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Superantigens from *Staphylococcus aureus* induce procoagulant activity and monocyte tissue factor expression in whole blood and mononuclear cells via IL-1 β

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Summary. *Background:* *Staphylococcus aureus* is one of the most common bacteria in human sepsis, a condition in which the activation of blood coagulation plays a critical pathophysiological role. During severe sepsis and septic shock microthrombi and multiorgan dysfunction are observed as a result of bacterial interference with the host defense and coagulation systems. *Objectives:* In the present study, staphylococcal superantigens were tested for their ability to induce procoagulant activity and tissue factor (TF) expression in human whole blood and in peripheral blood mononuclear cells. *Methods and results:* Determination of clotting time showed that enterotoxin A, B and toxic shock syndrome toxin 1 from *S. aureus* induce procoagulant activity in whole blood and in mononuclear cells. The procoagulant activity was dependent on the expression of TF in monocytes since antibodies to TF inhibited the effect of the toxins and TF was detected on the surface of monocytes by flow cytometry. In the supernatants from staphylococcal toxin-stimulated mononuclear cells, interleukin (IL)-1 β was detected by ELISA. Furthermore, the increased procoagulant activity and TF expression in monocytes induced by the staphylococcal toxins were inhibited in the presence of IL-1 receptor antagonist, a natural inhibitor of IL-1 β . *Conclusions:* The present study shows that superantigens from *S. aureus* activate the extrinsic coagulation pathway by inducing expression of TF in monocytes, and that the expression is mainly triggered by superantigen-induced IL-1 β release.

Keywords: IL-1 β , monocytes, superantigens, tissue factor.

Introduction

Staphylococcus aureus is one of the most common Gram-positive bacteria in sepsis [1]. During severe sepsis and septic shock, a massive release of cytokines and activation of the coagulation system result in disseminated intravascular coagulation (DIC) and multiorgan dysfunction syndrome [2–5].

Monocytes and endothelial cells possess potential thrombogenic properties through their ability to express tissue factor (TF). TF, a single-chain transmembrane protein composed of 263 amino acid residues, is recognized as the major physiological initiator of blood coagulation [6]. TF binds to plasma factor (F)VII, forming a potent procoagulant complex, which in its turn can rapidly activate FIX and FX. Activated FX converts prothrombin into thrombin that subsequently converts soluble fibrinogen into insoluble fibrin [7]. Monocytes do not constitutively express TF but can be stimulated to do so by lipopolysaccharides (LPS), and peptidoglycan, components of the outer cell wall of Gram-negative and Gram-positive bacteria, respectively [8,9]. Since monocytes are the only peripheral blood cells capable of expressing TF, there has been considerable interest in measuring the procoagulant activity (PCA) of these cells, under pathophysiological conditions [3,10].

Gram-positive bacteria can trigger sepsis and septic shock by at least two mechanisms. Whole bacteria or cell wall components such as peptidoglycan, teichoic acid and lipoteichoic acid can activate a sequence of processes culminating in septic shock [1]. Furthermore, many Gram-positive bacteria produce exotoxins that activate several pathways involved in sepsis [11]. *S. aureus* can release toxins that are classified as superantigens, meaning that they can induce broad-spectrum T-cell activation without antigen specificity. Bacterial superantigens interact directly with MHC class II molecules on antigen-presenting cells, and in this context they stimulate T cells bearing particular variable-region β -chain elements irrespective of the other variable components of the T-cell receptors. As a result of the binding of superantigens, both antigen-presenting cells and T cells become activated [12,13].

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Toxic shock syndrome toxin (TSST)-1 is considered the major cause of toxic shock syndrome, which is characterized by fever, hypotension, a scarlatiniform rash, and the involvement of at least three major organ systems [14–16]. TSST-1 is unique in its ability to pass through mucosal surfaces [17]. Other staphylococcal superantigens are the enterotoxins: A, B, Cn, D, E and M [17]. These cause staphylococcal food poisoning syndrome and some of them also toxic shock syndrome [1,14].

Recently, we showed that intact *S. aureus* bacteria and staphylococcal peptidoglycan induced PCA and TF expression in human monocytes [9]. Some *S. aureus* strains produce soluble superantigens that have potent effects on the immune system without any presence of bacteria in the circulating blood [17]. Therefore, the present investigation was stimulated by the hypothesis that staphylococcal superantigens could induce TF production in human monocytes. In this study, we demonstrate that enterotoxin A (EA), enterotoxin B (EB), and TSST-1 from *S. aureus* induce PCA and TF expression in human monocytes. Furthermore, we show that the induction of TF expression is mainly induced via released interleukin (IL)-1 β .

