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Post challenge inhibition of C3 and CD14 attenuates *Escherichia coli*-induced inflammation in human whole blood

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Abstract

Combined inhibition of CD 14 and complement, two main inducers of the inflammatory response, have proved particularly effective in attenuating Gram-negative bacteria-induced inflammation. Approaching possible clinical relevance, we investigated the effect of such inhibition in a post-challenge setting. Human whole blood was anti-coagulated with lepirudin. Anti-CD14, compstatin (C3 inhibitor) and the combination thereof were added 5 min prior to or 5, 15 or 30 min after adding *Escherichia coli*. Total incubation time with *Escherichia coli* was 120 min. Cytokines, myeloperoxidase (MPO) and the terminal complement complex (TCC) were measured using multiplex technology and ELISA. Delayed combined inhibition significantly attenuated the inflammatory response. IL-1 β , IL-8 and TNF- α were significantly inhibited in the range of 20–40%, even when adding the inhibitors with up to 30 min delay. IL-6 was significantly inhibited with 15 min delay, and MIP-1 α and MPO with 5 min delay. Complement activation (TCC) was blocked completely at each time point compstatin was added, whereas the cytokines and MPO increased steadily between the time points. The combined regimen was significantly more effective than single inhibition in the pre-challenge setting. The attenuation of *Escherichia coli*-induced inflammation in a post-challenge setting suggests a potential therapeutic window for this treatment in sepsis.

Keywords

CD 14; complement; cytokines; c3; *Escherichia coli*; human; post-challenge

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Conflict of interest

JD Lambris, is the holder of several patent applications on complement inhibitors and the founder of Amyndas Biopharmaceuticals, which is developing complement inhibitors for clinical applications. The other authors declare that they have no competing financial or other interest in relation to their work.

Introduction

The clinical syndrome of sepsis is still a major health problem with high morbidity and mortality.^{1–4} Numerous efforts trying to attenuate the inappropriate systemic inflammatory response induced by severe sepsis and septic shock have failed in clinical trials. A major reason for this may be that treatment in a clinical situation is started too late to reverse the disease process. Currently, no drug is approved for specific treatment of sepsis and systemic inflammatory response syndromes.⁵ The need to define new therapeutic approaches is therefore critical.^{6,7}

Among the complex network of activated inflammatory pathways seen in systemic inflammation, complement and the TLRs are central upstream sensor- and effector-systems. These branches of innate immunity have pattern recognition receptors (PRRs) recognizing conserved patterns of exogenous, as well as endogenous molecules, thus working as a forceful first line defence with several downstream effects.^{8–10}

TLR4 is a PRR for LPS of Gram-negative bacteria¹¹ TLR4 signalling is facilitated by the accessory molecules myeloid differentiation factor 2 (MD2) and CD14.^{12–14} Upon activation, the CD14/MD2/TLR4-complex induces activation of intracellular transcription factors required for transcriptions of a wide variety of immune responses, and inflammation-associated genes with subsequent synthesis and release of several pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6, IL-8, and macrophage inflammatory protein 1 α (MIP-1 α) and 1 β (MIP-1 β).^{15,16} CD14 is a particularly important molecule in the TLR family as it is a co-receptor for a number of TLRs and therefore an attractive target for inhibition.¹³

The complement system is a protein cascade system with versatile physiological effects playing a vital role in host defence and homeostasis.^{17,18} Activation is known to occur via the classical, the lectin and the alternative pathways, all leading to the cleavage of the central complement factor C3 to C3a and C3b, and C5 to C5a and C5b.^{19,20} C3a and, in particular, C5a are potent anaphylatoxins and induce inflammatory responses, while C3b is an important opsonin and C5b initiates assembly of TCC.²¹ When activated systemically, the complement system, and, in particular, C5a, becomes an important and dangerous part of the pathogenesis in sepsis, which may cause hypotension, organ failure and, ultimately, death.^{22–25}

