Chapter 8
The Effects of Selective Complement and CD14 Inhibition on the *E. coli*-Induced Tissue Factor mRNA Upregulation, Monocyte Tissue Factor Expression, and Tissue Factor Functional Activity in Human Whole Blood

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Abstract *Background*: The complement pathway and CD14 play essential roles in inflammation, but little is known about the relative roles of complement and CD14 in *E. coli*-induced tissue factor (TF) mRNA upregulation, expression by monocytes, and functional activity in human whole blood. *Methods*: Whole *E. coli* bacteria were incubated for up to 4 h in human whole blood containing the anticoagulant lepirudin, which does not affect complement activation. TF mRNA levels were analyzed using reverse transcription, quantitative real-time PCR (RT-qPCR), and the expression of TF on the cell surface was analyzed using flow cytometry. Complement was selectively inhibited using the C3 convertase inhibitor compstatin or a C5a receptor antagonist (C5aRa), while CD14 was blocked by an anti-CD14 $F(ab')_2$ monoclonal antibody. *Results*: The *E. coli*-induced TF mRNA upregulation was reduced to virtually background levels by compstatin, whereas anti-CD14 had no effect. Monocyte TF expression and TF activity in plasma microparticles were significantly reduced by C5aRa. Anti-CD14 alone only slightly reduced *E. coli*-induced monocyte TF expression but showed a modest additive effect when combined with the complement inhibitors. Inhibiting complement and CD14 efficiently reduced the expression of the *E. coli*-induced cytokines IL-1β, IL-6, IL-8, and platelet-derived growth factor bb. *Conclusion*: Our results indicate that *E. coli*-induced TF mRNA upregulation is mainly dependent on complement activation, while CD14 plays a modest role in monocyte TF expression and the plasma TF activity in human whole blood.

8.1 Introduction

Sepsis almost invariably leads to hemostatic abnormalities ranging from insignificant blood test changes to severe disseminated intravascular coagulation (DIC), which may cause bleeding or microvascular dysfunction and contributes to organ failure (Levi et al. 2006). Gram-negative bacteria such as *Escherichia coli* are important pathogens that cause urinary tract infections and sepsis in humans.

Tissue factor (TF) is the main initiator of blood coagulation in vivo. TF is a 47 kDa, type I transmembrane glycoprotein that initiates coagulation by binding to factor VII/VIIa and the active serine protease FVIIa (Siegbahn 2000). The TF/factor VIIa complex has both procoagulant capabilities and

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signaling activities (Mackman 2009). TF is constitutively expressed in the cells surrounding the blood vessels and large organs, forming a hemostatic barrier (Mackman 2009). The total lethality of homozygous TF knockout mouse embryos indicates that TF is indispensable for life (Siegbahn et al. 2005). During infection, bacteria and lipopolysaccharides (LPS) also induce TF expression in endothelial cells and monocytes (Siegbahn 2000). TF activity may also be present in plasma microparticles derived from activated platelets or apoptotic monocytes in the blood (Henriksson et al. 2005). These intravascular microparticles can fuse with activated platelets (Del Conde et al. 2005) and bind to endothelial cells, resulting in their concentration at sites of injury (Coughlin 2000). In healthy individuals, monocytes express traces of TF encrypted in the cell membrane (Osterud et al. 2008).

The most studied inducer of TF expression in vivo and in vitro is LPS, the main component of the outer membrane of Gram-negative bacteria (Hiller et al. 1977). LPS-induced TF expression by monocytes is dependent on the recognition molecule CD14 (Meszaros et al. 1994). LPS binds to LPS-binding protein (LBP) in the plasma, and the LBP-LPS complex binds to the cell surface receptor CD14 (Schumann et al. 1990). Membrane-bound CD14 then transports LPS to the co-receptor MD-2, which induces Toll-like receptor-4 (TLR-4) dimerization and activates a signaling cascade via myeloid differentiation protein 88 (MyD88) (Malarstig and Siegbahn 2007). Thereafter, the NF*k*B subunits p50 and p65 translocate into the nucleus for the initiation of transcription, leading to the upregulation of TF and many other inflammatory mediators, including a number of cytokines (Oeth et al. 1994). Other proinflammatory mediators, such as immune complexes (Lyberg and Prydz 1982), complement (Prydz et al. 1977), oxidized low density lipoprotein (Eligini et al. 2002), tumor necrosis factor-α (TNF)-α, IL-1α and IL-1β (Osnes et al. 1996), monocyte chemoattractant protein-1 (Ernofsson and Siegbahn 1996), platelet-derived growth factor bb (Ernofsson and Siegbahn 1996), C-reactive protein (Ernofsson et al. 1996), and tethering adhesion molecules (Kappelmayer et al. 1993), also induce TF expression in monocytes in vitro. In a whole blood environment, however, only LPS, immune complexes, and adhesion induce TF expression in monocytes (Osterud 1995).

The activation of the complement system is a hallmark of severe Gram-negative sepsis and is related to disease severity (Brandtzaeg et al. 1989). *E. coli* bacteria mainly activate the alternative pathway (Mollnes et al. 2002). Blocking the C5a-receptor during experimental, sepsis greatly improves survival in rodents, supporting a key role of complement activation in sepsis (Riedemann et al. 2003). LPS-induced septic shock in rats can be mimicked by the injection of C5a, and blocking C5a with an antibody attenuates LPS-induced responses (Ward 2004). However, whole *E. coli* bacteria activate the complement system more potently than *E. coli*-derived LPS (Brekke et al. 2007). *E. coli*-induced complement activation results in the release of C5a, rapidly upregulates CD11b and enhances the oxidative burst in human whole blood granulocytes and monocytes (Mollnes et al. 2002). This *E. coli*-induced CD11b upregulation and oxidative burst were completely blocked by inhibiting C5a and CD14 (Brekke et al. 2007).

