

THE ANTI-INFLAMMATORY EFFECT OF COMBINED COMPLEMENT AND
CD14 INHIBITION IS PRESERVED DURING ESCALATING BACTERIAL LOAD.

Kjetil H. Egge^a, Andreas Barratt-Due^a, Stig Nymo^b, Julie K. Lindstad^a, Anne Pharo^a,
Corinna Lau^b, Terje Espevik^c, Ebbe B. Thorgersen^a, and Tom E. Mollnes^{a,b,c}

^a Department of Immunology, Oslo University Hospital Rikshospitalet, and K. G.
Jebsen IRC, University of Oslo, Oslo, Norway.

^b Research Laboratory, Nordland Hospital and Faculty of Health Sciences, K. G. Jebsen
TREC, University of Tromsø, Tromsø, Norway.

^c Centre of Molecular Inflammation Research, and Department of Cancer Research and
Molecular Medicine, Norwegian University of Science and Technology, Trondheim,
Norway

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Corresponding author: Tom E. Mollnes,
Department of Immunology, Oslo University Hospital Rikshospitalet,
P.B. 4950 Nydalen, N-0424 Oslo, Norway.

Telephone +47 23073500. Telefax +47 23073510. Email: t.e.mollnes@med.uio.no

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ABSTRACT

Combined inhibition of complement and CD14 is known to attenuate bacterial-induced inflammation, but the dependency of the bacterial load on this effect is unknown. Thus, we investigated whether the effect of such combined inhibition on *Escherichia coli*- and *Staphylococcus aureus*-induced inflammation was preserved during increasing bacterial concentrations. Human whole blood was preincubated with anti-CD14, eculizumab (C5-inhibitor) or compstatin (C3-inhibitor), or combinations thereof. Then heat-inactivated bacteria were added at final concentrations of 5×10^4 - 1×10^8 /mL (*E. coli*) or 5×10^7 - 4×10^8 /mL (*S. aureus*). Inflammatory markers were measured using ELISA, multiplex technology and flow cytometry. Combined inhibition of complement and CD14 significantly ($P < 0.05$) reduced *E. coli*-induced IL-6 by 40-92% at all bacterial concentrations. IL-1 β , IL-8 and MIP-1 α were significantly ($P < 0.05$) inhibited by 53-100%, and the effect was lost only at the highest bacterial concentration. TNF and MIP-1 β were significantly ($P < 0.05$) reduced by 80-97% at the lowest bacterial concentration. Monocyte and granulocyte CD11b were significantly ($P < 0.05$) reduced by 63-91% at all bacterial doses. Lactoferrin was significantly ($P < 0.05$) attenuated to the level of background activity at the lowest bacterial concentration. For *S. aureus* similar effects were observed, but the attenuation was in general less pronounced. Compared to *E. coli*, much higher concentrations of *S. aureus* were required to induce the same cytokine responses. This study demonstrates generally preserved effects of combined complement and CD14 inhibition on Gram-negative and Gram-positive bacterial-induced inflammation during escalating bacterial load. The implications of these findings for future therapy of sepsis are discussed.

INTRODUCTION

Despite modern treatment including up to date intensive care, sepsis still has high morbidity and mortality [1;2]. Massive research and several apparently promising clinical trials have so far not led to effective mediator-directed therapy for the systemic inflammatory response syndrome (SIRS) potentially leading to severe sepsis and septic shock [3].

The cardinal trigger of the immune response which leads to SIRS, is the recognition of conserved patterns of exogenous as well as endogenous “danger” molecules by pattern recognition receptors (PRRs) of innate immunity [4;5]. Complement and Toll-like receptors (TLRs) are central upstream sensor- and eventually effector-systems, thus triggering and maintaining the complex and widespread inflammatory response [6;7].

The complement system is a protein cascade with the main goal of defending the host against pathogens and maintaining homeostasis [8]. Complement is activated via the classical, the lectin or the alternative pathways. The pathways converge on the central complement factor C3 which is cleaved to C3a and C3b. Activation of the terminal pathway leading to the formation of the terminal C5b-9 complement complex (TCC) is triggered as C5 is cleaved to C5a and C5b [9]. C5a is a biologically highly potent anaphylatoxin associated with morbidity and mortality in sepsis [10].

The TLR family is a group of PRRs which recognize a wide variety of evolutionary conserved molecular structures [11]. Lipopolysaccharides (LPS) of Gram-negative bacteria are recognized by TLR4, which is essential in inducing the Gram-negative

inflammatory response [12]. In Gram-positive bacteria several danger molecules have been identified, including various lipoproteins and lipoteichoic acid (LTA), and some of these danger molecules are recognized by TLR2 [13-15]. CD14 is a promiscuous co-receptor for a number of TLRs and facilitates the signalling of both TLR4 and TLR2 [11;15]. Therefore, CD14 is an attractive target for upstream inhibition of TLR-responses.

There is increasing evidence for close cross-talk between complement and TLRs, and recent research suggests possible synergistic anti-inflammatory effects when inhibiting both systems simultaneously [16]. Combined inhibition of C3 and CD14 has proven to be significantly more effective than single inhibition with either of these [17].