Materials and methods

Special reagents

Polymyxin B, LPS from *Escherichia coli* strain O111:B4, EA, EB, and TSST-1 (lot no 071K4107, 110K4080 and 071K4108, respectively) were purchased from Sigma (St Louis, MO, USA). The purity of the toxins was confirmed by SDS electrophoresis. No significant contamination of EA or EB was detected in the EB and EA preparations, respectively. In addition, toxin-induced PCA in peripheral blood mononuclear cells (PBMC) and whole blood was the same both in the absence and presence of polymyxin B during incubations, excluding the presence of contaminating endotoxins. Citrated blood and plasma were obtained from healthy volunteers after informed consent. Goat antihuman TF immunoglobulin G (IgG) was a kind gift from Dr M. Kjalke (Novo Nordisk, Copenhagen, Denmark), and IgG purified from a goat immunized with human β_2 -microglobulin was used as a control. Human recombinant tumor necrosis factor (TNF)- α , IL-1 β , interferon (IFN)- γ , IL-1 receptor antagonist (IL-1Ra) and neutralizing monoclonal antibodies (MoAb) against TNF- α were from R&D (Abingdon, UK).

Isolation of human PBMC

PBMC were isolated essentially as previously described [9]. In short, PBMC were removed by centrifugation over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). The PBMC consisted of $27 \pm 6\%$ (mean \pm SD) monocytes and $73 \pm 7\%$ lymphocytes, as judged by flow cytometry, using side scatter and forward scatter characteristics and labelling of the cells by a combination of CD45 and CD14 monoclonal antibodies (Dakopatts, Glostrup, Denmark). The viability of the cells was $>99\%$ as judged by trypan blue exclusion.

Stimulation of PBMC or whole blood and determination of PCA

PBMC were resuspended in CO₂-Independent Medium (Invitrogen, Lidingö, Sweden), and 250 μ L cell suspensions (2.5×10^6 cells mL^{-1}) to which 50 μ L of stimuli dissolved in medium were added, and incubated on a rotator at 37 °C for 8 h, if not otherwise indicated. To exclude endotoxin contamination during the experiment, the toxins were preincubated with polymyxin B at a final concentration of 20 $\mu\text{g mL}^{-1}$ for 30 min, before they were added to PBMC. After thawing, 100 μL of plasma were incubated with 100 μL of 30 mM CaCl₂ in 1% bovine serum albumin at 37 °C for 1 min in a coagulometer (Amelung, Lemgo, Germany). Thereafter, 100 μL of cell suspension were added, and PCA was determined. All samples were analyzed in duplicate.

Whole blood (600 μL) was incubated with the different toxins (preincubated with polymyxin B as mentioned above), or LPS, 50 μL in Eppendorf tubes, with holes in the coverings to allow gas exchange, in 5% CO₂ at 37 °C for different time periods according to Santucci *et al.* [18]. After vortexing, 300 μL of blood were transferred to the coagulometer and recalcified with 40 μL of 100 mM CaCl₂ and clotting time was determined in the coagulometer. The responsiveness of recalcified plasma and whole blood to TF-induced coagulation was confirmed by the use of relipidated TF (Sigma). Standard curves were performed by adding increasing concentrations of purified TF to recalcified plasma and whole blood, respectively, whereafter clotting time was determined in the coagulometer. All samples were analyzed in duplicate. In experiments including anti-TF IgG, PBMC or whole blood was incubated with stimulus for 8 and 24 h, respectively. Thereafter, anti-TF IgG or control IgG was added to the cell suspension or blood sample (at a final concentration of 55 $\mu\text{g mL}^{-1}$), and incubated for another 30 min before analysis of PCA.

Detection of TF expression by flow cytometry

PBMC were incubated as described above in the absence or presence of toxins for 16 h. After incubation, the cells were put on ice and fixed with paraformaldehyde at a final concentration of 1% (w/v). Thereafter, the cells were incubated with a mixture of a phycoerythrin-conjugated monoclonal antibody against CD14 (Dakopatts) and a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against TF (American Diagnostica, Greenwich, CT, USA) or, as a control, CD14 antibody and an isotype-matched FITC-conjugated irrelevant monoclonal antibody (monoclonal antibody IgG1; Immunotech, Marseille, France) at the same concentration as the TF antibody. Monocytes were gated by their characteristics in side scatter and forward scatter, and their identity was further confirmed by their characteristic expression of CD14. The mean fluorescence intensity (MFI) for the control antibody, representing background, was subtracted from the MFI for the TF antibody.