Therefore, the purpose of the present study was to examine the separate and combined effects of complement and CD14 inhibition on *E. coli*-induced TF mRNA upregulation, monocyte TF expression, and plasma TF activity in human whole blood. The anticoagulant lepirudin was used because, in contrast to heparin and calcium-binding anticoagulants, it does not affect complement activation (Mollnes et al. 2002).

8.2 Materials and Methods

8.2.1 Antibodies and Reagents

All equipment, including tubes, tips, and solutions, were endotoxin-free. Polypropylene tubes from Nalgene Nunc (Roskilde, DK) were used to obtain low background activation of complement. Sterile phosphate-buffered saline (PBS) with or without Ca^{2+} and Mg^{2+} was from Life Technologies (Paisley, UK).

Lepirudin (Refludan®) was obtained from Hoechst (Frankfurt am Main, Germany). Ultrapure (up) *E. coli* LPS (strain O111:B4) was purchased from InvivoGen (San Diego, CA). Recombinant human apo-tissue factor was purchased from Calbiochem® Merck Biosciences Gmbh (Swalbach, Germany). A potent analog (Ac-I[CV(1MeW)QDWGAHRC]T-NH_2) of the C3 convertase inhibitor compstatin (Katragadda et al. 2006), its corresponding control peptide (Ac-IAVVQDWGAHRAT-NH_2), and the specific C5a-receptor-antagonist (AcF[OPdChaWR]) (C5aRa) were synthesized, as previously described (Strey et al. 2003). Murine antihuman CD14 $F(ab')_2$ (clone 18D11) was obtained from Diatec AS (Oslo, Norway). The optimal inhibitor concentration was determined in separate dose-response experiments (data not shown). All inhibitors were checked for LPS contamination using an endotoxin kit from Cape Cod Inc. (East Falmouth, MA) or QCL-1000 from BioWhittaker (Walkersville, MD), with the final LPS contamination measuring in the low pg/mL range.

8.2.2 Bacterial Preparation

Nonopsonized *E. coli* strain LE392 (ATCC 33572) was obtained from the American Type Culture Collection (Manassas, VA). The bacteria were grown, heat-inactivated, and washed nine times in PBS without Ca^{2+} and Mg^{2+} to remove extracellular LPS, as previously described (Mollnes et al. 2002). A stock solution of 0.5×10^9 bacteria per mL PBS was stored at +4°C. The LPS concentration in the bacterial supernatant was 5 ng/mL and was diluted to 580 pg LPS/mL when the final *E. coli* concentration was 14.2×10^6 cells/mL.

8.2.3 Whole Blood Model of Sepsis

Blood from healthy adult donors was obtained according to guidelines from the Regional Ethics Committee after informed consent. Experiments with each blood sample were performed as single experiments at different time points. In brief, fresh venous human blood was drawn from an antecubital vein into sterile polypropylene tubes containing lepirudin (50 μg/mL). Aliquots (500 μL) of whole blood were then preincubated in PBS with Ca^{2+} and Mg^{2+} or antibodies or inhibitors in PBS (100 μL or 14.3% (v/v)) for 4 min in sterile polypropylene tubes as indicated. *E. coli, E. coli* LPS or PBS (100 μL or 14.3% (v/v)), was then added, and the samples were incubated at 37°C for the time indicated. After incubation, the samples were immediately processed for flow cytometry. The plasma was harvested by centrifugation ($3{,}220 \times g$, 15 min), and samples were stored at −80°C.

8.2.4 RT-qPCR of TF mRNA Expression

Total RNA was extracted from lepirudin-treated whole blood using the total RNA chemistry protocol from Applied Biosystems (ABI, Foster City, CA). Whole blood samples were centrifuged ($3{,}220 \times g$, 15 min), and two volumes of 1× nucleic acid purification lysis solution (ABI) were added to the blood cells. The lysate was stored at −70°C. Total RNA was extracted using an ABI PRISM™ 6100 Nucleic Acid PrepStation in accordance with the manufacturer's protocol. The RNA was quantified by UV spectroscopy using the Gene Quant II (Pharmacia Biotech, Cambridge, UK). Thereafter, cDNA was synthesized from approximately 0.2 μg RNA using the High-Capacity cDNA Archive Kit (ABI) and the 2720 Thermal Cycler (ABI). RT-qPCR was performed with TaqMan Universal PCR Master Mix (ABI) and predeveloped TaqMan® gene expression assays (ABI). The gene expression assays used were TF (Hs00175225_m1) and beta-2-microglobulin (assay ID 4333766F). Beta-2-microglobulin was used as the endogenous control. The PCR was performed in triplicate in a single-plex reaction

containing a 25-μL final volume using the 7500 Real-Time PCR System (ABI) in accordance with the manual. The threshold cycles (Ct) were determined by comparing all samples with the zero time calibrator (T0). The T0 value was set to one using the delta-Ct method for relative quantification.