In clinical sepsis the bacterial load may increase rapidly leading to an overwhelming inflammatory response. Although not equivalent to clinical sepsis, we have used an *ex vivo* human whole blood model to simulate bacterial-induced inflammation. In previous studies, using one bacterial load, the combined inhibition of complement and CD14 has proven effective. In the present study we aimed to test whether the effect of combined inhibition would depend on bacterial load. By incubating escalating loads of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) in the human whole blood model, we examined to which extent the anti-inflammatory effect of the combined inhibition was preserved.

MATERIALS AND METHODS

Equipment and reagents

Endotoxin-free tubes and tips were purchased from Thermo Fischer Scientific NUNC (Roskilde, Denmark). Sterile Phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} and Ethylenediaminetetraacetic acid (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 CD14 (clone 18D11; F(ab')₂ 3118, lot1383), which neutralizes CD14, was purchased from Diatec monoclonals AS (Oslo, Norway) and used in the *E. coli* experiments. The recombinant anti-human CD14 IgG2/4 Ab (r18D11) was used in the *S. aureus* experiments [18]. The complement C5 inhibitor, eculizumab (Soliris[®]) was obtained from Alexion Pharmaceuticals (Cheshire, CT). The compstatin analogue Cp40 (*d*Tyr-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-Sar-His-Arg-Cys]-mIle-NH₂; 1.7 kDa), a kind gift from Professor John Lambris, was produced by solid-phase peptide synthesis as previously described [19].

Bacteria

Heat-inactivated *E. coli* strain LE392 (ATCC 33572) and *S. aureus* Cowan Strain 1 (ATCC 12598) were obtained from American Type Culture Collection (Manassas, VA).

Ex vivo whole blood model

The whole blood model has been described in details previously [20]. In short, blood was drawn into 4.5 mL NUNC tubes containing the anticoagulant lepirudin (50 $\mu\text{g/mL}$), which only blocks thrombin and does not interfere with the remaining inflammatory network. All of the following experiments were performed with blood from six healthy donors. The different conditions described below were defined after careful pilot titration experiments to obtain optimal concentration and time intervals.

The E. coli experiments

Incubation of whole blood for final plasma analyses

The baseline sample (T0) was processed immediately after the blood was drawn and EDTA added to the whole blood. One tube was preincubated with PBS and served as the negative control. Four tubes were preincubated with PBS for five minutes at 37°C before adding *E. coli* to final concentrations of 5×10^4 , 5×10^5 , 5×10^6 and 5×10^7 bacteria/mL and served as positive controls. In the same manner, four tubes were preincubated with eculizumab only, four tubes with anti-CD14 only and four tubes with the combination of eculizumab and anti-CD14 before adding *E. coli*. All samples were incubated for a total of four hours before adding EDTA to a final concentration of 20 mM. The tubes receiving 5×10^5 , 5×10^6 and 5×10^7 bacteria/mL were used for measuring proinflammatory cytokines, whereas the tubes receiving 5×10^4 , 5×10^5 and 5×10^6 bacteria/mL were used for measuring chemokines. The tubes receiving 5×10^6 and 5×10^7 bacteria/mL were used for measuring TCC. Experiments for analysis of granulocyte activation markers were conducted in the same manner as described above, with three modifications: instead of eculizumab, compstatin Cp40 was used for inhibiting

complement, *E. coli* was added to final concentrations of 1×10^6 , 3×10^6 and 9×10^6 bacteria/mL, and all samples were incubated for a total of one hour. We consistently used Cp40 (a C3-inhibitor) to study the release of granulocyte enzyme release instead of eculizumab, since this effect has been shown to be C3 dependent, in contrast to the other inflammatory readouts studied [24].

Incubation of whole blood for CD11b analysis

Immediately after drawing the blood from the donor, the cells from a sample of the whole blood were fixed with 0.5% (v/v) paraformaldehyde in an equal volume for four minutes at 37°C to serve as a baseline (T0) sample. The subsequent bacterial activation of the whole blood was done as described in the experiments for cytokine readout, with two modifications: *E. coli* was added to a final concentration of 4×10^6 , 2×10^7 and 1×10^8 bacteria/mL, and incubated for 15 minutes. Following 15 minutes of incubation, the cells were fixed with 0.5% (v/v) paraformaldehyde in an equal volume for four minutes at 37°C, and then stained with anti-CD11b PE and anti-CD14 FITC (Becton Dickinson, San Jose, CA). The red cells were lysed and the samples washed twice using PBS with 0.1 % Rinder albumin (300 g, five minutes, 4 °C) before they were run on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with threshold on FSC to exclude debris. Monocytes were gated in a side scatter/CD14 dot plot, whereas granulocytes were gated in a forward scatter/side scatter dot plot. CD11b expression was measured as median fluorescence intensity (MFI).

The S. aureus experiments

Incubation of whole blood for final plasma analyses

The experiments were conducted in the same manner as described for *E. coli*, with two modifications: instead of *E. coli*, *S. aureus* was added to final concentrations of 5×10^7 , 1×10^8 and 2×10^8 bacteria/mL, and all samples were incubated for a total of two hours.