Although complement and TLRs represent different branches of pattern recognition, evidence suggests that the pathways are closely related through an extensive cross-talk.^{26,27} Previously, we have demonstrated in human *ex vivo* and animal *in vivo* models, that inhibition of CD 14 or key molecules of complement (C3 or C5), and, in particular, a combination thereof, profoundly attenuated Gram-negative-induced inflammation.^{28–30} These studies, evaluating the effect of inhibition when given prior to the induction of inflammation reflect a *proof of concept*, and support the hypothesis that combined inhibition of complement and CD 14 may be a future treatment regimen in sepsis.²⁴ However, it is important to evaluate the effect of inhibiting the inflammatory response after the onset, in an

effort to approach the clinical situation where treatment frequently would be delayed compared with the initial microbial insult. In the present study, we therefore examined the efficacy on *Escherichia coli* (*E. coli*)-induced inflammation by a delayed inhibition of C3, CD 14 and a combination thereof, compared with the pre-challenge regimen, in our well-established *ex vivo* whole blood model of inflammation.³¹

Materials and methods

Equipment and reagents

Endotoxin-free tubes and tips were purchased from Thermo Fischer Scientific NUNC (Roskilde, Denmark). Sterile PBS with Ca²⁺ and Mg²⁺, and EDTA were purchased from Sigma-Aldrich (Steinheim, Germany). Lepirudin 2.5 mg/ml (Refludan, Pharmion, Windsor, UK) was used as an anticoagulant.

Inhibitors

Azide-free mouse anti-human CD 14 [clone 18D11; F(ab')₂ 3118, lot1383] was purchased from Diatec monoclonals AS (Oslo, Norway). The compstatin analogue AMY-90, a cyclic peptide that inhibits cleavage of C3, comprised of the following sequence Ac-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-mle-NH₂ and a corresponding control peptide (AMY-101) were synthesized as described previously.³²

Bacteria

Heat-inactivated *E. coli* strain LE392 was from American Type Culture Collection (ATCC 33572; Manassas, VA, USA).

Ex vivo whole blood experiments

The whole blood model has been described, in detail, previously.³¹ Six healthy volunteers donated blood to this study. The blood was drawn into 4.5 ml NUNC tubes containing the anticoagulant lepirudin (50 µg/ml), which only blocks thrombin and does not interfere with the remaining inflammatory network. The following protocol was performed with blood from each of the six donors: the baseline sample (T0) was processed immediately after the blood was drawn. Two tubes were pre-incubated with inhibitors for 5 min at 37°C prior to adding *E. coli* to a final concentration of 10⁷/ml whole blood. Compstatin (final concentration 20 µM), anti-CD14 (final concentration 10 µg/ml), or a combination thereof (hereafter named 'combined inhibitors'), were added to separate tubes in duplicate at each of the following time points: 5, 15 and 30 min after adding *E. coli*. Two tubes served as positive controls and were incubated with *E. coli* and PBS instead of inhibitors. Two tubes served as negative controls and were incubated with PBS only. The samples were incubated for 60 and 120 min. Complement activation was stopped by placing the samples on crushed ice and adding EDTA to a final concentration of 20 mM. The samples were centrifuged for 15 min at 3220g at 4°C. Plasma was collected and stored at -70°C until analysed.

Multiplex analysis

Cytokines (TNF- α , IL-1 β , IL-6, IL-8, MIP-1 α and MIP-1 β) were measured using a multiplex cytokine assay (Bio-Rad Laboratories, Hercules, CA, USA).

Enzyme immunoassays

The soluble terminal C5b-9 complement complex (TCC) was measured in an enzyme immunoassay (EIA), as described previously and later modified.^{33,34} Briefly, the mAb aE11 reacting with a neo-epitope exposed in C9 after incorporation in the C5b-9 complex was used as capture Ab. A biotinylated anti-C6mAb (clone 9C4) was used as the detection Ab. The standard was normal human serum activated with zymosan and defined to contain 1000 arbitrary units (AU)/mL Zymosan activated human serum was used as a positive control. The buffer used as a diluent for the standards and samples and as a negative control was PBS containing EDTA and a detergent (Tween). The granulocyte activation marker myeloperoxidase (MPO) was quantified using a commercial EIA kit (Hycult Biotech, Uden, the Netherlands), performed in accordance to the instructions from the manufacturer.