8.2.5 Flow Cytometry

TF and CD14 were detected simultaneously using a FITC-conjugated murine antihuman TF antibody (product no. 4507CJ, clone VD8, American Diagnostica Inc., Stamford, CT) and a PE-conjugated anti-CD14 antibody (Becton Dickinson, San Jose, CA). A mouse IgG1-FITC antibody (Becton Dickinson) served as an isotype Fc gamma control. The antibodies were added to 25-μL blood and incubated for 15 min in the dark. Thereafter, 500-μL EasyLyse (Dako, Glostrup, DK) was added, and samples were incubated for another 15 min in the dark. The samples were centrifuged (290 × *g*, 5 min), 300-μL PBS was added, and samples were analyzed using a FACScalibur cytometer from BD. The monocytes were gated using a CD14 PE/SSC dot plot, and the median fluorescence intensity (MFI) for the gamma control was subtracted from the MFI indicating TF expression.

8.2.6 Quantification of TF Activity in Plasma Microparticles

Whole blood samples were centrifuged as described above, and the TF functional activity in plasma microparticles from platelet-poor plasma was analyzed as described by Engstad et al. (1995). Briefly, microparticles were isolated from previously isolated platelet-poor plasma by ultracentrifugation at 40,000 × *g* for 1 h 30 min at 4°C, followed by resuspension in 200-μL 0.15 M NaCl and freezing at −70°C. Samples were then thawed and tested for TF activity. TF was measured in a two-stage amidolytic assay based on the ability of TF to accelerate the activation of FX by FVIIa, followed by the FXa conversion of prothrombin to thrombin in the presence of activated FV. Thrombin was quantified using the Th-1 substrate, and the amount of color generated was determined spectrophotometrically at 405 nm using a microplate reader. Crude rabbit brain extract was used as a standard for TF activity, with an undiluted activity assigned at 1,000 mU/mL.

8.2.7 ELISA of the Terminal Complement Complex (TCC)

The activation of the complement cascade was analyzed by measuring the soluble C5b-9 terminal complement complex (TCC) using an ELISA as previously described (Mollnes et al. 1985). The monoclonal antibody aE11, which reacts with the C9 neoepitope exposed after incorporation in the C5b-9 complex, was used as the capture antibody, and a biotinylated anti-C6 monoclonal antibody (Quidel Corporation, San Diego, CA) was used as the detection antibody. The results were expressed in arbitrary units/mL (AU/mL), using human serum activated with zymosan as a positive control set to 1,000 AU/mL.

8.2.8 Cytokine Analysis

Lepirudin plasma was prepared by centrifugation, as described above. The cytokine levels in the plasma were analyzed using the microsphere-based Bio-Plex Human Cytokine 27-plex (27 different cytokines, interleukins, and growth factors) Assay (Bio-Rad, Hercules, CA), as previously described (Brekke et al. 2008).

8.2.9 Statistics

The data were analyzed using the SigmaStat version 3.5 statistical program (Systat Software Inc., Chicago, IL). The data were analyzed by one-way, repeated measures ANOVA using Dunnett's post hoc multiple comparisons. Data were compared to *E. coli* alone after 2 h. The data were transformed logarithmically if the normality test failed. $p < 0.05$ was considered statistically significant.

8.3 Results

8.3.1 The Effect of Selective Complement and CD14 Inhibition on E. coli-Induced TF mRNA Upregulation in Human Whole Blood

Incubation with *E. coli* (14.2×10^6/mL) for 2 h in fresh human whole blood increased TF mRNA levels approximately sixfold compared to the spontaneous control (Fig. 8.1). The C3 convertase inhibitor compstatin abolished *E. coli*-induced TF mRNA upregulation ($p < 0.05$). C5aRa also markedly reduced *E. coli*-induced TF mRNA upregulation, although the reduction did not reach statistical significance. Furthermore, inhibiting both complement and CD14 had no additional effect. In comparison, selective CD14 inhibition using anti-CD14 had no effect on *E. coli*-induced TF mRNA upregulation. Similar results were found using a higher *E. coli* concentration (data not shown). The incubation of whole blood with PBS only for 2 h led to the spontaneous upregulation of TF mRNA (Fig. 8.1, first left column) as compared to baseline levels at time zero, which were set to RQ = 1 in the assay.

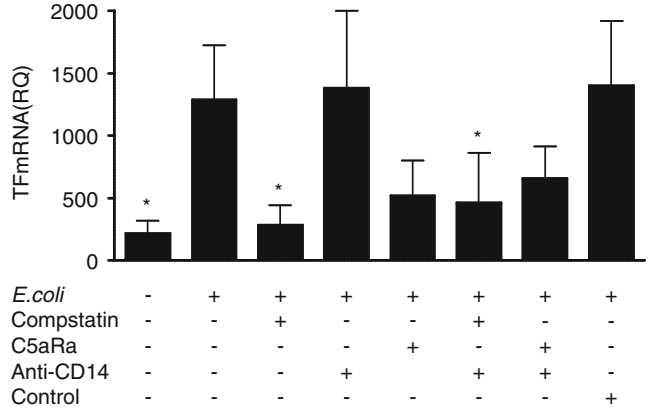

Fig. 8.1 *The effect of selective complement and CD14 inhibition on E. coli-induced TF mRNA upregulation in human whole blood. E. coli* (14.2×10^6/mL) was incubated with human whole blood in the presence of compstatin (25 μM), a C5a receptor antagonist (C5aRa, 10 μg/mL), anti-CD14 $F(ab')_2$ (anti-CD14, 50 μg/mL), or combination thereof. A control peptide (10 μg/mL) and a control $F(ab')_2$ (50 μg/mL) were added in combination as a control (Control). Total RNA was extracted from whole blood and TF expression was analyzed using RT-qPCR. The TF mRNA level in the spontaneous control after incubation with PBS only is indicated (first bar from the *left*). The fold change in TF mRNA relative to the baseline sample at time zero is expressed as relative quantitation (*RQ*). Values are means ±SD (n = 4) using separate blood donors in each experiment. $^*p < 0.05$ compared to *E. coli* in the absence of inhibitors

