ORIGINAL ARTICLE

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

E. W. SKJEFLO,*† D. CHRISTIANSEN,* H. FURE,* J. K. LUDVIKSEN,* T. M. WOODRUFF,‡
T. ESPEVIK,§ E. W. NIELSEN,†¶** O. L. BREKKE*† and T. E. MOLLNES*†§††

*Research Laboratory, Nordland Hospital, Bodø; †Faculty of Health Sciences, K. G. Jebsen TREC, UiT – The Arctic University of Norway, Tromsø, Norway; ‡School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland, Australia; §Center of Molecular Inflammation Research, and Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim; ¶Department of Anesthesiology, Nordland Hospital; **Faculty of Nursing and Health Sciences, Nord University, Bodø; and ††Department of Immunology, Oslo University Hospital and K. G. Jebsen IRC, University of Oslo, Oslo, Norway

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 $1 + 2$ ($PTF_{1 + 2}$) 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, $PTF_{1 + 2}$, 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 $PTF_{1 + 2}$ 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.

Correspondence: Tom Eirik Mollnes, Research Laboratory, Nordland Hospital, Bodø, Norway
Tel.: + 47 9063 0015
E-mail: t.e.mollnes@medisin.uio.no

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 systems 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 immune-mediated 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 polypropylene 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 $CaCl_2$ and $MgCl_2$ (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 1×10^8 mL^{-1}. In the dose–response experiments, 1×10^7 mL^{-1}, 3×10^7 mL^{-1} and 1×10^8 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 tubes were centrifuged for 15 min at $3220 \times g$ at 4 °C. Plasma was stored at − 80 °C until it was analyzed. Cell pellets were lysed and stabilized with PAXgene reagent (PreAnalytiX, Qiagen, UK), and the lysates were stored at − 80 °C until mRNA analysis was performed.

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 polypropylene tubes (4.5 mL; Nunc, Roskilde, Denmark).

Inhibitors and agonists

Anti-CD14 F(ab′)$_2$ (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 (IgG$_1$; 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 (PTF$_{1 + 2}$), whereas coincubation with infestin reduced PTF$_{1 + 2}$ 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 (E5564) 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* ultrapurified LPS (100 ng mL^{-1}) from Invivogen (San Diego, CA, USA) and the plasma tested for TNF (data not shown).

ELISAs

PTF$_{1 + 2}$ plasma levels were measured with the Enzygnost PTF$_{1 + 2}$ (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 × *g* for 15 min at ambient temperature. Plasma was centrifuged at 13 000 × *g* for 2 min, and supernatants were frozen at − 80 °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 FVII 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 (Dynex 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 β$_2$-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 Δ-Δ 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. IgG$_1$ 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 6.0h (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 1×10^7, 3×10^7 and 1×10^8 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 1×10^8 mL^{-1} induced higher levels than 1×10^7 mL^{-1} at 60 min and 120 min, but not than 3×10^7 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 1×10^8 staphylococci per milliliter of whole blood for an incubation time of 120 min in the following experiments.
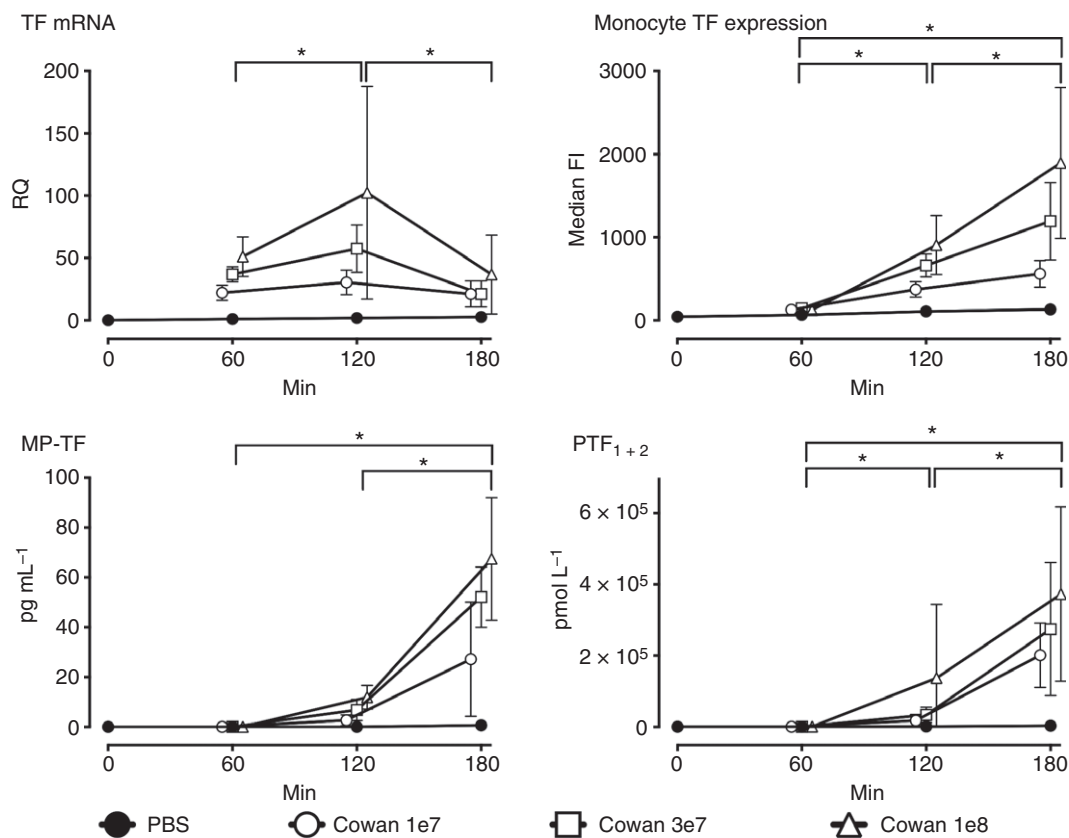