In addition, samples containing 1×10^8 and 2×10^8 bacteria/mL were incubated for a total of four hours for measuring TCC. Experiments for analysis of granulocyte activation markers were conducted in the same manner as described for *E. coli*, with one modification: instead of *E. coli*, *S. aureus* was added to final concentrations of 5×10^7 , 1×10^8 and 2×10^8 bacteria/mL.

Incubation of whole blood for CD11b analysis

The experiments were conducted in the same manner as described for *E. coli*, with one modification: Instead of *E. coli*, *S. aureus* was added to final concentrations of 1×10^8 , 2×10^8 and 4×10^8 bacteria/mL.

Multiplex analysis

Cytokines (TNF, IL-1 β , IL-6, IL-8, MIP-1 α and MIP-1 β) were measured using a multiplex cytokine assay from Bio-Rad Laboratories (Hercules, CA). The multiplex kit was used according to the instructions from the manufacturer.

Enzyme immunoassays

TCC

The soluble terminal C5b-9 complement complex (TCC) was measured in an enzyme immunoassay (EIA), as described previously and later modified [21;22]. Briefly, the monoclonal antibody (mAb) aE11 reacting with a neoepitope exposed in C9 after incorporation in the C5b-9 complex was used as capture antibody. A biotinylated anti-C6 mAb (clone 9C4) was used as detection antibody. The standard was Stock Complement Standard #2 [23], which is normal human serum activated with zymosan and defined to contain 1000 complement arbitrary units (CAU)/mL. Zymosan activated human serum was used as a positive control.

Granulocyte activation markers.

The granulocyte activation markers myeloperoxidase and lactoferrin were quantified using commercial EIA kits from Hycult Biotech (Uden, The Netherlands). The analyses were performed in accordance to the instructions from the manufacturer.

Data presentation and statistics

All results are given as mean and standard error of the mean (SEM). GraphPad Prism version 5.04 (San Diego, CA) was used for statistical analysis. One-way ANOVA with Dunnett's multiple comparison test was used to compare single and combined inhibition with positive controls. The background activation (negative control) was subtracted from the positive control and the intervention groups before calculating the percentage inhibition by the different inhibitors. 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 consent to participate in the study.

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RESULTS

The E. coli experiments

Effect of bacterial load on single and combined inhibition of C5 and CD14 on proinflammatory cytokines

IL-1 β

Single inhibition of both C5 and CD14 reduced IL-1 β by 93% ($P<0.01$) and 66% ($P<0.001$) respectively, when whole blood was incubated with 5×10^5 *E. coli*/mL (Figure 1, upper left panel). No significant reduction of IL-1 β was seen by single inhibition when using 5×10^6 and 5×10^7 *E. coli*/mL.

Combined inhibition of C5 and CD14 reduced IL-1 β by 91% ($P<0.001$) and 53% ($P<0.05$) when whole blood was incubated with 5×10^5 and 5×10^6 *E. coli*/mL, respectively (Figure 1, upper left panel). No significant reduction of IL-1 β was seen when using 5×10^7 *E. coli*/mL.

IL-6

Single inhibition of C5 had no significant effect on IL-6 at any of the bacterial concentrations, whereas single inhibition of CD14 reduced IL-6 by 76% ($P<0.001$) and 34% ($P<0.05$) when whole blood was incubated with 5×10^5 and 5×10^6 *E. coli*/mL, respectively (Figure 1, upper middle panel). No significant reduction of IL-6 was seen by single inhibition of CD14 when using 5×10^7 *E. coli*/mL.

Combined inhibition reduced IL-6 by 92% ($P<0.001$), 62% ($P<0.001$), and 40% ($P<0.05$) when whole blood was incubated with 5×10^5 , 5×10^6 and 5×10^7 *E. coli*/mL, respectively (Figure 1, upper middle panel).

TNF

Single inhibition of C5 had no significant effect on TNF at any of the bacterial concentrations, whereas single inhibition of CD14 reduced TNF by 41% ($P<0.01$) when whole blood was incubated with 5×10^5 *E. coli*/mL (Figure 1, upper right panel). No significant reduction of TNF was seen by single inhibition of CD14 when using 5×10^6 and 5×10^7 *E. coli*/mL.

Combined inhibition reduced TNF by 80% ($P<0.001$) when whole blood was incubated with 5×10^5 *E. coli*/mL (Figure 1, upper right panel). No significant reduction of TNF was seen when using 5×10^6 and 5×10^7 *E. coli*/mL.

Effect of bacterial load on single and combined inhibition of C5 and CD14 on chemokines

IL-8

Single inhibition of C5 and CD14 had no significant effect on IL-8 at any of the bacterial concentrations.

Combined inhibition of C5 and CD14 reduced IL-8 by 100% ($P<0.05$) when whole blood was incubated with 5×10^5 *E. coli*/mL (Figure 1, lower left panel). No significant reduction of IL-8 was seen when using 5×10^4 and 5×10^6 *E. coli*/mL.

MIP-1 α

Single inhibition of C5 reduced MIP-1 α by 60% ($P<0.05$) when whole blood was incubated with 5×10^4 *E. coli*/mL, whereas no significant reduction was seen when using higher concentrations of *E. coli* (Figure 1, lower middle panel).