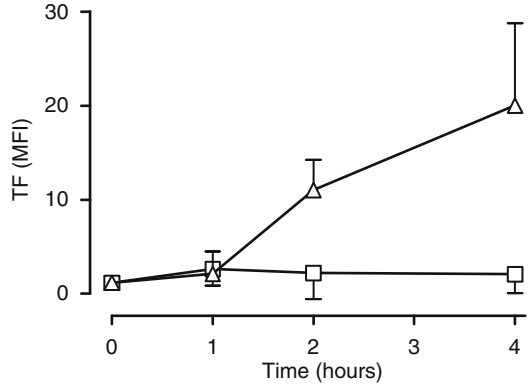

Fig. 8.2 *Time course of E. coli-induced TF expression in whole blood monocytes*. Human whole blood was incubated in the absence (*open square*) or presence (*open triangle*) of *E. coli* (14.2×10^6/mL). The expression of TF on the surface of monocytes was analyzed using flow cytometry at the time points indicated and is expressed as MFI. Values are means ±SD from two to three separate experiments using different blood donors each time

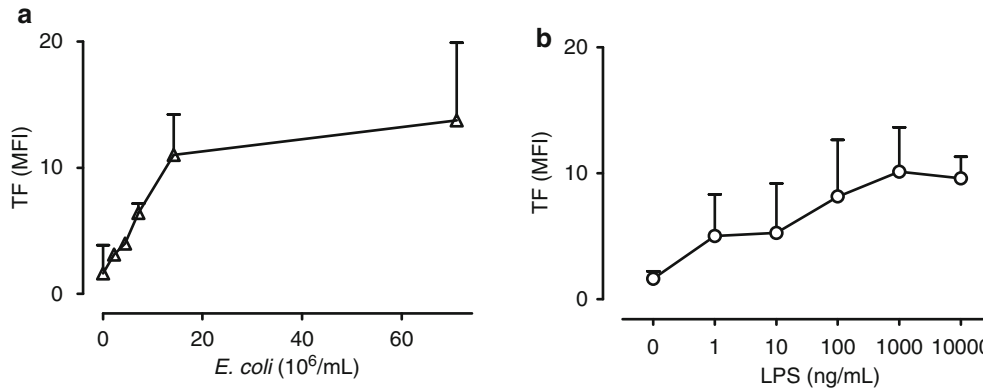

Fig. 8.3 *The dose-response effect of E. coli bacteria and ultra-purified E. coli LPS on monocyte TF expression in human whole blood*. (**a**) The dose-response effect of *E. coli* bacteria (*open triangles*) and (**b**) ultra-purified *E. coli* LPS (*open circles*) on monocyte TF expression. TF surface expression was analyzed using flow cytometry and is expressed as MFI. Values are means ±SD from two to three separate experiments using different blood donors each time

8.3.2 Time Course of E. coli-Induced TF Cell Surface Expression on Monocytes and Dose-Response Effect of LPS

The incubation of whole blood with *E. coli* (14.2×10^6/mL) was continued for up to 4 h (Fig. 8.2). A time-dependent increase in the monocyte TF cell surface expression was observed after 1 h (Fig. 8.2). TF expression by monocytes did not increase in whole blood incubated in the absence of bacteria (Fig. 8.2). The expression of TF on the surface of whole blood granulocytes was not detected using flow cytometry (data not shown); however, cytoplasmic TF staining was not performed in this study.

E. coli dose dependently increased monocyte expression of TF (Fig. 8.3a). Ultra-purified LPS was then added to whole blood at doses ranging from 1 to 10,000 ng/mL, and the incubation continued for

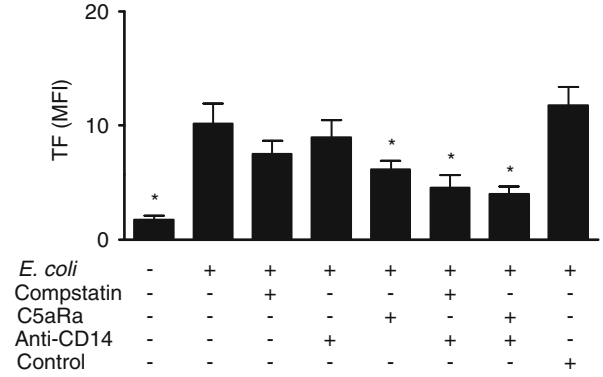

Fig. 8.4 *The effect of selective complement and CD14 inhibition on E. coli-induced TF expression by human monocytes*. *E. coli* (14.2×10^6/mL) was added to human whole blood in the presence of compstatin, C5aRa, anti-CD14, or a combination thereof as indicated. The concentrations and abbreviations of inhibitors and controls are the same as indicated in the legend for Fig. 8.1. The expression of TF on the surface of monocytes after 2 h of incubation was analyzed using flow cytometry and is expressed as MFI. The TF expression in the baseline sample after 2 h of incubation with PBS only is shown in the first bar from the left. Values are given as means ±SD (n = 6) using separate blood donors each time. $^*p < 0.05$ compared to *E. coli* in the absence of inhibitors

4 h (Fig. 8.3b). LPS dose dependently increased TF expression by human whole blood monocytes (Fig. 8.3b). Notably, LPS at a dose below 1,000 ng/mL did not activate complement measured as TCC in plasma (data not shown). In comparison, *E. coli* bacteria significantly increased the TCC levels threefold compared to the PBS control after 2 h of incubation (data not shown), which is in accordance with previous findings (Brekke et al. 2007).