Fig. 1. Effects of *Staphyloccus aureus* Cowan on tissue factor (TF) upregulation and coagulation in human whole blood. TF mRNA levels, TF surface expression on monocytes, the activity of TF in microparticles (MP-TF) and the levels of prothrombin fragment 1 + 2 ($PTF_{1 + 2}$) were all increased in response to *S. aureus* Cowan, and correlated with both increasing doses of bacteria (final concentrations 1×10^7, 3×10^7 and 1×10^8 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.

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 PTF_{1+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 PTF_{1+2} levels also increased in response to three strains. Collectively, the different parameters increased five-fold to 55-fold, but the ratios of the functional assays (MP-TF and PTF_{1+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 PTF_{1+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 PTF_{1+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 PTF_{1+2} (Table 1). In extension of this, C5a, TCC and TF surface expression were all positively correlated with MP-TF and PTF_{1+2} (Fig. 2, left panel). However, little correlation was seen between TF mRNA levels and MP-TF or PTF_{1+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).

Complement 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 PTF_{1+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 PTF_{1+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 PTF_{1+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		Wood		Newman	
	Fold change	*P*-value	Fold change	*P*-value	Fold change	*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
PTF_{1+2} (pmol L^{-1})	11 ± 3	0.0163	8 ± 2	0.0258	37 ± 14	0.0411