Determination of TNF- α and IL-1 β by ELISA

PBMC were incubated in the presence or in the absence of toxins as described above. Thereafter, the cells were spun down (10 000 g, 10 min) and the supernatants were collected and stored at -70°C until analyzed for their content of TNF- α and IL-1 β in the supernatants by ELISA (R&D). The detection limit of both assays was 4 pg mL $^{-1}$.

Statistical analysis

For statistical analysis, one-way repeated measures analysis of variance was used, followed by Dunnett's-test when appropri-

ate. A value of $P < 0.05$ was considered to be statistically significant.

Results

EA, EB, and TSST-1 induce PCA in whole blood

EA, EB, TSST-1, and LPS were incubated with whole blood at various concentrations for 24 h. Subsequently, the blood was recalcified and the clotting time was determined. EA, EB and TSST-1 all induced PCA. EB and TSST-1 were weaker stimuli than EA (Fig. 1A). The lowest concentrations of toxins that caused significantly increased PCA were 100 ng mL $^{-1}$ (EA,

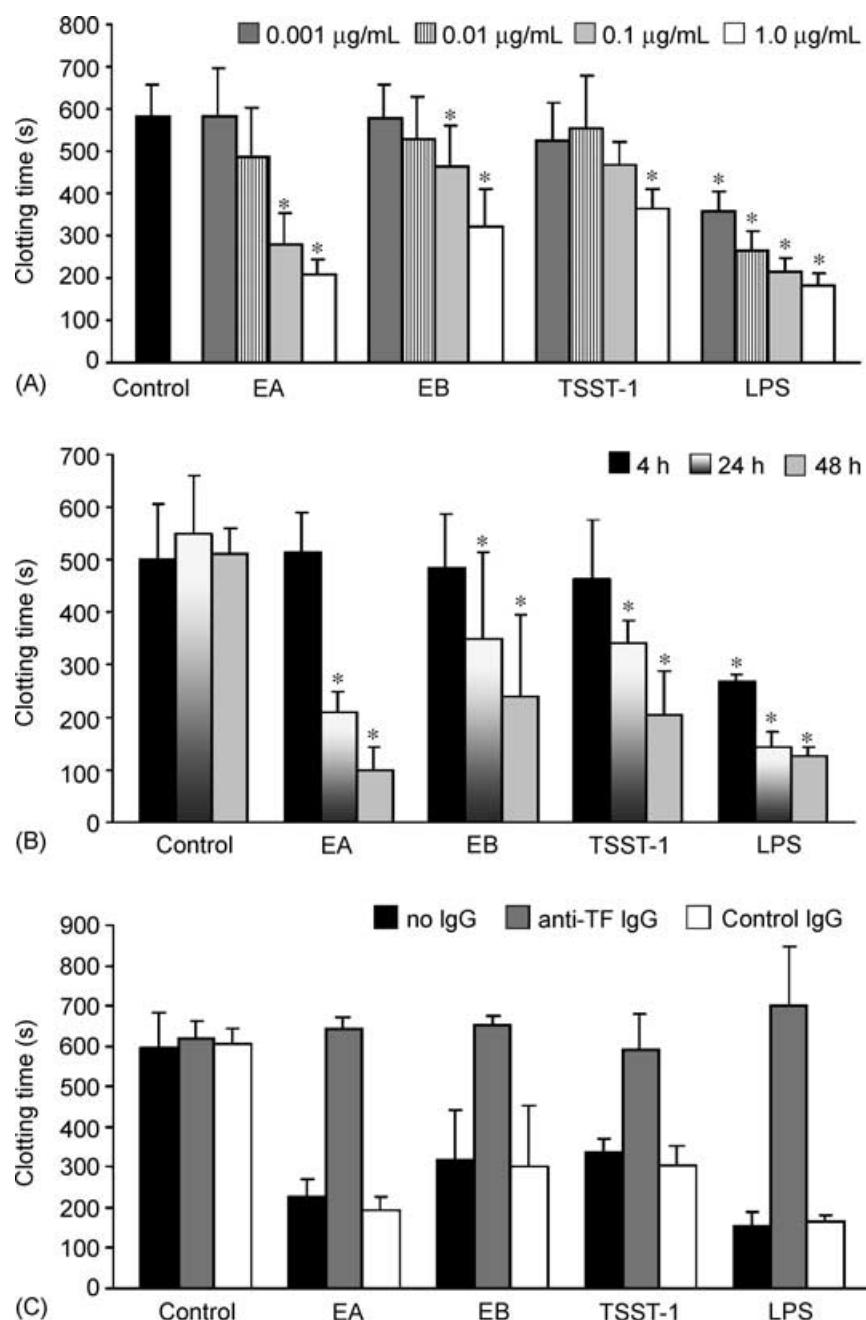


Fig. 1. Enterotoxin A (EA), enterotoxin B (EB) and toxic shock syndrome toxin (TSST-1) induce procoagulant activity (PCA) and tissue factor (TF) expression in whole blood. (A) Whole blood was incubated with medium alone or the indicated concentrations of EA, EB, TSST-1, or lipopolysaccharide (LPS) for 24 h. Clotting time was determined after recalcification of the blood. (B) Whole blood was incubated with medium alone or EA, EB, TSST-1, or LPS (1 $\mu\text{g mL}^{-1}$). Clotting time was determined after 4, 24, and 48 h of incubation. (C) Whole blood was incubated with medium alone or EA, EB, TSST-1, or LPS (1 $\mu\text{g mL}^{-1}$). After 24 h of incubation, TF-neutralizing goat IgG or control goat IgG was added to the cells, followed by another 30 min of incubation before determination of clotting time. Values are means \pm SD ($n=3$). * $P < 0.05$.

EB) and $1 \mu\text{g mL}^{-1}$ (TSST-1). LPS, used as a positive control, was much more potent on a weight basis than the toxins, showing a significantly increased PCA already at 1 ng mL^{-1} .