To confirm the specificity of the flow cytometry used to detect the expression of TF in monocytes, recombinant human TF (rhTF) was added to human whole blood. The addition of increasing concentrations of rhTF dose dependently blocked the *E. coli*-induced TF MFI signal detected by flow cytometry (data not shown), confirming the specificity of this antibody in this particular method.

8.3.3 The Effect of Selective Complement and CD14 Inhibition on E. coli-Induced TF Surface Expression in Monocytes

Incubating monocytes with *E. coli* for 2 h significantly ($p < 0.05$) enhanced monocyte TF (MFI) expression (Fig. 8.4). The selective inhibition of complement using compstatin or CD14 using anti-CD14 nonsignificantly reduced *E. coli*-induced TF expression by 31% and 14%, respectively. In comparison, treating monocytes with C5aRa alone significantly reduced *E. coli*-induced TF expression by 47% ($p < 0.05$). The combination of compstatin and anti-CD14 significantly reduced the *E. coli*-induced expression of TF in monocytes by 67% ($p < 0.05$), and the combination of C5aRa and anti-CD14 significantly reduced the *E. coli*-induced TF expression by 73% ($p < 0.05$) (Fig. 8.4). Similar effects were also obtained when TF expression was calculated as the percent of all CD14 positive cells that were also TF positive (data not shown). Thus, treatment with anti-CD14 and the complement inhibitors exhibited an additive effect when TF expression was calculated either as MFI or the percentage of TF + cells. The combined effect of increasing doses of compstatin and anti-CD14 on *E. coli*-induced TF expression was then examined. Compstatin alone and compstatin plus anti-CD14 both dose dependently reduced *E. coli*-induced TF expression by monocytes in human whole blood (data not shown).

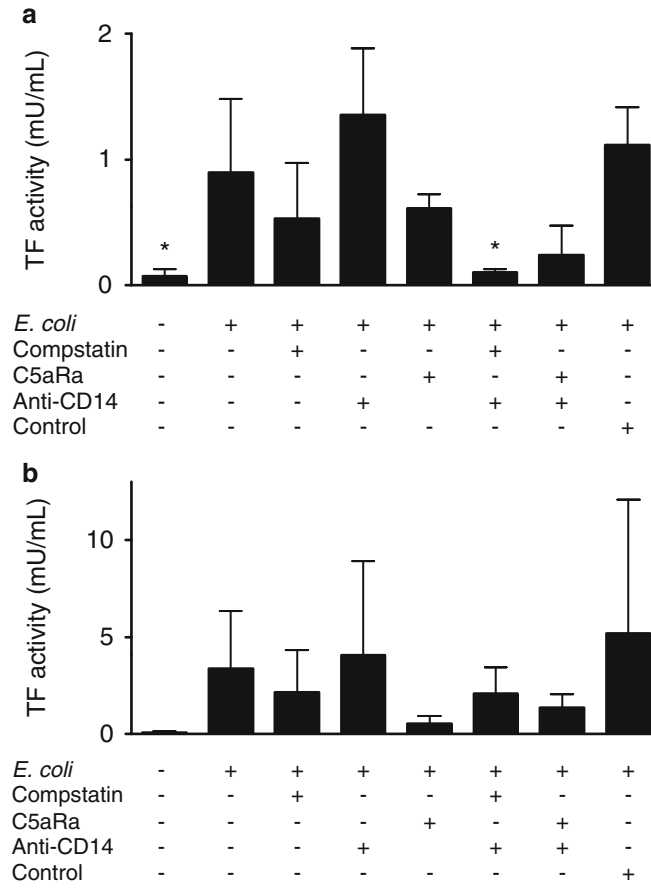

Fig. 8.5 *The effect of selective complement and CD14 inhibition on E. coli-induced TF functional activity in plasma microparticles*. (**a**) A low dose (14.2×10^6/mL) or (**b**) high dose (71×10^6/mL) of *E. coli* was added to human whole blood in the presence of compstatin, C5aRa, anti-CD14, or a combination thereof. The concentrations of inhibitors and controls are indicated in the legend for Fig. 1. The TF activity in plasma microparticles from platelet-poor plasma was analyzed in a two-stage amidolytic assay. The TF activity after 2 h of incubation with PBS only is shown in the first bar from the left. TF activity is expressed as mUnits/mL (mU/mL). Values are means ±SD (n = 2–4). $^*p < 0.05$ compared to *E. coli* in the absence of inhibitors

8.3.4 The Effect of Selective Complement and CD14 Inhibition on TF Functional Activity in Plasma Microparticles

Very low baseline levels of TF activity in plasma microparticles were detected at time zero (data not shown). There was no increase in TF activity in samples incubated with PBS between baseline and two hours (Fig. 8.5a, b, first left column). After 2 h of incubation, a low dose of *E. coli* (14.2×10^6/mL) enhanced TF activity 13-fold compared to the spontaneous control (Fig. 8.5a). Selective complement inhibition using compstatin or C5aRa reduced *E. coli*-induced TF activity in plasma microparticles by 44% and 34%, respectively. However, compstatin plus anti-CD14 reduced low-dose *E. coli*-induced TF activity in plasma microparticles by 96%. In comparison, C5aRa and anti-CD14 in combination reduced the *E. coli*-induced TF activity by 79%. In contrast, anti-CD14 alone had no effect on *E. coli*-induced TF activity in plasma microparticles. A high dose of *E. coli* (71×10^6/mL) enhanced TF functional activity approximately 41-fold (Fig. 8.5b). C5aRa was the most efficient treatment, reducing *E. coli*-induced TF activity by approximately 86% ($p < 0.05$). Compstatin and anti-CD14 in combination reduced high-dose *E. coli*-induced TF activity by 38%. In comparison, the combination of C5aRa and anti-CD14 reduced *E. coli*-induced TF activity by 60%. Again, anti-CD14 alone had no effect, indicating that CD14 does not play a major role in *E. coli*-induced TF activity in plasma.