PTF_{1+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.
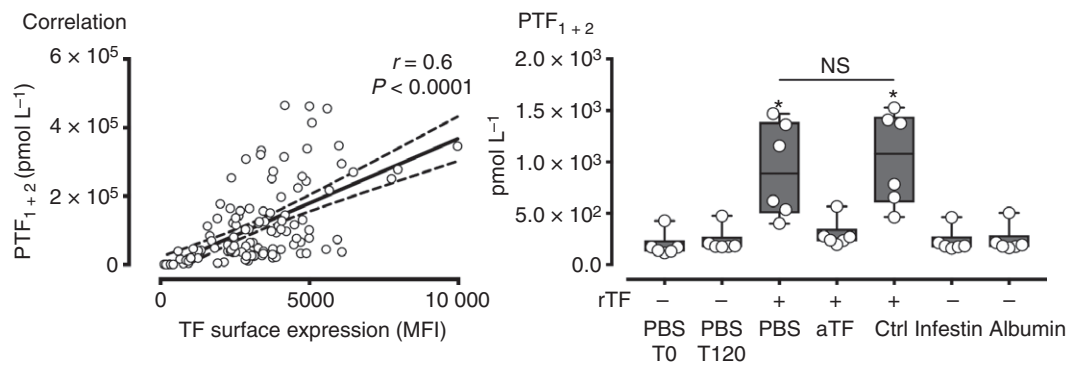
Fig. 2. Correlation between tissue factor (TF) surface expression and prothrombin fragment 1 + 2 (PTF_{1+2}) levels in whole blood, and quantification of the PTF_{1+2} generation by recombinant TF (rTF) in plasma. Left panel: TFsurface expression in response to live *Staphylococcus aureus* Cowan after 120 min was plotted against PTF_{1+2} levels from the same experiment. The two correlated significantly ($P < 0.0001$), as seen by the linear regression line and its associated 95% confidence band. $N = 66$. Right panel: lepirudin-anticoagulated human plasma was incubated with or without rTF (2.25 $\mu g\ mL^{-1}$) for 120 min, and the level of PTF_{1+2} was assayed. Coincubation with anti-TF (aTF; 10 $\mu g\ mL^{-1}$) reduced the otherwise strong increase in PTF_{1+2} levels, whereas IgG_1 (10 $\mu g\ mL^{-1}$), serving as a negative control (Ctrl) for aTF, did not. Neither infestin (400 $\mu g\ mL^{-1}$) nor the rTF control, albumin (10 $\mu g\ mL^{-1}$), affected the PTF_{1+2} levels. 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 Tukey's multiple comparisons *post hoc* test. MFI, median fluorescence intensity; NS, not significant; PBS, phosphate-buffered saline.

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_{1+2} was generated as a result of increased TF levels

To study whether coagulation, quantified as PTF_{1+2}, 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_{1+2} 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_{1+2} levels. Importantly, PTF_{1+2} generation was not completely abolished by blocking of TF. In a follow-up experiment conducted in lepirudin-anticoagulated plasma, recombinant TF induced PTF_{1+2} generation in a similar fashion to the whole bacteria (Fig. 2, right panel). This PTF_{1+2} 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_{1+2} 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_{1+2} than dead bacteria, whereas the levels of TF mRNA, TF surface expression and MP-TF increased comparably in