In order to study the kinetics of the increase in PCA induced by the toxins, whole blood was incubated with toxins or LPS ($1 \mu\text{g mL}^{-1}$) for different time intervals before recalcification of the blood and determination of clotting time. After 4 h incubation, no significant change in clotting time was observed in blood samples incubated with toxins, in contrast to LPS-stimulated blood, where a reduction in clotting time of 50% was observed (Fig. 1B). After 24 h of incubation, there was an increased PCA in blood stimulated with EA, EB and TSST-1, being most pronounced for EA. After another 24 h, i.e. 48 h of incubation, the PCA had increased further in staphylococcal toxin-stimulated blood, while LPS-stimulated blood reached a plateau with regard to PCA after 24 h of incubation. In the control, whole blood incubated with medium alone, no significant effects were observed.

Staphylococcal toxins induce PCA in PBMC

EA, EB, or TSST-1 at various concentrations, were incubated with human PBMC ($2.5 \times 10^6 \text{ cells mL}^{-1}$) for 8 h. Subsequently, the suspension was added to recalcified human plasma, and the clotting time was analyzed. Figure 2A shows that PCA was induced in a concentration-dependent fashion by the staphylococcal toxins with a threshold concentration at

1 ng mL^{-1} . In accordance with the experiments using whole blood, LPS was again a more potent inducer of PCA compared with the toxins. To investigate the kinetics of the PCA induced by the toxins, PBMC were incubated with $1 \mu\text{g mL}^{-1}$ of the toxins for different time intervals before determination of the clotting time. The data show that the toxin-induced PCA by PBMC is a rapid process with significant changes of PCA after 4 h of incubation (Fig. 2B). In order to exclude that endotoxins were contaminating the purified preparations of superantigens, thereby causing the PCA, the toxins were preincubated with polymyxin B (an antibiotic which neutralizes endotoxin) before the addition of toxins to PBMC or blood. Polymyxin B effectively inhibited the effect of 100 ng mL^{-1} LPS in the PCA assay but did not affect the PCA induced by the toxins (data not shown).

Expression of TF by monocytes is necessary for the PCA induced by staphylococcal toxins in PBMC and whole blood

To investigate whether TF was responsible for the PCA, TF-neutralizing IgG or control IgG was added to toxin-stimulated PBMC or whole blood after 4 h and 24 h of incubation, respectively, and thereafter incubated for another 30 min followed by determination of PCA. Anti-TF IgG effectively inhibited the increased PCA of toxin-stimulated PBMC and whole blood, showing that the PCA was dependent on TF expression (Fig. 1C). Clotting time of plasma, which had been incubated with EA-stimulated PBMC in the presence of medium, anti-TF IgG