Single inhibition of CD14 reduced MIP-1 α by 78% ($P<0.01$) and 78% ($P<0.001$) when whole blood was incubated with 5×10^4 and 5×10^5 *E. coli*/mL, respectively (Figure 1, lower middle panel). No significant reduction of MIP-1 α was seen by single CD14 inhibition when using and 5×10^6 *E. coli*/mL.

Combined inhibition reduced MIP-1 α by 97% ($P<0.001$) and 93% ($P<0.001$) when whole blood was incubated with 5×10^4 and 5×10^5 *E. coli*/mL, respectively (Figure 1, lower middle panel). No significant reduction of MIP-1 α was seen when using 5×10^6 *E. coli*/mL.

MIP-1 β

Single inhibition of C5 had no significant effect on MIP-1 β at any of the bacterial concentrations, whereas single inhibition of CD14 reduced MIP-1 β by 82% ($P<0.05$) when whole blood was incubated with 5×10^4 *E. coli*/mL (Figure 1, lower right panel).

No significant reduction of MIP-1 β was seen by single CD14 inhibition when using 5×10^5 and 5×10^6 *E. coli*/mL.

Combined inhibition reduced MIP-1 β by 97% ($P<0.05$) when whole blood was incubated with 5×10^4 *E. coli*/mL (Figure 1, lower right panel). No significant reduction of MIP-1 β was seen when using 5×10^5 and 5×10^6 *E. coli*/mL.

Effect of bacterial load on single and combined inhibition of C5 and CD14 on the expression of CD11b

Monocytes

Single inhibition of C5 had no significant effect on CD11b expression at any of the bacterial concentrations, whereas single inhibition of CD14 reduced CD11b expression by 49% ($P<0.05$), 55% ($P<0.01$) and 29% ($P<0.01$) when whole blood was incubated with 4×10^6 , 2×10^7 and 1×10^8 *E. coli*/mL, respectively (Figure 2, upper left panel).

Combined inhibition of C5 and CD14 reduced CD11b expression by 66% ($P<0.01$), 77% ($P<0.01$) and 63% ($P<0.001$) when whole blood was incubated with 4×10^6 , 2×10^7 and 1×10^8 *E. coli*/mL, respectively (Figure 2, upper left panel).

Granulocytes

Single inhibition of C5 reduced CD11b expression by 77% ($P<0.05$), 75% ($P<0.01$) and 59% ($P<0.01$) when whole blood was incubated with 4×10^6 , 2×10^7 and 1×10^8 *E. coli*/mL, respectively (Figure 2, upper right panel). Single inhibition of CD14 had no significant effect on CD11b expression at any of the bacterial concentrations.

Combined inhibition reduced CD11b expression by 77% ($P<0.05$), 91% ($P<0.001$) and 84% ($P<0.001$) when whole blood was incubated with 4×10^6 , 2×10^7 and 1×10^8 *E. coli*/mL, respectively (Figure 2, upper right panel).

Effect of bacterial load on single and combined inhibition of C3 and CD14 on granulocyte enzyme release

We used a C3 inhibitor to study the release of granulocyte enzyme release, since this effect was shown to be C3 dependent, in contrast to the other inflammatory readouts studied, which were mainly C5-dependent [24].

Myeloperoxidase

Single and combined inhibition of C3 and CD14 had no significant effect on myeloperoxidase at any of the bacterial concentrations.
(Figure 2, lower left panel).

Lactoferrin

Single inhibition of C3 and CD14 had no significant effect on lactoferrin at any of the bacterial concentrations.

Combined inhibition of C3 and CD14 reduced the release of lactoferrin to the level of background activity when whole blood was incubated with 1×10^6 *E. coli*/mL ($P<0.05$) (Figure 2, lower right panel).

Effect of bacterial load on single and combined inhibition of C5 and CD14 on TCC concentration

Combined inhibition of C5 and CD14 and single inhibition of C5, reduced the formation of TCC by 98-99% ($P<0.001$) and 99% ($P<0.001$) when whole blood was incubated with 5×10^6 and 5×10^7 *E. coli*/mL, respectively (Table 1), confirming that C5 inhibition was efficient over the whole range of bacterial concentrations. As expected, no reduction of TCC was seen with selective inhibition of CD14 (Table 1).

Taken together, these data indicate that the effect of combined inhibition of complement and CD14 in general is well preserved by increasing doses of *E. coli*, though with some reduced effect on cytokines and chemokines in the highest bacterial concentration.

The S. aureus experiments

Effect of bacterial load on single and combined inhibition of C5 and CD14 on proinflammatory cytokines

IL-1 β

Single and combined inhibition of C5 and CD14 had no significant effect on IL-1 β at any of the bacterial concentrations (Figure 3, upper left panel).

IL-6

Single inhibition of C5 and CD14 had no significant effect on IL-1 β at any of the bacterial concentrations.

Combined inhibition reduced IL-6 by 93% ($P<0.05$) when whole blood was incubated with 5×10^7 *S. aureus*/mL (Figure 3, upper middle panel). No significant reduction of IL-6 was seen when using 1×10^8 and 2×10^8 *S. aureus*/mL.