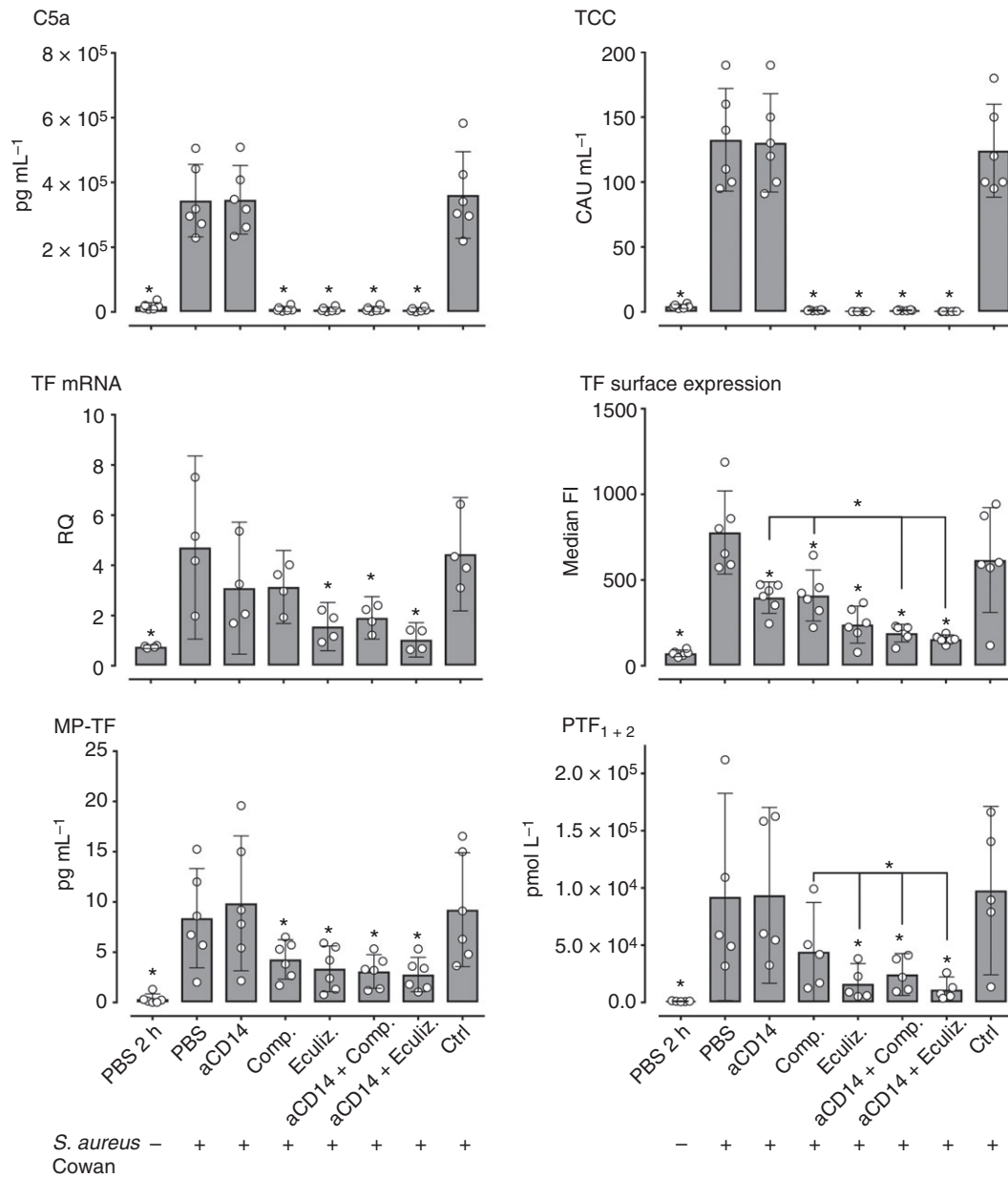**Fig. 3.** Responses to *Staphylococcus aureus* Cowan in human whole blood. Heat-inactivated *S. aureus* Cowan (1×10^8 mL^{-1}) increased the levels of complement fragment C5a and terminal complement complex (TCC) in plasma, and tissue factor (TF) mRNA, monocyte surface expression of TF, TF activity in microparticles (MP-TF) and prothrombin fragment 1 + 2 (PTF$_{1 + 2}$) levels in humans after 120 min of incubation as compared with the phosphate-buffered saline (PBS) control. All of these 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 TF monocyte surface expression only. The anti-CD14 antibody (aCD14) reduced TF surface expression, and this affect was potentiated by using one of the two complement inhibitors. 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 (four donors for TF mRNA data, as two samples were lost in analysis), 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 and Tukey's multiple comparisons *post hoc* tests. CAU, complement activating units; Ctrl, control; FI, fluorescence intensity; RQ, relative quantification.