Data presentation and statistics

All results are given as mean and SEM. GraphPad Prism version 5.04 (GraphPad, San Diego, CA, USA) was used for statistical analysis. When comparing the effect of combined inhibition to the effect of anti-CD14 and compstatin, the data were analysed using one-way ANOVA with repeated measurements, then post-tested using Dunnett's multiple comparison test. When comparing the effect of inhibiting at different time points with the positive control group, the results were analysed using a paired *t*-test. Percentage inhibition of the positive control by the different inhibitors is given related to the negative control as a basic value. A *P*-Value < 0.05 was considered statistically significant for all analyses.

Ethics

The study was approved by the Regional Ethical Committee and all blood donors gave written informed consents to participate in the study.

Results

Effect of delayed inhibition of C3 and CD14 on pro-inflammatory cytokines

IL-1 β —After 60 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited IL-1 β by 86% (*P* = 0.047) (Figure 1, upper left panel). Five min delayed addition of the combined inhibitors significantly inhibited IL-1 β by 53% (*P* = 0.037) (Figure 1, upper left panel). Pre-incubation with compstatin alone did not significantly inhibit IL-1 β , whereas anti-CD14 alone significantly inhibited IL-1 β by 74% (*P* = 0.049) (data not shown). Delayed addition of compstatin or anti-CD14 alone did not inhibit IL-1 β significantly (data not shown).

After 120 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited IL-1 β by 85% (*P* = 0.017) (Figure 1, lower left panel). Up to 30 min delayed addition of the combined inhibitors significantly inhibited IL-1 β in the range of 42–45% (*P* = 0.031) (Figure 1, lower left panel). Pre-incubation with compstatin alone

significantly inhibited IL-1 β by 45% ($P=0.025$), whereas anti-CD14 alone significantly inhibited IL-1 β by 54% ($P=0.024$) (data not shown).

IL-6—After 60 min incubation with *E. coli*, IL-6 was not released (Figure 1, upper middle panel).

After 120 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited IL-6 by 90% ($P=0.019$) (Figure 1, lower middle panel). Up to 15 min delayed addition of the combined inhibitors significantly inhibited IL-6 in the range of 22–25% ($P=0.047$) (Figure 1, lower middle panel). Pre-incubation with compstatin alone did not significantly inhibit IL-6, whereas anti-CD14 alone significantly inhibited IL-6 by 71% ($P=0.014$) (data not shown). Delayed addition of compstatin or anti-CD14 alone did not significantly inhibit IL-6 (data not shown).

TNF- α —After 60 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited TNF- α by 94% ($P=0.003$) (Figure 1, upper right panel). Up to 15 min delayed addition of the combined inhibitors significantly inhibited TNF- α in the range of 28–59% ($P=0.005$) (Figure 1, upper right panel). Pre-incubation with compstatin alone significantly inhibited TNF- α by 28% ($P=0.029$), whereas anti-CD14 alone significantly inhibited TNF- α by 78% ($P=0.002$) (data not shown). Delayed addition of compstatin alone did not significantly inhibit TNF- α , whereas 5 min delayed addition of anti-CD14 alone significantly inhibited TNF- α by 28% ($P=0.011$) (data not shown).

After 120 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited TNF- α by 70% ($P=0.012$) (Figure 1, lower right panel). Up to 30 min delayed addition of the combined inhibitors significantly inhibited TNF- α in the range of 19–32% ($P=0.035$) (Figure 1, lower right panel). Pre-incubation with compstatin alone significantly inhibited TNF- α by 36% ($P=0.011$), whereas anti-CD14 alone significantly inhibited TNF- α by 44% ($P=0.047$) (data not shown). Up to 15 min delayed addition of compstatin alone significantly inhibited TNF- α in the range of 37–44% ($P=0.022$) (data not shown), whereas up to 15 min delayed addition of anti-CD14 alone significantly inhibited TNF- α in the range of 11–20% ($P=0.020$) (data not shown).

Effect of delayed inhibition of C3 and CD14 on chemokines

IL-8—After 60 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited IL-8 by 92% ($P=0.001$) (Figure 2, upper left panel). Up to 15 min delayed addition of the combined inhibitors significantly inhibited IL-8 in the range of 29–60% ($P=0.001$) (Figure 2, upper left panel). Pre-incubation with compstatin alone did not significantly inhibit IL-8, whereas anti-CD14 alone significantly inhibited IL-8 by 72% ($p=0.002$) (data not shown). Delayed addition of compstatin or anti-CD14 alone did not significantly inhibit IL-8 (data not shown).