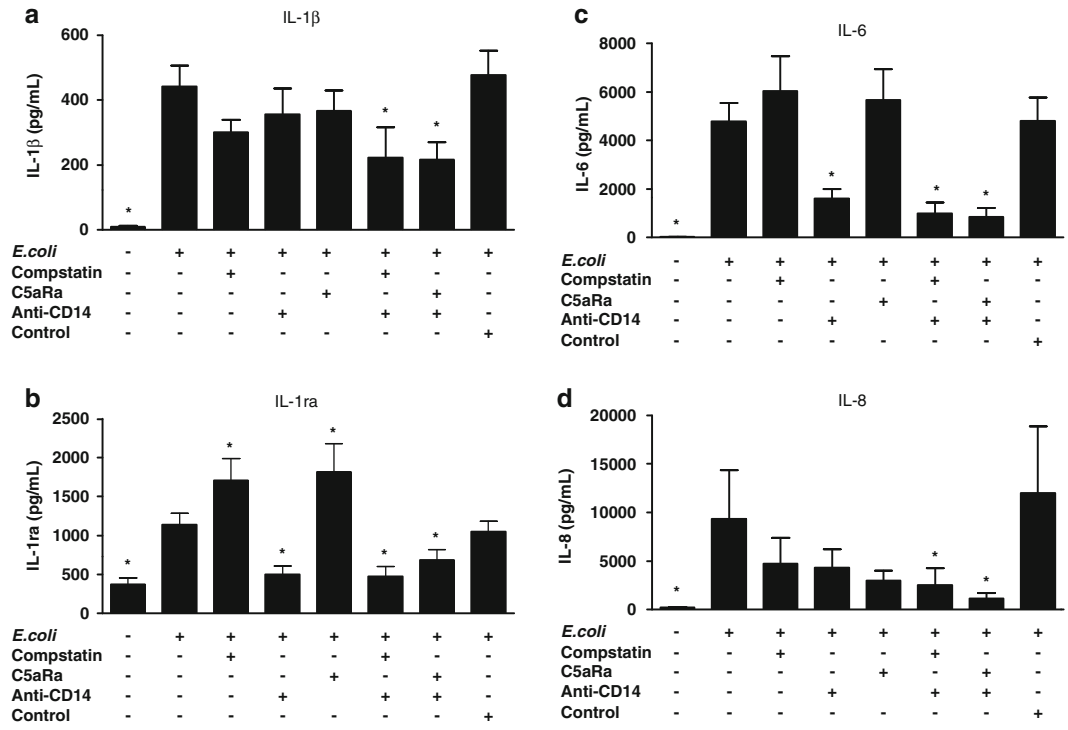

Fig. 8.6 *The effect of selective complement and CD14 inhibition on E. coli-induced IL-1β, IL-1ra, IL-6, and IL-8 synthesis in whole blood.* A low dose of *E. coli* (14.2×10^6/mL) was added to human whole blood in the presence of compstatin, C5aRa, anti-CD14, or a combination thereof and incubated for 2 h. The concentrations of inhibitors and controls are indicated in the legend for Fig. 8.1. (**a**) IL-1β, (**b**) IL-1ra, (**c**) IL-6, and (**d**) IL-8 levels in plasma were analyzed using a multiplex ELISA. Results are expressed as pg/mL. Values are means ±SD (n = 6). $^*p < 0.05$ compared to *E. coli* in the absence of inhibitors

8.3.5 *The Effect of Selective Complement and CD14 Inhibition on E. coli-Induced Cytokine Synthesis*

We next examined the effect of complement and CD14 inhibition on *E. coli* (14.2×10^6/mL)-induced cytokine levels in the plasma from six healthy blood donors after 2 h of incubation (Fig. 8.6). The *E. coli*-induced synthesis of IL-1β was only reduced by compstatin or C5aRa and anti-CD14 in combination (Fig. 8.6a). The C3 convertase inhibitor compstatin and C5aRa both significantly reduced *E. coli*-induced IL-8 synthesis but had no effect on the other cytokines examined (Fig. 8.6d). In comparison, anti-CD14 significantly reduced *E. coli*-induced IL-1ra, IL-6, IL-8 (Fig. 8.6b–d), and IP-10 synthesis (Fig. 8.7c). The combination of complement and CD14 inhibition almost completely blocked *E. coli*-induced cytokine synthesis (Figs. 8.6 and 8.7).

8.4 Discussion

This chapter indicates an essential role of complement in *E. coli*-induced TF mRNA upregulation, a combined role of complement and CD14 in TF surface expression by human whole blood monocytes and a major role of complement in TF functional activity in plasma.