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).
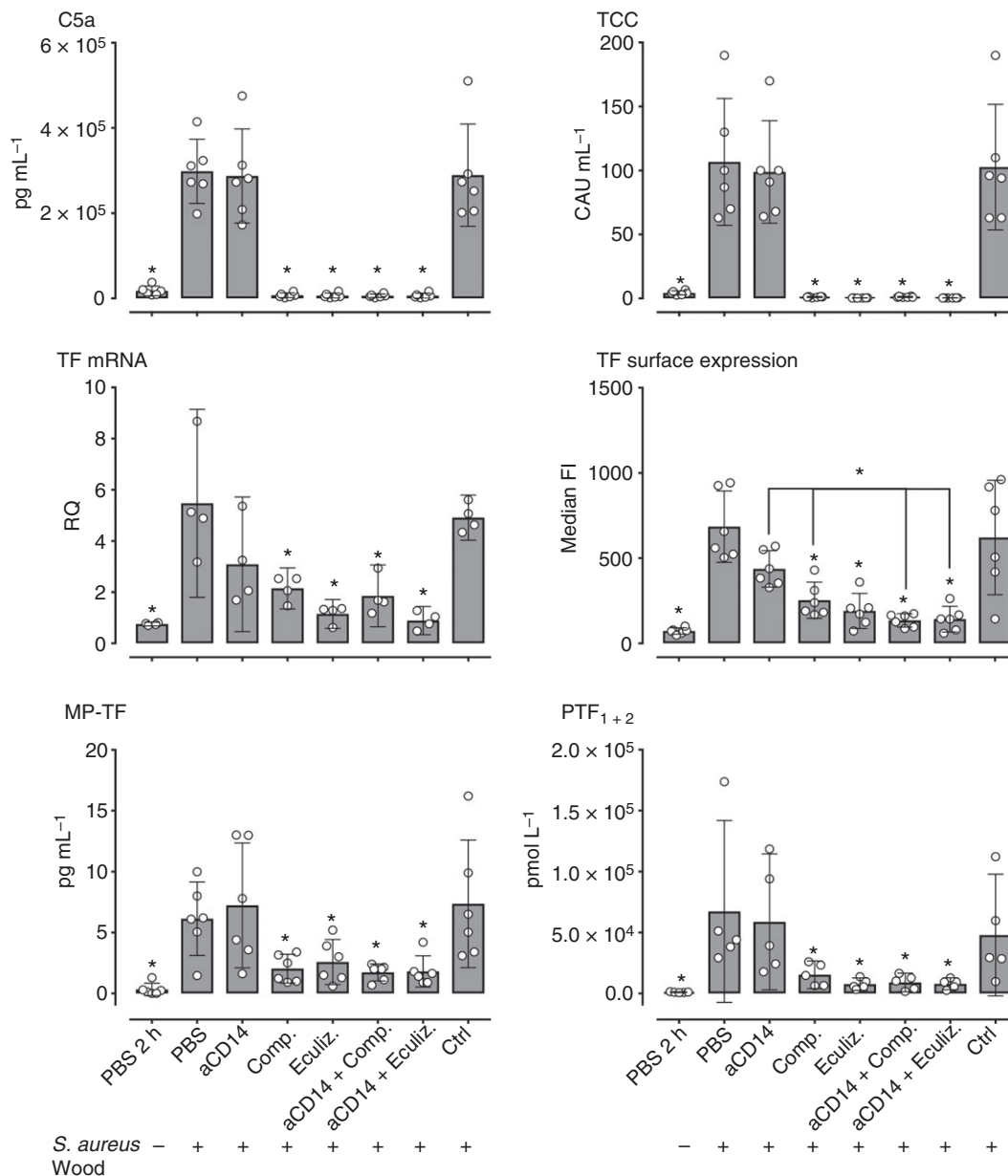
Fig. 4. Responses to *Staphylococcus aureus* Wood in human whole blood. Heat-inactivated *S. aureus* Wood (1×10^8 mL^{-1}) increased the levels of complement fragment C5a and terminal complement complex (TCC) in plasma, and tissue factor (TF) mRNA, monocyte surface expression of TF, TF activity in microparticles (MP-TF) and prothrombin fragment 1 + 2 (PTF$_{1 + 2}$) levels in human whole blood after 120 min of incubation as compared with the phosphate-buffered saline (PBS) control. All of these were reduced towards baseline levels when whole blood was preincubated with either the C3-inhibitory peptide compstatin (Comp.) or the C5-inhibitory antibody eculizumab (Eculiz.), as well as by the combination of either compstatin or eculizumab with the anti-CD14 antibody (aCD14). Although aCD14 exerted no inhibitory effect on its own, combined inhibition with compstatin and aCD14 had a significantly stronger effect on TF surface expression than compstatin alone. 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 PTF$_{1 + 2}$ 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; FI, fluorescence intensity; RQ, relative quantification.