TNF

Single inhibition of C5 significantly reduced TNF by 79% ($P<0.05$) when whole blood was incubated with 5×10^7 *S. aureus*/mL (Figure 3, upper right panel). No significant reduction of TNF was seen when using 1×10^8 and 2×10^8 *S. aureus*/mL. Single inhibition of CD14 had no significant effect on TNF at any of the bacterial concentrations.

Combined inhibition reduced TNF by 86% ($P<0.05$) when whole blood was incubated with 5×10^7 *S. aureus*/mL (Figure 3, upper right panel). No significant reduction of TNF was seen when using 1×10^8 and 2×10^8 *S. aureus*/mL.

Effect of bacterial load on single and combined inhibition of C5 and CD14 on chemokines

IL-8

Single inhibition of C5 and CD14 had no significant effect on IL-1 β at any of the bacterial concentrations.

Combined inhibition of C5 and CD14 reduced IL-8 by 80% ($P<0.05$) when whole blood was incubated with 1×10^8 *S. aureus*/mL (Figure 3, lower left panel). No significant reduction of IL-8 was seen when using 5×10^7 and 2×10^8 *S. aureus*/mL.

MIP-1 α

Single and combined inhibition of C5 and CD14 had no significant effect on MIP-1 α at any of the bacterial concentrations (Figure 3, lower middle panel).

MIP-1 β

Single and combined inhibition of C5 and CD14 had no significant effect on MIP-1 β at any of the bacterial concentrations (Figure 3, lower right panel).

Effect of bacterial load on single and combined inhibition of C5 and CD14 on CD11b expression.*Monocytes*

Single inhibition of C5 reduced CD11b expression by 79% ($P<0.05$) and 74% ($P<0.01$) when whole blood was incubated with 1×10^8 and 2×10^8 *S. aureus*/mL, respectively (Figure 4, upper left panel). No significant reduction of CD11b expression was seen when using 4×10^8 *S. aureus*/mL. Single inhibition of CD14 had no significant effect on CD11b expression at any of the bacterial concentrations.

Combined inhibition of C5 and CD14 reduced CD11b expression by 70% ($P<0.05$), 87% ($P<0.001$) and 48% ($P<0.05$) when whole blood was incubated with 1×10^8 , 2×10^8 and 4×10^8 *S. aureus*/mL, respectively (Figure 4, upper left panel).

Granulocytes

Single inhibition of C5 reduced CD11b expression by 73% ($P<0.05$), 83% ($P<0.001$) and 61% ($P<0.01$) when whole blood was incubated with 1×10^8 , 2×10^8 and 4×10^8 *S.*

aureus/mL, respectively (Figure 4, upper right panel). Single inhibition of CD14 had no significant effect on CD11b expression at any of the bacterial concentrations.

Combined inhibition reduced CD11b expression by 83% ($P<0.05$), 95% ($P<0.001$) and 77% ($P<0.001$) when whole blood was incubated with 1×10^8 , 2×10^8 and 4×10^8 *S. aureus*/mL, respectively (Figure 4, upper right panel).

Effect of bacterial load on single and combined inhibition of C3 and CD14 on granulocyte enzyme release.

We used a C3 inhibitor to study the release of granulocyte enzyme release, since this effect was shown to be C3 dependent, in contrast to the other inflammatory readouts studied, which were mainly C5-dependent [24].

Myeloperoxidase

Single and combined inhibition of C3 and CD14 had no significant effect on myeloperoxidase at any of the bacterial concentrations (Figure 4, lower left panel).

Lactoferrin

Single and combined inhibition of C3 and CD14 had no significant effect on lactoferrin release at any of the bacterial concentrations (Figure 4, lower right panel).

Effect of bacterial load on single and combined inhibition of C5 and CD14 on TCC concentration

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Combined inhibition of C5 and CD14 and single inhibition of C5 reduced the formation of TCC by 98-99% ($P<0.05$) and 99% ($P<0.001$) when whole blood was incubated with 1×10^8 and 2×10^8 *S. aureus*/mL, respectively (Table 1), confirming that C5 inhibition was efficient over the whole range of bacterial concentrations. As expected, no reduction in TCC formation was seen with single inhibition of CD14.

Taken together, these data indicate that the preserved inhibitory effect of combined inhibition of complement and CD14 seen by increasing doses of the Gram-positive bacterium *S. aureus* was less pronounced as compared to what was found with the Gram-negative bacterium *E. coli*.

DISCUSSION

The results of this study consistently show a relation between the increased bacterial load and the inflammatory response induced by *E. coli* and *S. aureus*. The findings correspond well to the clinical setting in which an increased level of cytokines is correlated with the severity of sepsis [25]. Notably, combined inhibition of complement and CD14 efficiently attenuated bacterial-induced inflammation over a broad range of bacterial concentrations. This effect was quantitatively preserved for most of the inflammatory mediators although fewer significant results were observed for *S. aureus* as compared with *E. coli*, and slightly lesser effects were seen for some of the proinflammatory cytokines at the highest bacterial concentration. The latter result is consistent with previous data on *Neisseria meningitides* [26]. Evidence suggests that high bacterial doses lead to a “point of no return” after which the inflammatory cascade cannot be affected by treatment [27].