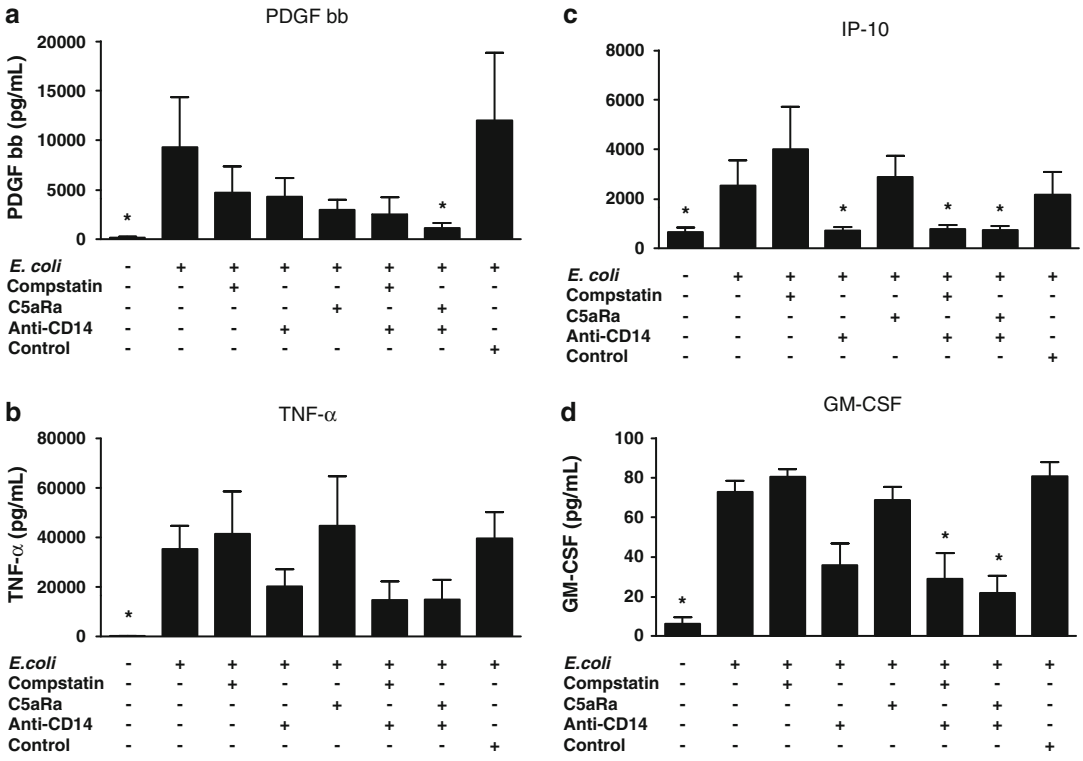

Fig. 8.7 *The effect of selective complement and CD14 inhibition on E. coli-induced PDGFbb, TNF-α, IP-10, and GM-CSF*. A low dose of *E. coli* (14.2×10^6/mL) was added to human whole blood in the presence of compstatin, C5aRa, anti-CD14, or a combination thereof and incubated for 2 h. The concentrations of inhibitors and controls are indicated in the legend for Fig. 8.1. (**a**) PDGFbb, (**b**) TNF-α, (**c**) IP-10, and (**d**) GM-CSF levels in plasma were analyzed using a multiplex ELISA. Results expressed as pg/mL. Values are means ±SD (n = 6). $^*p < 0.05$ compared to *E. coli* in the absence of inhibitors

We analyzed TF mRNA upregulation, TF expression on the surface of monocytes, and TF functional activity in plasma microparticles and found a distinct TF signal after 2 h of incubation with *E. coli*. The time course for *E. coli*-induced TF expression suggests that new TF is synthesized by increased transcription and potentially the translation of nascent TF molecules as well. This result is supported by the *E. coli*-induced TF mRNA upregulation we observed in whole blood and is in agreement with previous data generated using LPS (Gregory et al. 1989). The upregulation of TF mRNA was analyzed in total mRNA from whole blood in this study, and it is therefore not possible to determine whether this upregulation occurred in monocytes, granulocytes (Kambas et al. 2008), or other cell types.

The significant effect that compstatin had on *E. coli*-induced TF mRNA upregulation indicates that complement activation plays a major role in TF mRNA upregulation. Furthermore, the effect of C5aRa on *E. coli*-induced TF mRNA upregulation suggests that the anaphylatoxin C5a may be involved. The spontaneous increase in TF mRNA during the incubation of otherwise untreated samples indicates that whole blood exposed to polypropylene plastic tubes upregulates TF mRNA in a manner similar to other bio-incompatibility reactions of immunocompetent cells (Nilsson et al. 2007). However, this spontaneous TF mRNA upregulation was not followed by a spontaneous increase in TF expression by monocytes. We also found that anti-CD14 did not inhibit *E. coli*-induced TF mRNA upregulation. Compared to the significant effect compstatin had on this process, our data suggest that the *E. coli*-induced TF