After 120 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited IL-8 by 79% ($P<0.001$) (Figure 2, lower left panel). Up to 30 min delayed addition of the combined inhibitors significantly inhibited IL-8 in the range of 31–43% ($P=0.011$) (Figure 2, lower left panel). Pre-incubation with compstatin alone

significantly inhibited IL-8 by 40% ($P < 0.001$), whereas anti-CD14 alone significantly inhibited IL-8 by 33% ($P = 0.001$) (data not shown). Up to 30 min delayed addition of compstatin alone significantly inhibited IL-8 in the range of 27–37% ($P = 0.004$), whereas up to 30 min delayed addition of anti-CD14 alone significantly inhibited TNF- α in the range of 18–32% ($P = 0.040$) (data not shown).

MIP-1 α —After 60 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited MIP-1 α by 95% ($P = 0.012$) (Figure 2, upper middle panel). Five min delayed addition of the combined inhibitors significantly inhibited MIP-1 α by 46% ($P = 0.027$) (Figure 2, upper middle panel). Pre-incubation with compstatin alone did not significantly inhibit MIP-1 α , whereas anti-CD14 alone significantly inhibited MIP-1 α by 81% ($P = 0.011$) (data not shown). Delayed addition of compstatin or anti-CD14 alone did not significantly inhibit MIP-1 α (data not shown).

After 120 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited MIP-1 α by 81% ($P = 0.005$) (Figure 2, lower middle panel). Five min delayed addition of the combined inhibitors significantly inhibited MIP-1 α by 36% ($P = 0.046$) (Figure 2, lower middle panel). Pre-incubation with compstatin alone significantly inhibited MIP-1 α by 35% ($P = 0.016$), whereas anti-CD14 alone significantly inhibited MIP-1 α by 61% ($P = 0.002$) (data not shown). Up to 30 min delayed addition of compstatin alone significantly inhibited MIP-1 α in the range of 26–12% ($P = 0.050$), whereas delayed addition of anti-CD14 alone did not significantly inhibit MIP-1 α (data not shown).

MIP-1 β —After 60 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited MIP-1 β by 92% ($P = 0.002$) (Figure 2, right upper panel). Five min delayed addition of the combined inhibitors significantly inhibited MIP-1 β by 28% ($P = 0.013$) (Figure 2, right upper panel). Pre-incubation with compstatin alone did not significantly inhibit MIP-1 β , whereas anti-CD14 alone significantly inhibited MIP-1 β by 79% ($P = 0.002$) (data not shown). Delayed addition of compstatin or anti-CD14 alone did not significantly inhibit MIP-1 β (data not shown).

After 120 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited MIP-1 β by 64% ($p = 0.005$) (Figure 2, lower right panel). Delayed addition of the combined inhibitors did not significantly inhibit MIP-1 β (Figure 2, lower right panel). Pre-incubation with compstatin alone significantly inhibited MIP-1 β by 31% ($P = 0.002$), whereas anti-CD14 alone significantly inhibited MIP-1 β by 47% ($P = 0.006$) (data not shown). Up to 30 min delayed addition of compstatin alone significantly inhibited MIP-1 β in the range of 22–31% ($P = 0.029$), whereas up to 15 min delayed addition of anti-CD14 alone significantly inhibited MIP-1 β in the range of 33–34% ($P = 0.004$) (data not shown).

Effect of delayed inhibition of C3 and CD14 on the granulocyte activation marker MPO

MPO—After 60 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited MPO by 84% ($P = 0.002$) (Figure 3, left upper panel). Five min delayed addition of the combined inhibitors significantly inhibited MPO by 46% ($P = 0.035$)

(Figure 3, left upper panel). Pre-incubation with compstatin or anti-CD14 alone did not significantly inhibit MPO. Delayed addition of compstatin or anti-CD14 alone did not significantly inhibit MPO.

After 120 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited MPO by 62% ($P = 0.027$) (Figure 3, left lower panel). Five min delayed addition of the combined inhibitors significantly inhibited MPO by 39% ($P = 0.042$) (Figure 3, left lower panel). Pre-incubation with compstatin or anti-CD14 alone did not significantly inhibit MPO. Delayed addition of compstatin or anti-CD14 did not significantly inhibit MPO.