Taking a “point of no return” into consideration, the administration of anti-inflammatory drugs must be done at an early stage, or maybe even before the onset of sepsis in patients at high risk. As the microorganism is seldom known in early stages of clinical sepsis, we equally importantly showed that the combined inhibition was effective in reducing Gram-negative and Gram-positive induced inflammation. However, as compared to *E. coli*, much higher concentrations of *S. aureus* were required to induce the same inflammatory responses; the *E. coli*-induced inflammatory responses, especially chemokine responses and granulocyte enzyme release, were also more efficiently attenuated by the combined inhibition of complement and CD14.

The inflammatory response in Gram-positive sepsis is more complement driven as compared to the Gram-negative response which is largely LPS dependent and therefore more TLR4 driven [28]. Although both complement and CD14 play vital roles mediating *E. coli*- as well as *S. aureus*-induced inflammation, it is uncertain whether the anti-CD14 effects observed on the *S. aureus*-induced responses reflect the involvement of TLR2, TLR9 or other TLRs [24;29]. The bacteria causing the infection are most often unknown at the time sepsis develops [30], and quite often the infecting microbe remains unknown. A treatment with combined inhibition of complement and CD14 is meant for early attenuation of the inflammatory response, before the cytokine storm emerges. Thus, from a clinical perspective the combined approach therefore seems sensible, provided proper antibiotic therapy is given. Furthermore, no single downstream anti-inflammatory treatment has been able to achieve increased survival in sepsis [31], whereas a cecal ligation and puncture-induced polymicrobial sepsis study in mice clearly showed increased survival in the group receiving combined inhibition [32]. The anti-inflammatory effects observed in the current study are thus linked to *in vivo* effects, strengthening possible clinical implications by the combined inhibition.

The proinflammatory cytokines and chemokines included in the present study were selected based on their implication in the pathogenesis of sepsis. Plasma TNF, IL-1 β , IL-6 and IL-8, are important mediators of the inflammatory response in SIRS and sepsis and correlate partially with mortality and severity in septic patients [33-35]. However, several large clinical trials show that increased survival has not been achieved by targeting these cytokines selectively [36-38]. By targeting upstream recognition molecules of innate immunity, i.e. complement and CD14, we observed a significant

concurrent reduction of all four cytokines in escalating bacterial-induced inflammation, implicating benefits of upstream as opposed to downstream single agent anti-inflammatory treatments, even in relatively high bacterial concentrations. Interestingly, the release of IL-6 was significantly inhibited even at the highest concentration of *E. coli*. The inhibitory effect was still significant for IL-1 β , IL-8 and MIP-1 α at bacterial concentrations close to the highest dose. For *S. aureus* there were less statistically significant effects observed as compared with *E. coli*, corresponding with a greater variation in the inflammatory response to the Gram-positive bacterium.

C5a has for a long time been reported to induce IL-8 [39], which may explain the relative complement dependent IL-8 release observed in the current study. IL-8 is one of the major mediators of the inflammatory response in sepsis with the main function to induce chemotaxis of neutrophils to the site of inflammation [25]. As the level of IL-8 correlate to mortality in septic patients [33], the use of complement inhibition in sepsis is thus logical.

E. coli-induced surface expression of CD11b on granulocytes was significantly reduced to background levels by the combined inhibition even at the highest bacterial concentration. The release of myeloperoxidase was not significantly changed, whereas combined inhibition significantly reduced lactoferrin at the lower dose. In presence of Gram-positive bacteria, however, this leukocyte inflammatory response was gradually less affected by combined inhibition as the bacterial load increased. Interestingly, *E. coli*-induced granulocyte CD11b expression was essentially complement dependent, whereas for monocytes it was mainly CD14 dependent. In contrast, when activating

whole blood with *S. aureus*, CD11b expression seemed to be complement dependent for both subsets of leukocytes. These results are in line with previous studies and the concept of cross-talk between complement and TLRs [24;40].

In conclusion, this study showed significant broad anti-inflammatory effects by the combined inhibition of complement and CD14 by increasing Gram-negative as well as Gram-positive bacteria to high concentrations. A “point of no return” in the inflammatory response emerges at last, underscoring the relevance of applying such treatment at an early stage of the syndrome. The results of the present study strengthen the hypothesis that the combined inhibition of complement and CD14 may become a future mediator directed therapy in sepsis.

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CONFLICT OF INTEREST STATEMENT

All authors declare that they have no competing financial or other interest in relation to their work.