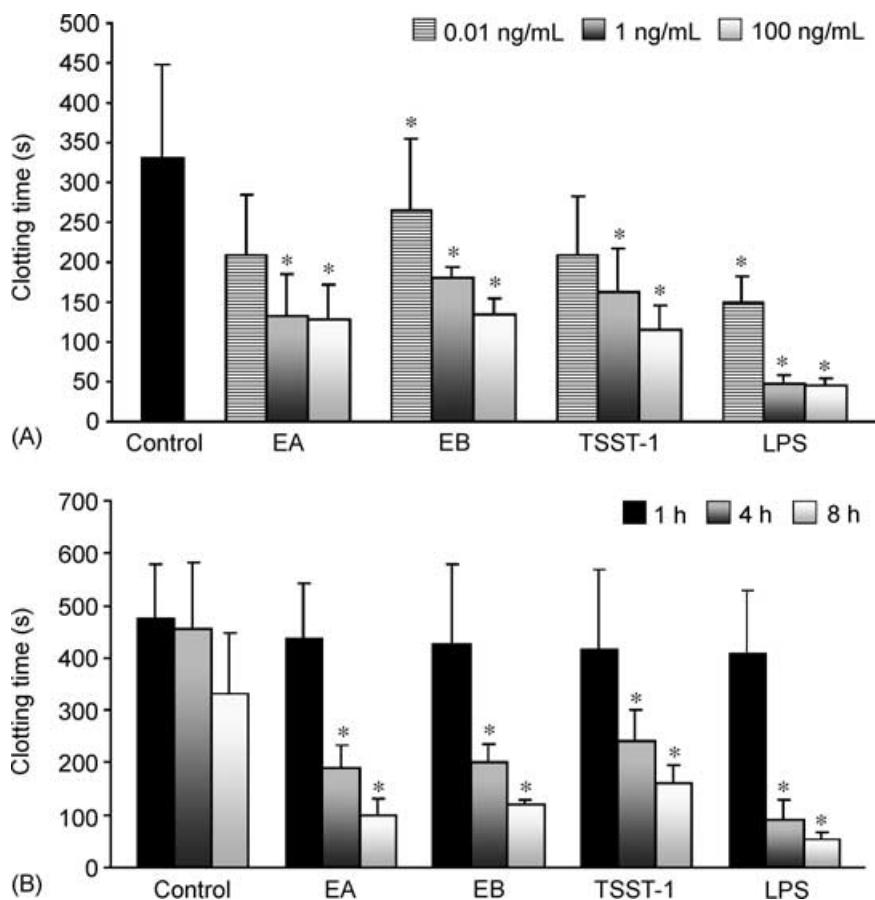


Fig. 2. Enterotoxin A (EA), enterotoxin B (EB) and toxic shock syndrome toxin (TSST)-1 induce procoagulant activity (PCA) and tissue factor (TF) expression in peripheral blood mononuclear cells (PBMC). (A) PBMC were incubated with medium alone or the indicated concentrations of EA, EB, TSST-1, or lipopolysaccharide (LPS) for 8 h. Clotting time was determined after exposure of the cells to recalcified plasma. (B) PBMC were incubated with medium alone or EA, EB, TSST-1, or LPS ($1 \mu\text{g mL}^{-1}$) for the indicated time periods. Subsequently, the cells were exposed to recalcified human plasma, and the clotting time was determined. Values are means \pm SD ($n = 3$). * $P < 0.05$.

