PAPER IV
Combined Inhibition of Complement and CD14 Attenuates Bacteria-Induced Inflammation in Human Whole Blood More Efficiently Than Antagonizing the Toll-like Receptor 4–MD2 Complex

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**Background.** Single inhibition of the Toll-like receptor 4 (TLR4)–MD2 complex failed in treatment of sepsis. CD14 is a coreceptor for several TLRs, including TLR4 and TLR2. The aim of this study was to investigate the effect of single TLR4-MD2 inhibition by using eritoran, compared with the effect of CD14 inhibition alone and combined with the C3 complement inhibitor compstatin (Cp40), on the bacteria-induced inflammatory response in human whole blood.

**Methods.** Cytokines were measured by multiplex technology, and leukocyte activation markers CD11b and CD35 were measured by flow cytometry.

**Results.** Lipopolysaccharide (LPS)–induced inflammatory markers were efficiently abolished by both anti-CD14 and eritoran. Anti-CD14 was significantly more effective than eritoran in inhibiting LPS-binding to HEK-293E cells transfected with CD14 and Escherichia coli–induced upregulation of monocyte activation markers (P < .001). Combining Cp40 with anti-CD14 was significantly more effective than combining Cp40 with eritoran in reducing E. coli–induced interleukin 6 (P < .05) and monocyte activation markers induced by both E. coli (P < .001) and Staphylococcus aureus (P < .01). Combining CP40 with anti-CD14 was more efficient than eritoran alone for 18 of 20 bacteria-induced inflammatory responses (mean P < .0001).

**Conclusions.** Whole bacteria–induced inflammation was inhibited more efficiently by anti-CD14 than by eritoran, particularly when combined with complement inhibition. Combined CD14 and complement inhibition may prove a promising treatment strategy for bacterial sepsis.

**Keywords.** sepsis; complement; CD14; eritoran; treatment; TLR.
Toll-like receptors (TLRs) induce responses through the activation of specific intracellular signaling pathways [4]. TLR4 and TLR2 are the most studied receptors, with TLR4 being the key receptor for lipopolysaccharide (LPS) from gram-negative bacteria and TLR2 detecting lipoproteins from gram-positive bacteria. CD14 serves as a coreceptor for TLR4, where it presents the LPS molecule to the LPS-binding site on the TLR4-MD2 complex [5]. CD14 has also been shown to interact with other TLRs, including TLR2, TLR3, TLR7, and TLR9 [6–8]. Recently, we produced a recombinant version of the neutralizing monoclonal mouse anti-human CD14 antibody 18D11 [9]. This antibody is endowed with a human immunoglobulin G2/4 (IgG2/4) hybrid heavy chain and is thus inert with respect to the major IgG effector functions (ie, complement activation and Fcγ-receptor binding) and therefore does not deplete leukocytes.

Eritoran is a synthetic lipid A analogue that binds to the LPS binding site on MD2 and prevents further signaling via the TLR4-MD2 complex. Recent reports suggest that eritoran also binds directly to CD14 and might partly inhibit the LPS-CD14 interaction [10]. Although eritoran effectively inhibits LPS-induced inflammatory responses, no reduction in 28-day mortality was achieved in a phase 3 study, in which eritoran was administered as an adjunct to standard sepsis treatment [11].

Combined inhibition with a neutralizing CD14 antibody and a complement inhibitor is effective in attenuating the inflammatory process by inhibiting cytokine release and expression of activation markers on leukocytes to a greater extent than single inhibition with either agent alone [12–14]. Murine and porcine in vivo sepsis models have demonstrated this strategy to be superior to single inhibition with either of the inhibitors alone, with respect to both attenuation of the inflammatory response and increased survival [15–17].

The aim of the present study was to elucidate the differential effects of eritoran and anti-CD14, alone and in combination with a complement inhibitor, on the inflammatory process induced by LPS, gram-negative bacteria, and gram-positive bacteria.

**MATERIALS AND METHODS**

**Equipment and Reagents**

Endotoxin-free Cryo tubes were purchased from Nunc (Roskilde, Denmark). Ethylenediaminetetraacetic acid (EDTA), parafomaldehyde, and sterile phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, Missouri). Lepirudin (Refludan) was purchased from Celgene (Marburg, Germany). Dimethyl sulfoxide and Alexa Fluor 488 were obtained from Invitrogen Molecular Probes (Eugene, Oregon). Anti-CD11b-PE (clone D12), anti-CD35-FITC (clone E11), anti-CD45-PerCP (clone 2D1), anti-CD45-PerCP, and anti-CD14-PE (clone Mo9P) and the isotype controls PE-mouse IgG2a (clone X59) and FITC-mouse IgGlk (clone MOPC-21) were obtained from Becton, Dickinson, and Company (San Jose, California).