Effect of delayed inhibition of C3 or CD14 on complement activation

TCC—After 60 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited TCC ($P < 0.001$) (Figure 3, right upper panel). Up to 30 min delayed addition of the combined inhibitors significantly inhibited TCC ($P = 0.050$) (Figure 3, upper right panel). Pre-incubation with compstatin alone significantly inhibited TCC ($P < 0.001$) (data not shown). Anti-CD14 alone did not inhibit TCC; thus, the effect of compstatin was responsible for the inhibition of TCC. Up to 30 min delayed addition of compstatin alone significantly inhibited TCC ($P = 0.003$) (data not shown).

After 120 min incubation with *E. coli*, pre-incubation with the combined inhibitors significantly inhibited TCC ($P < 0.001$) (Figure 3, lower right panel). Up to 30 min delayed addition of the combined inhibitors significantly inhibited TCC ($P < 0.001$) (Figure 3, lower right panel). Pre-incubation with compstatin alone significantly inhibited TCC ($P < 0.001$) (data not shown), whereas anti-CD14 alone did not inhibit TCC. Up to 30 min delayed addition of compstatin alone significantly inhibited TCC ($P = 0.022$) (data not shown). Notably, in contrast to the cytokines, the TCC formation was completely blocked at each time point and no further activation took place after addition of compstatin.

Comparison of single versus combined inhibition of C3 and CD14 in the pre-challenge setting

Previously it has been shown that the combined inhibition of C3 and CD14 in the pre-challenge model is highly effective,^{28–30} although it has never been demonstrated that the combined inhibition is significantly more effective than single inhibition. Thus, we investigated this for the biomarkers included in the present study.

IL-1 β was significantly inhibited by compstatin ($P = 0.025$), anti-CD14 ($p = 0.024$) and the combined inhibitors ($P = 0.017$) (Figure 4). The combined inhibition was significantly more effective than inhibition with compstatin alone ($P < 0.05$) and anti-CD14 alone ($p < 0.01$).

IL-6 was not inhibited by compstatin, but by anti-CD14 ($P = 0.014$) and the combined inhibitors ($P = 0.019$) (Figure 4). The combined inhibition was not significantly more effective than anti-CD14 alone.

TNF- α was significantly inhibited by compstatin ($P = 0.011$), anti-CD14 ($P = 0.047$) and the combined inhibitors ($P = 0.012$) (Figure 4). The combined inhibition was significantly more effective than compstatin alone ($P < 0.01$) and anti-CD14 alone ($P < 0.05$).

IL-8 was significantly inhibited by compstatin ($P < 0.001$), anti-CD14 ($P = 0.001$) and the combined inhibitors ($P < 0.001$) (Figure 4). The combined inhibition was significantly more effective than compstatin alone ($P < 0.01$) and anti-CD14 alone ($P < 0.01$).

MIP-1 α was significantly inhibited by compstatin ($P = 0.016$), anti-CD14 ($P = 0.002$) and the combined inhibitors ($P = 0.005$) (Figure 4). The combined inhibition was significantly more effective than inhibition with compstatin alone ($P < 0.01$), but not significantly more effective than anti-CD14 alone.

MIP-1 β was significantly inhibited by compstatin ($P = 0.002$), anti-CD14 ($P = 0.006$) and the combined inhibitors ($P = 0.005$) (Figure 4). The combined inhibition was significantly more effective than compstatin alone ($P < 0.05$), but not significantly more effective than anti-CD14 alone.

MPO was not inhibited by compstatin or anti-CD14 alone, but significantly inhibited by the combination of the two ($P = 0.027$) (Figure 4).

TCC was significantly inhibited by compstatin ($P < 0.001$), but inhibiting CD14 had no effect (Figure 4). The combined inhibition was equally effective as single inhibition by compstatin.

Discussion

In this study, we show, for the first time, that *E. coli*-induced inflammation in human whole blood is significantly attenuated by inhibition of C3 and CD 14 in a post-challenge model. Despite the discrepancy between the clinical setting of sepsis and an *ex vivo* model, the data strengthens the hypothesis that targeting CD 14 and complement in combination might be a therapeutic approach in Gram-negative sepsis and opens a possible therapeutic window by this regimen.