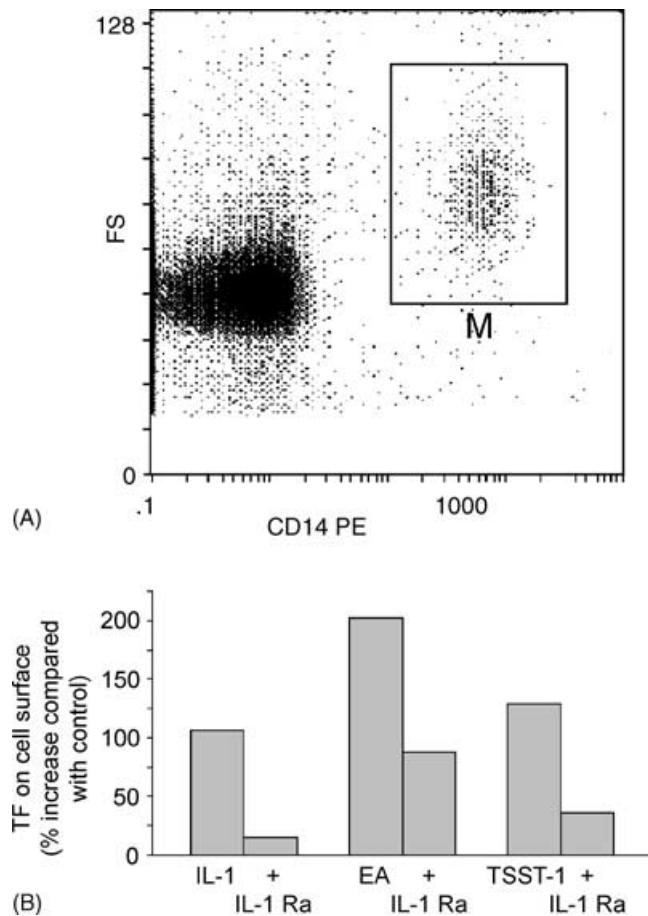


Fig. 3. Enterotoxin A (EA) and toxic shock syndrome toxin (TSST)-1 induce tissue factor (TF) expression on the surface of monocytes. Inhibition by interleukin-1 receptor antagonist (IL-1Ra). Peripheral blood mononuclear cells (PBMC) were incubated in medium alone, EA ($1 \mu\text{g mL}^{-1}$), TSST-1 ($1 \mu\text{g mL}^{-1}$), or IL-1 β (500 pg mL^{-1}) in the absence or presence of IL-1Ra (100 ng mL^{-1}) for 16 h. Cells were then incubated with phycoerythrin-conjugated CD14, FITC-conjugated TF, or isotype-matched control antibodies and assayed by flow cytometry. (A) Monocytes ($\text{M}\phi$) were gated using their characteristics in forward scatter and expression of CD14. (B) EA and TSST-1 increased TF expression on the surface of monocytes compared with cells incubated in medium alone. Addition of IL-1Ra reduced the increase in TF expression caused by EA and TSST-1, showing that TF expression is dependent on IL-1. As a control, cells were incubated with IL-1 β , in the absence or presence of IL-1Ra, showing that IL-1 β induces TF expression in monocytes and that IL-1Ra inhibits the effect. One experiment representative of five is shown.

or control IgG was 217 ± 104 , 555 ± 82 and 226 ± 75 s (mean \pm SD, $n = 4$), respectively. Using flow cytometry, it was found that TF was expressed on the surface of EA- and TSST-1-stimulated monocytes (EB was not tested). CD14 was used to verify the phenotype of monocytes, confirming that only monocytes expressed TF after stimulation with EA and TSST-1 (Fig. 3).

Toxin-induced TF expression in monocytes is dependent on the release of IL-1 β

Since superantigens from *S. aureus* can induce expression of TNF- α , IL-1 β and IFN- γ in human PBMC, and these cytokines

have the ability to induce TF expression in monocytes [15,19–22], it is a possibility that the toxin-induced PCA is caused by auto- and paracrine effects from these cytokines. To investigate this, PBMC ($2.5 \times 10^6 \text{ cells mL}^{-1}$) were incubated with human recombinant TNF- α , IL-1 β , or IFN- γ for 4 h. In our hands, TNF- α and IL-1 β both induced PCA in PBMC in a concentration-dependent fashion, while IFN- γ did not, despite the high concentrations tested (33 ng mL^{-1}) (Fig. 4A). TNF- α and IL-1 β were equally potent and about 500 pg mL^{-1} of the cytokines reduced the clotting time by 50% compared with the control (PBMC without stimulus). Thereafter, the cytokine content of supernatants from toxin-stimulated PBMC was investigated. PBMC were stimulated with toxins ($1 \mu\text{g mL}^{-1}$) for 4 h, subsequently TNF- α and IL-1 β of the supernatants were determined by ELISA. EA, EB, and TSST-1 all induced IL-1 β release from PBMC (Fig. 4B). Surprisingly, we could detect only low levels of TNF- α (in the order of 20 – 60 pg mL^{-1}) in the supernatants (data not shown).

To determine the role of IL-1 β in toxin-induced TF expression in human monocytes, PBMC were incubated with IL-1 β (500 pg mL^{-1}), EA ($1 \mu\text{g mL}^{-1}$), or TSST-1 ($1 \mu\text{g mL}^{-1}$) in the presence or absence of IL-1Ra (100 ng mL^{-1}), the naturally occurring inhibitor of IL-1 [23]. IL-1Ra was an efficient inhibitor of the PCA induced by IL-1 β (control), EA and TSST-1 (Figs 3 and 4C), suggesting that the induction of PCA by staphylococcal toxins is mainly mediated via released IL-1 β .

Discussion

Recent years of research have shown that coagulation is part of the innate immune system and during the progression of an infection, components released from the microbe can modulate the procoagulant and anticoagulant equilibrium of the host [24]. This sometimes leads to severe complications, such as disseminated intravascular coagulation combined with high mortality rates [25]. Most studies addressing this problem have been performed with LPS from the Gram-negative pathogen *E. coli*. In contrast, the molecular mechanisms behind Gram-positive sepsis are poorly understood. Thus, the present study aimed at investigating staphylococcal superantigens in their ability to trigger TF activity in monocytes. In the present study, we show that superantigens from *S. aureus* induce PCA in PBMC mainly via the release of IL-1 β , thereby linking the inflammatory response elicited by the superantigens to expression of TF and procoagulant activity in monocytes.