mRNA upregulation in human whole blood is almost completely dependent on complement and is CD14 independent. This conclusion is supported by the finding that compstatin alone inhibited the upregulation of TF mRNA better than the combination of compstatin and anti-CD14. Studies using LPS as a stimulant make it difficult to identify the important role played by complement in *E. coli*-induced TF upregulation because LPS only activates complement at high concentrations (Brekke et al. 2007). Compstatin binds to and neutralizes native C3, thereby inhibiting the C3 convertase-mediated cleavage of C3 to C3a and C3b (Sahu et al. 1996). The finding that the combination of compstatin and anti-CD14 significantly reduced TF expression by monocytes indicates that anti-CD14, which did not significantly reduce TF expression on its own, inhibits a different pathway leading to TF expression. Recent studies have shown that TF receives several posttranslational modifications, and we speculate that these processes may be affected by LPS (Mohan Rao and Mackman 2010). Anti-CD14 probably blocks the binding of LPS to membrane-bound CD14 on monocytes, although its effects on soluble CD14 in the plasma may also be involved. The finding that we more than doubled the inhibitory effect on monocyte TF expression by combining compstatin and anti-CD14 suggests that complement and CD14 synergistically affect TF surface expression. The mechanism behind this combined complement and CD14 inhibition is not known, although previous data indicates that it blocks both phagocytosis of *E. coli* and cytokine synthesis (Brekke et al. 2008). In addition, CD14 is an important recognition molecule associated with the functions of TLR2 and TLR3. The significant effect that inhibiting complement and CD14 has on *E. coli*-induced cytokine expression was confirmed by this report after we switched to using Nunc tubes (Brekke et al. 2008). The finding that the C5aRa efficiently reduced TF expression supports the idea that complement plays an important role in mediating TF expression by monocytes. However, treating cells with C5aRa and anti-CD14 in combination further reduced the *E. coli*-induced TF expression by monocytes, suggesting a synergistic cross talk between the C5aR and the TLR-4/CD14/MD2 receptor complex. The main mechanism functioning in this process is probably the complement-mediated inhibition of TF mRNA upregulation. The complement-dependent expression of TF in monocytes that we identified is in accordance with a previous study showing that *N. meningitidis*-induced TF expression was more than 50% dependent on complement (Lappegard et al. 2009). Because the C3 convertase inhibitor compstatin completely blocked *E. coli*-induced mRNA upregulation but only slightly reduced *E. coli*-induced TF expression by monocytes, it is possible that presynthesized TF is exposed on the monocyte surface in response to *E. coli* in a process that is partly dependent on C3 activation. However, the C5aRa significantly reduced *E. coli*-induced TF expression, suggesting that C5a might be involved. Because compstatin inhibits complement and is a small nonimmunogenic peptide, it is a potential therapeutic agent (Janssen et al. 2007). The role of excessive complement activation in experimental sepsis models is further supported by the pivotal role of the C5a–C5a receptor interaction in the development of the systemic inflammatory response and multiorgan failure (Ward 2004; Huber-Lang et al. 2002). In human sepsis, harmful outcomes have been correlated with increased plasma levels of the complement activation products C3a, C4a, and C5a (Nakae et al. 1996). The potential beneficial effects of complement inhibitors in the prevention of tissue factor-induced thrombosis in vivo remain to be elucidated.

Huber-Lang et al. have described a new pathway of complement activation that is C3-independent (Huber-Lang et al. 2006). Their study showed that in the genetic absence of C3, thrombin substitutes for the C3-dependent C5 convertase. The potential role of this pathway in our in vitro sepsis model is difficult to assess due to the fact that we used lepirudin, a thrombin inhibitor, as the anticoagulant. It is possible that lepirudin inhibits this new pathway by using thrombin as an alternative C5 convertase. Whether TF or related factors are activated concomitantly with complement to convert prothrombin to thrombin remains to be determined.

This study indicates that anti-CD14 alone has no effect on *E. coli*-induced TF mRNA upregulation, TF expression by monocytes, or TF activity in whole blood. This may be due to the low concentration of free LPS in our *E. coli* bacteria preparation, which was at the pg/mL level. However, ultra-purified LPS at high concentrations significantly increased TF expression on monocytes, as expected. A study

by Steinemann et al. showed that anti-CD14 inhibited LPS-induced TF expression on monocytes, supporting that CD14 is important in LPS-induced TF expression (Steinemann et al. 1994). Although crude LPS preparations were used in their study, Osterud et al. previously showed that complement may play a role in LPS-induced TF (Osterud et al. 1984). However, the addition of low doses of *E. coli* LPS did not stimulate complement activation (Brekke et al. 2007). To the best of our knowledge, there is no previous report on the relative roles of complement and CD14 on *E. coli*-induced TF expression in human whole blood using lepirudin as an anticoagulant.

The combined inhibition of complement and anti-CD14 efficiently reduced *E. coli*-induced cytokine release. The release of IL-1β in the plasma indicates that caspase-1 is activated through the inflammasome, resulting in the subsequent synthesis of pro-IL-1β and the release of IL-1β into the plasma (Van de Veerdonk et al. 2011). The inhibitory effect that compstatin or C5aRa and anti-CD14 in combination have on *E. coli*-induced IL-1β and TF expression indicates that complement activation acts upstream of inflammasome activation. Interestingly, the C3 convertase inhibitor compstatin and C5aRa both increased the synthesis of IL-1ra, which binds to and thereby inhibits the effect of IL-1β (Gabay et al. 2010). We speculate that the C3 convertase inhibitor compstatin partly inhibits inflammation through increased IL-1ra synthesis in addition to inhibiting complement activation. Furthermore, the level of platelet-derived growth factor bb (PDGF bb) was also reduced by the combination of C5aRa and anti-CD14, indicating that adding *E. coli* to whole blood activates platelets and that this activation is reduced by blocking C5aR and CD14. However, the reduction of PDGF bb levels by compstatin or C5aRa alone was not statistically significant. In summary, the inhibition of complement and CD14 significantly reduced the levels of *E. coli*-induced IL-1β and other cytokines in addition to reducing TF expression, suggesting that complement and CD14 block signaling pathways that are upstream of inflammasome activation.

In conclusion, the present data indicate that complement is essential for *E. coli*-induced TF mRNA upregulation and that complement participates in *E. coli*-induced TF expression by monocytes. Adding CD14-inhibition to complement inhibition adds moderately to the expression of TF on human monocytes and, to some extent, plasma TF functional activity. This process seems to be upstream of the actual coagulation cascade itself. The potentially beneficial effects that inhibiting complement has on TF expression during *E. coli* sepsis requires clarification in future studies.

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Conflict of Interest Statement The author Professor J.D. Lambris has submitted several patent applications on complement inhibitors. None of the other authors have conflicts of interest.

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