columns to the means with 95% confidence intervals and all points shown. The TF mRNA levels were from four donors, as two samples were lost in analysis, and the PTF1 + 2 levels were from five donors, as one donor was censored by outlier removal. *P < 0.05, when analyzed with repeated measures, one-way ANOVA with Dunnett’s and Tukey’s multiple comparisons post hoc tests. Ctrl, control; RQ, relative quantification.
coreceptor CD14 exerted very little effect on its own, indicating that CD14 is generally more important as a coreceptor in the immunological response induced by Gram-negative bacteria [30]. However, anti-CD14 did reduce the monocyte surface expression of TF in response to the Cowan strain, in line with previous findings obtained with LPS [31], indicating that there is also some cross-talk between TLRs and coagulation in response to Gram-positive bacteria. Anti-TLR2 exerted similar effects as anti-CD14, as shown by the responses to specific TLR2 agonists [30], as well as staphylococcal exotoxins [32] and the Cowan strain used in this study [33]. In contrast to TLR2 inhibition, TLR4 inhibition did not affect coagulation in our study. However, both CD14 and

Fig. 6. Effect of a neutralizing anti-tissue factor antibody on Staphylococcus aureus-induced coagulation in human whole blood. Heat-inactivated S. aureus (1 × 10^8 mL^{-1}) increased prothrombin fragment 1 + 2 (PTF1 + 2) levels when added to human whole blood after 120 min of incubation as compared with the phosphate-buffered saline (PBS) control. The levels were reduced towards baseline levels by preincubating the whole blood with a neutralizing mAb against tissue factor (aTF). The rightmost column (Ctrl) represents whole blood incubated with an isotype-matched control antibody. Data were from six consecutive and independent experiments using blood from six different donors, and are represented as columns to the means with 95% confidence intervals and all points shown. *P < 0.05, when analyzed with repeated measures, one-way ANOVA with Dunnett’s multiple comparisons post hoc test, and all columns compared with the second column (S. aureus + PBS).
complement are involved in Gram-positive and Gram-negative bacteremia, and our data indicate that combined inhibition of complement and CD14/TLR2 broadly attenuates the downstream effects of *S. aureus* bacteremia with regard to both inflammation and coagulation. Indeed, CD14 is a documented coreceptor for TLR2, underscoring it as a suitable target for broad inhibition of TLR activation [34].

Apparently, compstatin had a less pronounced effect on reducing coagulation than eculizumab. However, an older version of compstatin (CP20) was used in this study, and it is reasonable to suggest that the somewhat lesser effect is attributable to some remnant C3 activity, generating small amounts of C5a leading to coagulation. Given the consistent effect of C5 inhibition on coagulation, we argue that C5a is the main inducer of TF generation. Furthermore, as both eculizumab and PMX53 efficiently and equally reduced the levels of TF and coagulation, we argue that coagulation is mainly driven by activation of the C5a–C5aR1 axis. Importantly, we document that the PTF1+2 generation seen in response to *S. aureus* is caused by a complement-dependent increase in TF expression, mediated by thrombin generation, whereas the contact-dependent pathway of coagulation at
the level of FXIIa is not involved. Likewise, the increased levels of PTF1 + 2 were efficiently reduced by the neutralizing anti-TF mAb, which returned PTF1 + 2 levels back towards baseline. Also, PTF1 + 2 generation was both correlated with TF expression on monocytes, and induced by recombinant TF, underscoring that TF was the main driver of coagulation. We also show that this is the case for live staphylococci (Cowan strain).

However, the substantial but incomplete blockade of PTF1 + 2 in response to S. aureus, as well as the variation between the molecular and functional assays of coagulation in response to the different strains, suggest that the functional assays of coagulation activation (although TF-driven, as shown in Fig. 5) are systematically sensitive to some factor other than TF expression. Indeed, the Newman strain, which also had the greatest impact on complement activation as quantified by the TCC/C5a ratio, generated the greatest amount of PTF1 + 2. Also, TF inhibition completely blocked PTF1 + 2 generation in response to recombinant TF in lepirudin-anticoagulated plasma, whereas some remnant PTF1 + 2 generation was apparent in response to the bacteria in whole blood. Collectively, these findings indicate that complement activation enhances the functional coagulation measurements, possibly by decryption of TF, or perhaps more likely by generating more procoagulant negatively charged phospholipid membrane via activation of platelets or monocytes. FXIIa inhibition, however, did not affect PTF1 + 2 levels, indicating that contact activation is not involved, but potential activation of FXI and FIX by serine proteases of the complement system cannot be ruled out.

A justified objection to our claims concerns the limitations of our whole blood model, which must be fully acknowledged. First, the human whole blood model is limited both in time and place by the absence of endothelium, blood flow, replenished cellular components, and acute-phase reactants. Second, lepirudin specifically blocks thrombin, both limiting the available assays of coagulation and masking the contributions of thrombin itself. Thrombin has been postulated to activate complement directly [35], although a very recent study performed in septic baboons did not detect any complement activation by thrombin (or plasmin) [36]. Likewise, the recent focus on the cross-talk between the many plasma cascade systems in thromboinflammation has revealed that thrombin can affect both platelet and endothelial cell function [13]. Another objection concerns the time of intervention, as the potential ‘treatment’ is added before the ‘disease’ is induced by the bacteria.

However, our whole blood model leaves all other plasma cascade systems and blood cells intact, and thus gives a holistic picture of the responses that we argue can be seen at localized foci of infection, where pathogens constantly interact with complement and TLRs, including the important CD14 molecule. This is an obvious strength as compared with isolated cell systems, for example. Equally, the high amounts of staphylococci used may exceed the concentrations seen systemically, but may be apparent at localized sites of infection. Comparable amounts of bacteria have been measured in the clinic through qPCR [37,38], although studies aimed at quantifying the bacteremia in sepsis are generally lacking [39].

Indeed, it would be interesting to establish more complex models of staphylococcal infection and sepsis, and to examine clinical cases, to verify our findings. Likewise, it is important to examine the role of platelets more closely, as this was beyond the scope of the present study. Thus far, our findings corroborate and extend the scope of earlier studies documenting coagulation downstream of complement activation [15–17,40]. We here document coagulation in response to complement activation induced by staphylococci. In turn, this can allow us to better assess the responses to S. aureus in larger, more complex and life-like models, ultimately leading to new therapeutic alternatives.

Conclusion
In conclusion, the inhibition of complement reduced the otherwise powerful coagulation in response to three different strains of staphylococci. The S. aureus-induced coagulation in human whole blood was chiefly attributable to C5aR1 activation, although TLR2 and its coreceptor CD14 contributed to the increased surface expression of TF on monocytes. These data underscore complement as a key player in S. aureus-induced coagulation, and complement inhibition could potentially limit S. aureus-induced thromboinflammation and full-blown sepsis.

Addendum
E. W. Skjeflo, E. W. Nielsen, O. L. Brekke, T. Espevik, and T. E. Mollnes contributed to the concept and design of the study. T. M. Woodruff provided intellectual input and key reagents. E. W. Skjeflo, D. Christiansen, H. Fure, and J. K. Ludviksen performed the experiments and analyzed the data. E. W. Skjeflo and T. E. Mollnes wrote the paper. All authors critically revised the manuscript and approved the final version.

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Disclosure of Conflict of Interests
The authors state that they have no conflict of interest.
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Quantification of staphylococcal load as colony-forming units (CFUs) in human whole blood culture.

Fig. S2. Comparison of coagulation induced by live and dead S. aureus.

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