The clotting assays used reflect fibrin formation in whole blood and plasma. Purified relipidated TF produced a typical dose–response curve, showing that the assays indeed are sensitive to TF (data not shown). Toxin stimulation of both whole blood and PBMC resulted in a shortening of clotting times, probably reflecting increased TF activity. Addition of neutralizing TF antibodies confirmed that the shortening in clotting times was certainly caused by toxin-stimulated TF activity. The advantage of the clotting assay is that functional TF is determined while FACS and ELISA measure the TF antigen levels

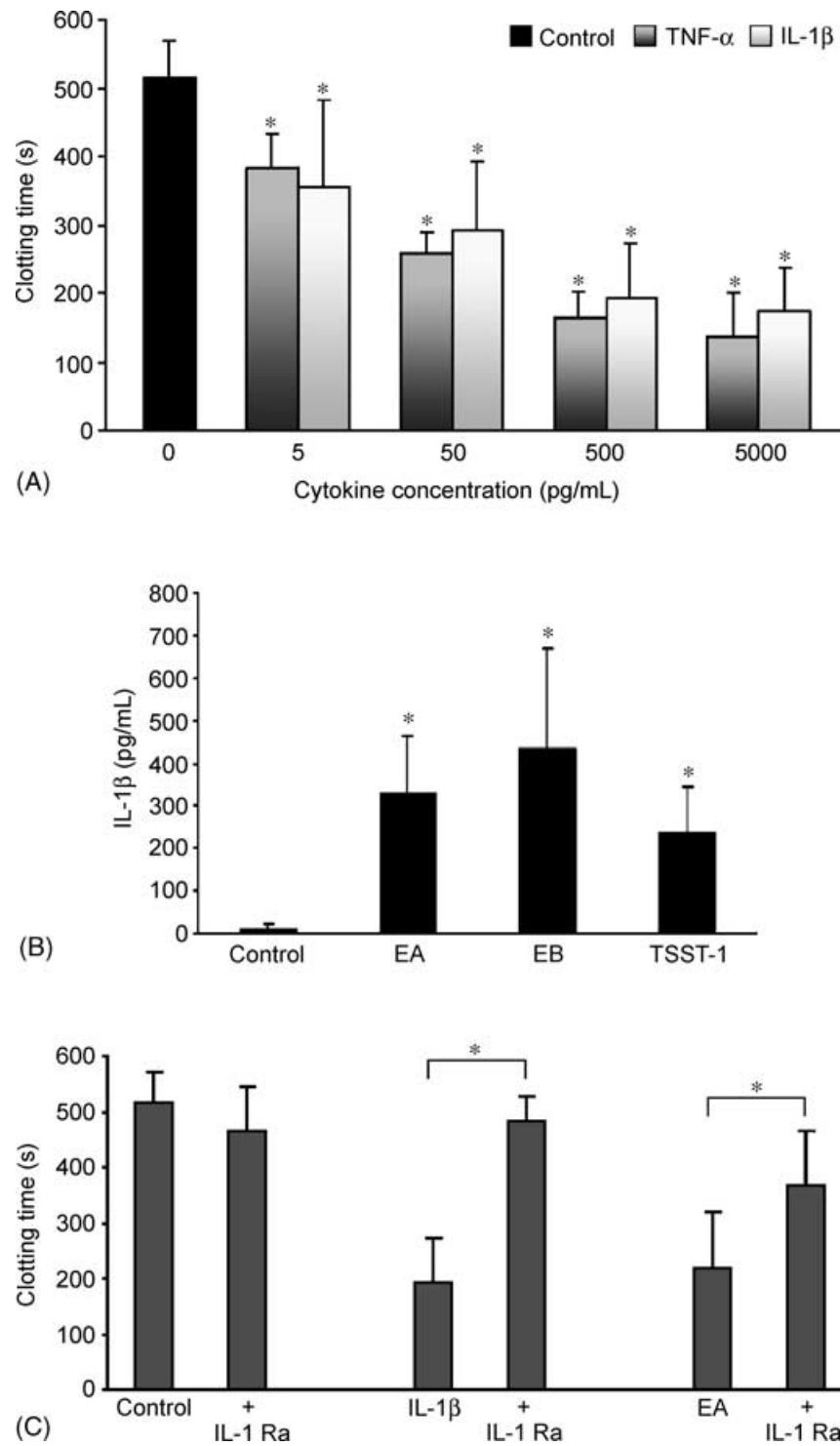


Fig. 4. Staphylococcal toxin-induced procoagulant activity (PCA) in peripheral blood mononuclear cells (PBMC) is mainly induced via interleukin (IL)-1 β . (A) PBMC were incubated with different concentrations of recombinant tumour necrosis factor (rTNF)- α or rIL-1 β for 4 h. Clotting time was determined in recalcified human plasma incubated with the cells. (B) PBMC were incubated with medium alone, enterotoxin A (EA), enterotoxin B (EB), or toxic shock syndrome toxin (TSST)-1 (1 μ g mL^{-1}) for 4 h. IL-1 β in the supernatants was determined by ELISA (pg mL^{-1}). (C) PBMC were incubated with medium alone, IL-1 β (500 pg mL^{-1}), or EA (1 μ g mL^{-1}) in the absence or in the presence of IL-1Ra (100 ng mL^{-1}) for 4 h. Thereafter clotting time was determined in recalcified human plasma. Values are means \pm SD ($n = 3$). * $P < 0.05$.