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 PTF$_{1 + 2}$. Complement inhibition at the level of C5,
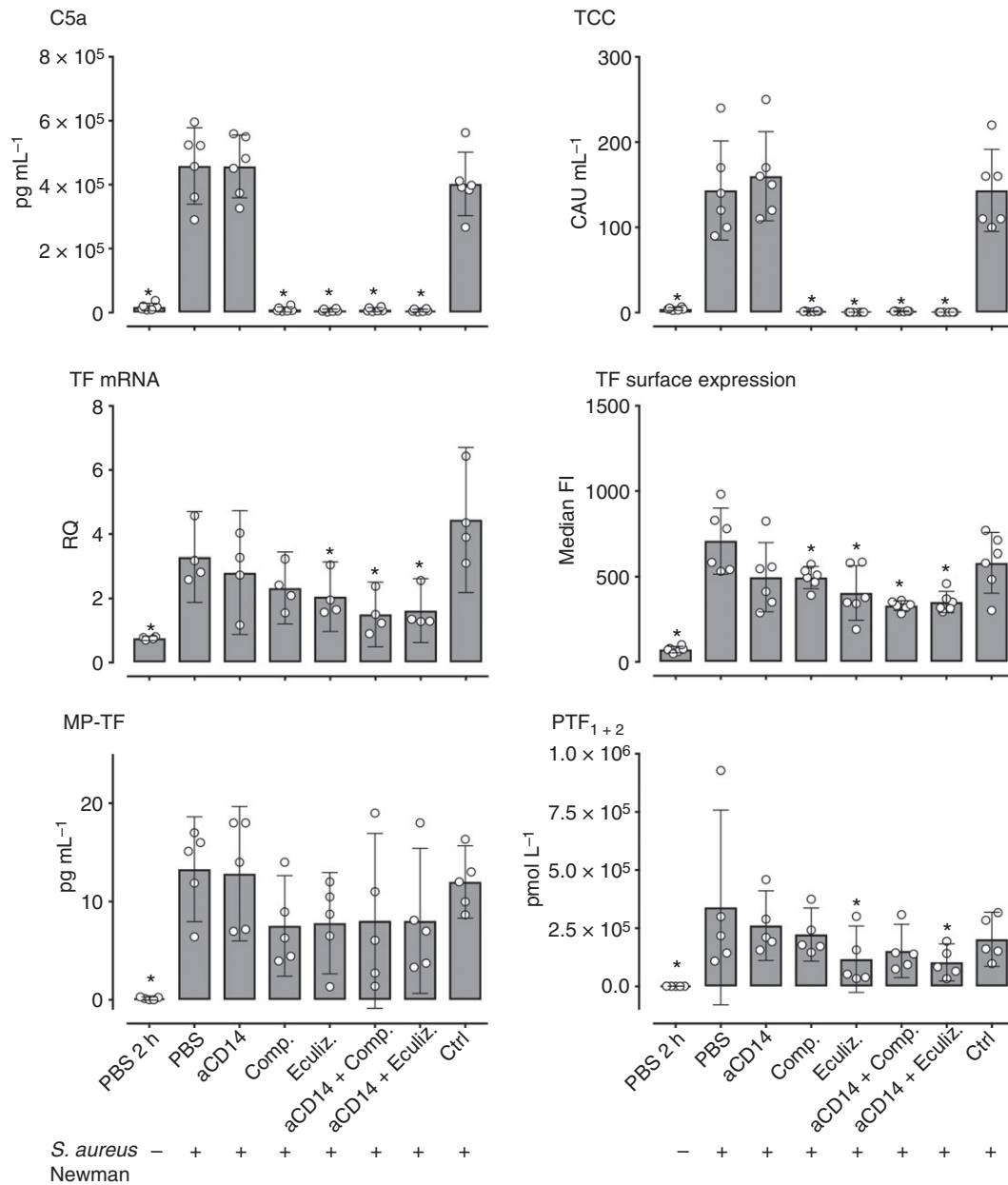
Fig. 5. Responses to *Staphylococcus aureus* Newman in human whole blood. Heat-inactivated *S. aureus* Newman (1×10^8 mL^{-1}) increased the levels of complement fragment C5a and terminal complement complex (TCC) in plasma, and tissue factor (TF) mRNA, monocyte surface expression of TF, TF activity in microparticles (MP-TF) and prothrombin fragment 1 + 2 (PTF$_{1 + 2}$) 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 PTF$_{1 + 2}$ 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.