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FIGURE LEGENDS

Figure 1. Effect of single and combined complement and CD14 inhibition on plasma concentrations of proinflammatory cytokines (upper panels) and chemokines (lower panels) with increasing bacterial load in the *E. coli* experiments. Data are presented as mean and standard error of the mean. The background activation (negative control) was subtracted from the positive control and the intervention groups before calculating the percentage inhibition by the different inhibitors. Significances are between the positive control group (*E. coli*) and single and combined inhibition of C5 (eculizumab) and CD14. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

Figure 2. Effect of single and combined complement and CD14 inhibition on CD11b expression on monocytes and granulocytes in whole blood (upper panels) and plasma concentrations of granulocyte enzyme release (lower panels) with increasing bacterial load in the *E. coli* experiments. Data are presented as mean and standard error of the mean. The background activation (negative control) was subtracted from the positive control and the intervention groups before calculating the percentage inhibition by the different inhibitors. Significances are between the positive control group (*E. coli*) and single and combined inhibition of either C5 (eculizumab in the CD11b experiments) and CD14, or C3 (compstatin in the granulocyte enzyme release experiments) and CD14. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

Figure 3. Effect of single and combined complement and CD14 inhibition on plasma concentrations of proinflammatory cytokines (upper panels) and chemokines (lower

panels) with increasing bacterial load in the *S. aureus* experiments. Data are presented as mean and standard error of the mean. The background activation (negative control) was subtracted from the positive control and the intervention groups before calculating the percentage inhibition by the different inhibitors. Significances are between the positive control group (*S. aureus*) and single and combined inhibition of C5 (eculizumab) and CD14. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

Figure 4. Effect of single and combined complement and CD14 inhibition on CD11b expression on monocytes and granulocytes in whole blood (upper panels) and plasma concentrations of granulocyte enzyme release (lower panels) with increasing bacterial load in the *S. aureus* experiments. Data are presented as mean and standard error of the mean. The background activation (negative control) was subtracted from the positive control and the intervention groups before calculating the percentage inhibition by the different inhibitors. Significances are between the positive control group (*S. aureus*) and single and combined inhibition of either C5 (eculizumab in the CD11b experiments) and CD14, or C3 (compstatin in the granulocyte enzyme release experiments) and CD14. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

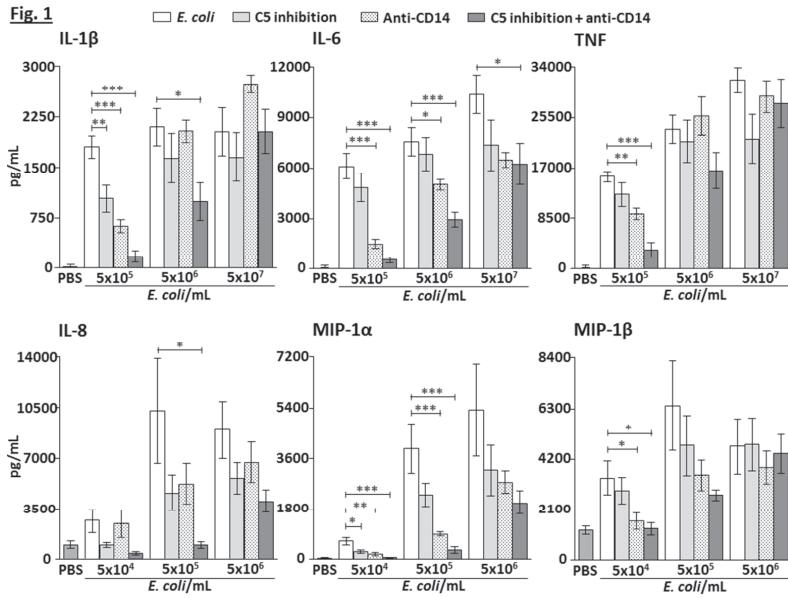
TABLE 1 Effect of complement C5 inhibition (eculizumab) and CD14 inhibition on the concentration of the terminal complement complex (TCC)

Bacteria (per mL)	Positive control (CAU) ^a	Anti-CD14 (%) ^b	C5 inhibition (%) ^{b, c}	C5 inhibition and anti-CD14 (%) ^{b, c}
<i>E. coli</i> 5x10 ⁶	31	0	98	98
<i>E. coli</i> 5x10 ⁷	116	19	100	100
<i>S. aureus</i> 1x10 ⁸	76	21	96	99
<i>S. aureus</i> 2x10 ⁸	171	7.6	100	100

^a TCC measured as Complement Arbitrary Units (CAU)

^b Reduction compared to positive control

^c All results $P < 0.05$

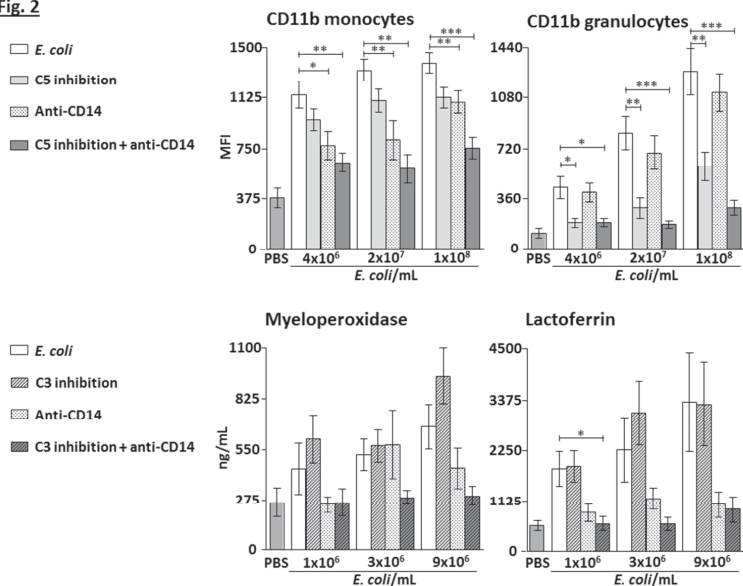


Effect of combined and single complement and CD14 inhibition on plasma concentrations of proinflammatory cytokines (upper panels) and chemokines (lower panels) with increasing bacterial load in the *E. coli* experiments. Data are presented as mean and standard error of the mean. Significances are between the positive control group (*E. coli*) and combined and single inhibition of C5 (eulizumab) and CD14. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