Complement and the TLR system are thoroughly-described parts of innate immunity, activated rapidly by microbial surface molecules to initiate an inflammatory response as part of first line host defence mechanisms.^{19,35,36} In previous preclinical and experimental studies on sepsis we have explored the efficacy of inhibiting CD 14 and complement when priming the biological system with the inhibitors prior to the insult of Gram-negative bacteria.^{28,29,37,38} We have documented consistently that combined inhibition is more effective than single therapy, but the present study is the first where it is documented that the combined regimen inhibited the inflammatory response statistically significantly more than the single treatments.

In the clinical setting of sepsis, it is unlikely to be able to start any interventions before the bacteria have elicited an inflammatory response. When the inflammatory response has evolved too far, attempts to inhibit it have been shown to have little effect.^{39,40} Thus, it is

crucially important to select candidates for such a treatment regimen very early in the disease process.

In this post-challenge model, the overall results display a significant attenuation of inflammation. The results are somewhat diversified depending on the duration of the incubation. Thus, after 60 min incubation the combined inhibition attenuated pro-inflammatory cytokines, as well as chemokines and MPO, time dependently. Compstatin and anti-CD14 used as single inhibitors also displayed a time-dependent pattern, but the attenuation was much less efficient than the combined inhibition. A 'point-of-no-return', after which the inhibitors had lost their efficacy, was reached at an earlier stage for the single inhibitors than the combined inhibition. After incubation for 120 min the picture changed somewhat as the time-dependent difference in the attenuating effect between inhibiting at 5, 15 or 30 min seen at 60 min virtually disappeared. Nevertheless, a statistically significant attenuating effect of approximately 40% for IL-1 β , 35% for IL-8 and 20% for TNF- α was observed when the combined inhibitors were added delayed for as long as 30 min. The fact that the attenuation of inflammation was almost as evident whether the combined inhibition of CD14 and complement C3 was delayed for 5 or 30 min may indicate that the long-term inflammatory response is susceptible to manipulation for quite some time after the initial triggering of the immune system, leaving open the possibility for a clinical therapeutic window.

As expected, the production of TCC is not influenced by inhibition of CD 14, and inhibition of complement at the level of C3 seems to be almost complete and responsible for the inhibition of TCC seen with the combination. The post-challenge model showed that compstatin completely blocked further activation of the complement cascade as soon as it was added. These results are consistent with previous findings on the efficacy of compstatin used as a single inhibitor. This means that no further activation products are generated after each addition of compstatin, but those fragments already formed might continue to induce secondary effects, although to a lower, and gradually declining, extent as they are cleared and inactivated.

The harmful role of the anaphylatoxin C5a in sepsis has been well described,^{22,25,40,41} and C5a receptor antagonists have been proposed as a possible treatment regimen in sepsis. Promising results from a study using an *in vivo* cecal ligation and puncture (CLP) model in rats showed an increased survival rate among the animals being pre-treated with the C5a-receptor antagonist. The protective effect and increased survival rate was maintained in animals given the C5a-receptor antagonist 6 or 12 h after the CLP-procedure, but the protective effect was lost when the injection of the Abs was delayed for 24 h.⁴¹ However, another study using a C5a-receptor antagonist in a murine CLP model showed no increased survival when the C5a receptor antagonist was given 6h after the CLP procedure.⁴⁰ The loss of increase in survival rates in the post-challenged group in this study might be viewed as an argument for upstream inhibition of the complement system, rather than inhibition downstream at the receptor level. Furthermore, in view of our results in the present and previous studies, one can speculate that a combined regimen inhibiting CD 14 in addition to a key molecule of the complement system is necessary in order to achieve increased survival in a post-challenge treatment regimen.