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
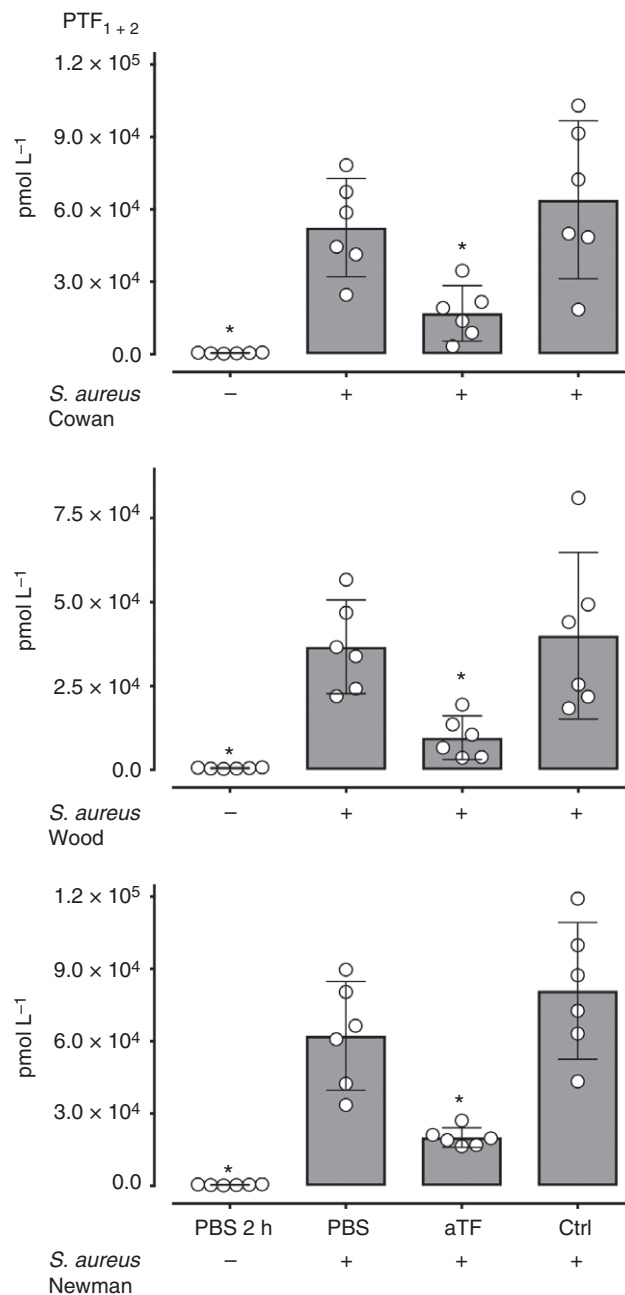
Fig. 6. Effect of a neutralizing anti-tissue factor antibody on *Staphylococcus aureus*-induced coagulation in human whole blood. Heat-inactivated *S. aureus* (1×10^8 mL^{-1}) increased prothrombin fragment $1 + 2$ (PTF$_{1+2}$) levels when added to human whole blood after 120 min of incubation as compared 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).