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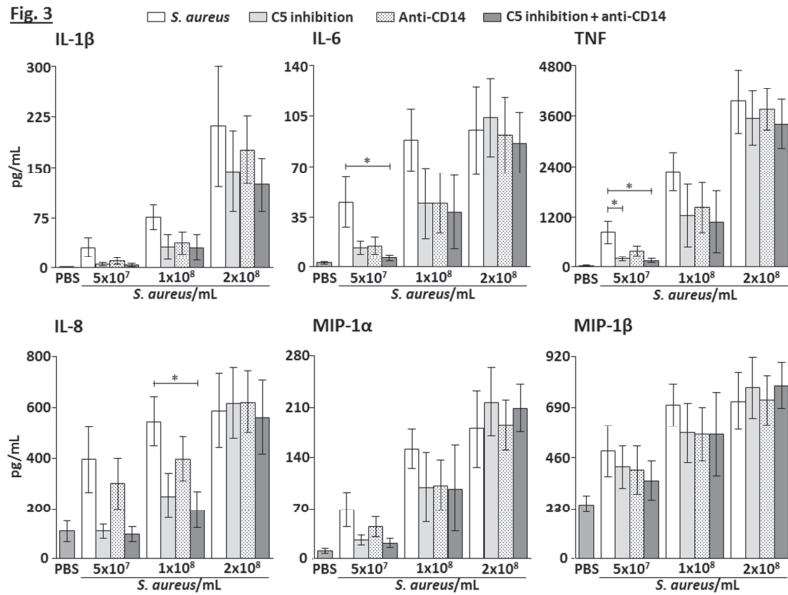
Fig. 2



Effect of combined and single complement and CD14 inhibition on CD11b expression on monocytes and granulocytes in whole blood (upper panels) and plasma concentrations of granulocyte enzyme release (lower panels) with increasing bacterial load in the *E. coli* experiments. Data are presented as mean and standard error of the mean. Significances are between the positive control group (*E. coli*) and the combined and single inhibition of either C5 (eculizumab in the CD11b experiments) and CD14, or C3 (compstatin in the granulocyte enzyme release experiments) and CD14. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

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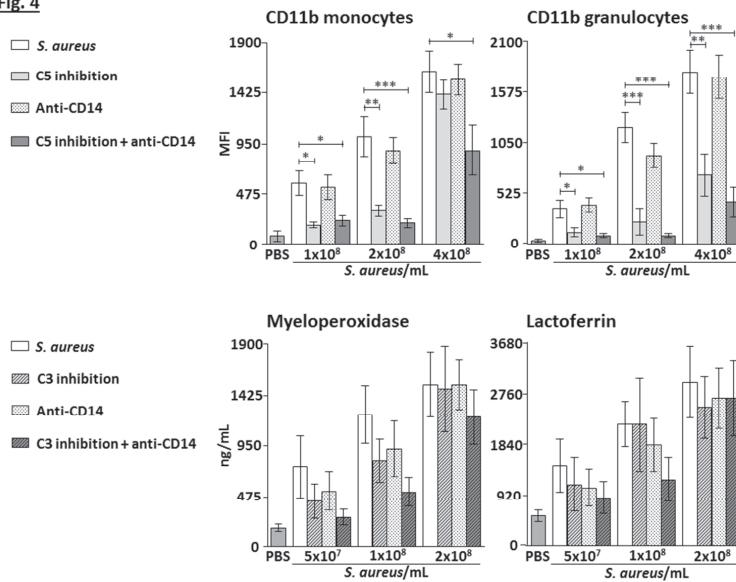


Effect of combined and single complement C5 and CD14 inhibition on plasma concentrations of proinflammatory cytokines (upper panels) and chemokines (lower panels) with increasing bacterial load in the *S. aureus* experiments. Data are presented as mean and standard error of the mean. Significances are between the positive control group (*S. aureus*) and the combined and single inhibition of C5 (eculizumab) and CD14. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.

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Fig. 4



Effect of combined and single complement and CD14 inhibition on CD11b expression on monocytes and granulocytes in whole blood (upper panels) and plasma concentrations of granulocyte enzyme release (lower panels) with increasing bacterial load in the *S. aureus* experiments. Data are presented as mean and standard error of the mean. Significances are between the positive control group (*S. aureus*) and combined and single inhibition of either C5 (eculizumab in the CD11b experiments) and CD14, or C3 (compstatin in the granulocyte enzyme release experiments) and CD14. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$.
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