One of the reasons for combining the inhibition of CD14 and complement is the synergistic interactions suggested and observed in several studies.^{29,42,43} Although complement and the TLR family act as partly independent branches of innate immunity, evidence indicates a considerable crosstalk, implying that they can either compensate, synergize or antagonize each other. Combined inhibition of these pathways therefore has a profound anti-inflammatory potential with possible synergistic properties. In our study the effective attenuation of MPO release by combined inhibition is intriguing as neither anti-CD14 nor compstatin had an attenuating effect on the MPO release when used as single inhibitors. This phenomenon was evident in the pre-challenge, as well as the post-challenge model, hence suggestive of a synergistic interaction between the complement system and CD14. Furthermore, this confirms the redundancy of host defence; when the complement system is blocked, the CD14 pathway may still elicit MPO release and *vice versa*, but, when both systems are inhibited, the MPO-release is attenuated effectively; in fact, it is almost completely abrogated after 60 min incubation when adding the inhibitors prior to *E. coli*. In a previous study, MPO release has been shown to be dependent on C3 as compstatin completely abolished its release.³⁰ It was also shown that anti-CD14 reduced the MPO release by more than 50%. The dosage of *E. coli* in the present study was five times higher than in the aforementioned study. This could explain the difference seen, as the inflammation would be more pronounced. If so, it favours the combined treatment regimen, as single inhibition seemed to be inefficient with higher bacterial concentrations. In addition, the enhanced inhibition of IL-6 by the combined regimen is another example of the regimen's potency. Used alone, compstatin had no effect on IL-6 release, whereas combined with anti-CD14 the inhibition was enhanced as compared to anti-CD14 alone, as also shown previously in several *ex vivo* experiments.^{30,44,45}

The present *ex vivo* model of Gram-negative sepsis is dependent largely on LPS and CD14. As our results show in the pre-challenge model, after 120 min incubation, inhibition of CD 14, C3 or the combination thereof attenuate the formation of IL-1 β substantially. If the secretion of cytokines was dependent only on LPS stimulation, inhibition of CD14 would expectably abrogate the secretion. This is, however, not the case, and is in accordance with previous findings in an article on another Gram-negative bacteria, *Neisseria meningitides*.⁴² In this study on *N. meningitides* LPS was shown to be the sole inflammation-inducing molecule when the bacterial load was 10⁵ bacteria/ml. When the bacterial concentration reached 10⁷ bacteria/ml or higher, non-LPS components were shown to elicit TLR2-mediated inflammation, and, in fact, even TLR4 has been shown to be activated CD14-independently at these concentrations.⁴⁴ If this also holds for similar bacterial concentrations of *E. coli*, it would explain why cytokine secretions are not totally abrogated—not even when pre-incubating the whole blood with the combined inhibition of complement C3 and CD 14. The fact that none of the cytokine secretions were completely abolished when combining the inhibitors may imply that non-LPS and non-complement-related activation also play a role in this model, supporting redundancy as a general phenomenon of host defence. Nevertheless, the combined inhibition of CD 14 and C3, as shown in the present study, inhibited all mediators tested statistically more significant than either one or both of the inhibitors did alone.

In conclusion, this study demonstrated that post-challenge inhibition of C3 and CD 14 substantially attenuated *E. coli*-induced inflammation in human whole blood. The effect of this treatment regimen indicates a potential therapeutic window and it seems imperative to proceed with *in vivo* studies exploring the efficacy of a similar regimen.

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Figure 1.

Plasma concentration of pro-inflammatory cytokines. Combined inhibition of C3 and CD14 are shown in all intervention groups. The upper panels display the results after incubation for 60 min. The lower panels display the results during the whole 120-min incubation period. The results are given as mean $\pm$ SEM. Significances are between the positive control group and one or more of the intervention groups. *E.c.*: *E. coli*.

Figure 2.

Plasma concentration of chemokines. Combined inhibition of C3 and CD14 are shown in all intervention groups. The upper panels display the results after incubation for 60 min. The lower panels display the results during the whole 120-min incubation period. The results are given as mean $\pm$ SEM. Significances are between the positive control group and one or more of the intervention groups. *E.c.*: *E. coli*.

Figure 4.

Comparison of single versus combined inhibition of C3 and CD14 in the pre-challenge model. All intervention groups were pre-incubated with either compstatin alone (Comp), anti-CD14 alone (aCD14) or the combined inhibitors (CI). * and ** indicate significance between the combined inhibitors and anti-CD14, and/or between the combined inhibitors and compstatin. # and ## indicate significance between the positive control and the intervention. E.c.: *E. coli*.