that may not correlate with levels of functional TF activity due to encryption [26]. The increase in PCA induced by the staphylococcal toxins should therefore reflect an upregulation of TF on the cell surface. However, a remodeling of the phospholipid composition of the outer leaflet of the cells by the toxins or IL-1 β could contribute to the effect [26]. However, a clear distinction is not possible at the moment and further experiments are required to address this question.

The threshold dose of toxin needed to generate PCA was higher in whole blood compared with PBMC incubations. Also the time kinetics was different comparing toxin-stimulated PBMC and whole blood, with PCA being more rapidly induced in PBMC. Further, LPS showed a difference between its induction of PCA in PBMC and whole blood, but it was not as pronounced as for the toxins. Whole blood represents of course the most *in vivo*-like situation and experimental set-ups

using isolated cells rarely replicate the complex networks of interactions between humoral and cellular agents. The fact that specific antibodies to EA, EB and TSST-1 are present in most normal sera [27] could explain why higher concentrations of the toxins are needed to induce TF activity in whole blood, where neutralizing antibodies are present. In contrast, PBMC were stimulated by the toxins in artificial medium that lacks antibodies. Further, the whole blood assay contains coagulation factors and anticoagulant factors that are absent during the stimulatory phase of the PBMC assay. The continuous presence of coagulation factors in the whole blood assay could result in a change of their properties, or alternatively, there may be an activation of anticoagulant pathways. The first suggestion is, however, unlikely since the clotting time remained unchanged in the controls during incubation time as long as 48 h. The finding that peptidoglycan induced the same PCA (dose-response and time kinetics) in the two assays rules out that minor differences in the ratio citrate and CaCl_2 in the assays should play a role (data not shown).

Since staphylococcal exotoxins can induce TNF- α , IL-1 β , and IFN- γ expression in PBMC and these cytokines can activate coagulation, we raised the question whether the toxin-induced PCA was a direct effect on the monocytes or was mediated by cytokines. TNF- α and IL-1 β induced PCA in PBMC in a dose-dependent manner, while IFN- γ did not. Further, we were able to detect IL-1 β in the supernatants from toxin-stimulated PBMC, but surprisingly only low amounts of TNF- α . To investigate possible roles for the cytokines further, toxin-stimulated PBMC were incubated in the absence or presence of IL-1Ra or neutralizing MoAb against TNF- α . The effect on PCA induced by the staphylococcal toxins was almost abrogated in the presence of IL-1Ra (Fig. 4c), but was not changed in the presence of MoAb against TNF- α (data not shown). This result explains findings from *in vivo* studies. While TNF- α can induce TF expression on monocytes *in vivo*, it is not a key player in the activation of coagulation during sepsis. This has become evident from studies where pretreatment of volunteers and baboons with anti-TNF- α before exposure to endotoxin did not affect activation of coagulation but suggest an important function for TNF- α in triggering the fibrinolytic system [28,29]. In contrast, administration of IL-1Ra resulted in inhibition of both the coagulation cascade and fibrinolysis in patients with sepsis [30]. These *in vivo* data emphasize the role of IL-1 β as an activator of the coagulation system, in parallel to what is found in the *in vitro* experiments in the present study.

Clinical studies have illustrated that the inflammatory response and coagulation are linked systems in sepsis, and strict control of both systems is vital to the host [31,32]. In these patients, activation of the extrinsic coagulation pathway triggered by TF induced increased coagulation activity [33]. TF pathway inhibitor, a naturally occurring inhibitor of TF, has proven to improve significantly the mortality rate in models of superantigen-induced shock, and recently protein C, a naturally occurring inhibitor of proinflammatory cytokines and thrombin, infused to patients with severe sepsis resulted in a reduction of mortality rate [31–33].

The present investigation indicates that the superantigens EA, EB and TSST-1 from *S. aureus* induce little or no direct PCA in monocytes on their own, but act indirectly via the induction of IL-1 β release from human PBMC. Greater knowledge of the host response against bacterial superantigens may elucidate novel therapeutic strategies in severe *S. aureus* infection.

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