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
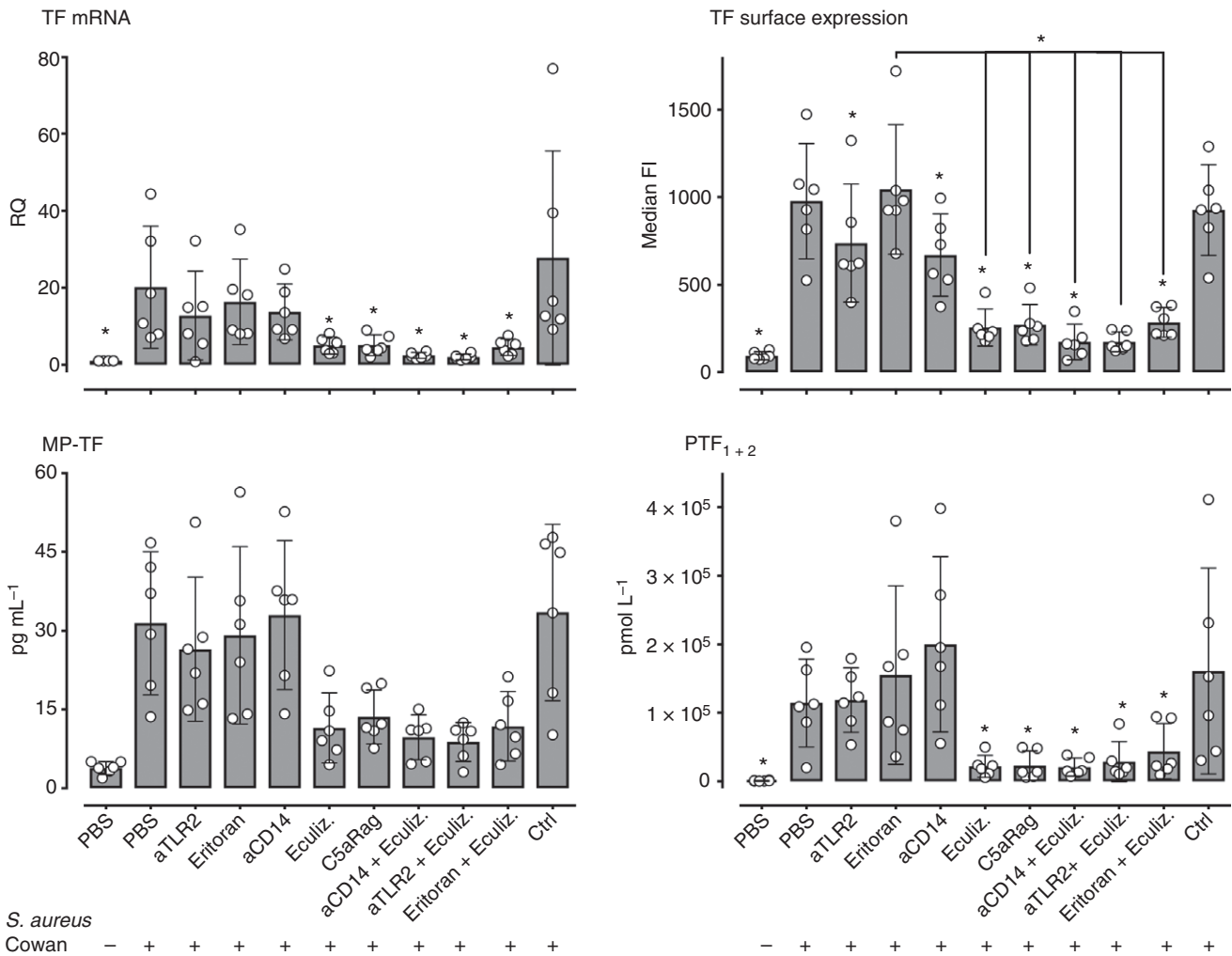
Fig. 7. Effects of selective C5a receptor 1, C5, Toll-like receptor (TLR) 2 and CD14 inhibition on *Staphylococcus aureus*-induced tissue factor (TF) generation and coagulation in human whole blood. Heat-inactivated *S. aureus* Cowan (1×10^8 mL^{-1}) increased TF mRNA, monocyte TF surface expression, TF activity in microparticles (MP-TF) and prothrombin fragment 1 + 2 (PTF$_{1 + 2}$) levels in human whole blood after 120 min of incubation as compared with the phosphate-buffered saline (PBS) control. All of these were reduced towards baseline levels when whole blood was preincubated with either the C5-inhibitory antibody eculizumab (Eculiz.) or the C5a receptor 1 antagonist (C5aRag). Both the anti-CD14 antibody (aCD14) and the anti-TLR2 mAb (aTLR2) significantly reduced the levels of TF expression on monocytes, but not any other parameter. Additional complement inhibition potentiated the effect of aCD14 and aTLR2 on the reduced TF surface expression. TLR4 blockade with eritoran had no inhibitory effect on the parameters measured. When combined with either complement inhibitor, all three TLR inhibitors significantly reduced the readings of all parameters measured. The rightmost column (control) represents stimulated whole blood preincubated with PBS containing 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 and Tukey's multiple comparisons *post hoc* tests. Ctrl, control; RQ, relative quantification.

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 PTF$_{1 + 2}$ 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 PTF_{1+2} were efficiently reduced by the neutralizing anti-TF mAb, which returned PTF_{1+2} levels back towards baseline. Also, PTF_{1+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 PTF_{1+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 PTF_{1+2}. Also, TF inhibition completely blocked PTF_{1+2} generation in response to recombinant TF in lepirudin-anticoagulated plasma, whereas some remnant PTF_{1+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 PTF_{1+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

Acknowledgements

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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