Inhibitors

The C3-inhibitor compstatin analogue Cp40 (D-Tyr-Ile-[Cys-Val-1MeTrp-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-meIle) and the control peptide (Sar-Sar-Trp[Me]-Ala-Ala-Asp-Ile-His-Val-Gln-Arg-mile-Trp-Ala-NH2) were produced in the lab of one of the authors (J. D. L.), as previously described [18]. Eritoran (E5564) was kindly provided by Eisai (Andover, Massachusetts). Recombinant anti-human CD14 IgG2/4 antibody (r18D11) and an IgG2/4 isotype control antibody were produced in our laboratory as previously described [9].

**LPS and Bacteria**

Ultrapure LPS from *E. coli* strain 0111:B4 (smooth type) was purchased from InvivoGen (San Diego, California) and Cy5-labelled for the human embryonic kidney (HEK) 293E cell–based experiments as described previously [19]. *E. coli* strain LE392 with smooth LPS (ATCC 33572) and *S. aureus* Cowan strain 1 (ATCC 12598) were obtained from American Type Culture Collection (ATCC; Manassas, Virginia). The bacteria were inactivated by heat and counted by flow cytometry as previously described [12, 14].

**HEK-293E Cell–Based Experiments**

HEK-293E cells do not express CD14, TLR4, or MD2 and were transfected with human CD14, using GeneJuice transfection reagent (Novagene, Darmstadt, Germany). The cells were preincubated for 5 minutes with increasing doses of anti-CD14 (0.007, 0.07, and 0.7 µM), eritoran (0.1, 1, and 10 µM), or an IgG2/4 isotype control antibody (0.007, 0.07, and 0.7 µM) before addition of Cy5-labeled LPS (500 ng/mL). Cy5-LPS binding to CD14 was measured by flow cytometry after 1 hour of incubation (37°C). The samples were washed in cold PBS twice and detached in Accutase solution (Sigma) before the addition of 2% fetal calf serum in PBS. The cell pellets were harvested by centrifugation (at 470g for 5 minutes) and resuspended in PBS before analysis on a BD LSR II flow cytometer. Data analysis was performed with FlowJo software (Ashland, Oregon). Results are given as mean fluorescence intensity.

**Whole-Blood Model of Inflammation**

The whole-blood model is an in vitro system for studying inflammation activity and has been described in detail previously [20]. Briefly, blood specimens were collected from healthy volunteers (both sexes were evaluated, with an age range of 30 to 58 years) and anticoagulated with the thrombin inhibitor lepirudin (50 µg/mL). Blood specimens were preincubated with the inhibitors compstatin Cp40 (20 µM), anti-CD14 (0.1 µM), eritoran (1 µM), control peptide (20 µM), IgG2/4 isotype control antibody (0.1 µM), or PBS in sterile polypropylene tubes for 5 minutes (37°C). The optimal inhibitor concentrations were determined in separate dose-response experiments (data not shown). The concentration of eritoran was comparable to the plasma concentration in a phase 2 clinical study [21], using a similar dose as that in the ACCESS sepsis trial [11]. Subsequently,
blood specimens were stimulated with LPS, E. coli, or S. aureus at concentrations specified below and incubated with gentle rotation (at 37°C) for the appropriate time, depending on which inflammation markers were to be studied.

**Cytokine Analysis**

LPS (10 ng/mL), E. coli (1 × 10⁸ bacteria/mL), or S. aureus (1 × 10⁷ bacteria/mL) were added, and samples were incubated for 120 minutes. After incubation, EDTA was added (10 mM), and the samples placed on ice and centrifuged (at 1800g for 15 minutes at 4°C); the resulting plasma was frozen (−70°C) until further analyzed. Analyses of tumor necrosis factor (TNF), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8) were performed with Bio-Plex Pro technology (Bio-Rad Laboratories, Hercules, California), using standard procedures from the manufacturer.

Leukocyte Activation Markers CD11b and CD35

LPS (10 ng/mL), E. coli (1 × 10⁷ bacteria/mL), or S. aureus (1 × 10⁸ bacteria/mL) were added, and samples were incubated for 20 minutes. Cells were fixed with 0.5% paraformaldehyde and incubated with anti-CD11b-PE and anti-CD35-FITC or with isotype controls, in addition to anti-CD14-PerCP. Samples were lysed, centrifuged (at 300g for 5 minutes at 4°C), and analyzed on a FACSCalibur flow cytometer. Data analysis was performed with FlowJo software. Monocytes and granulocytes were gated by side scatter and CD14 expression. Results are given as median fluorescence intensity.

Phagocytosis

As previously described, whole-blood specimens were incubated with a final concentration of Alexa Fluor 488–stained E. coli (5 × 10⁷ bacteria/mL) and S. aureus (1 × 10⁸ bacteria/mL) for 15 minutes, and phagocytosis was assessed by Phagotest (Glycootope Biotechnology, Heidelberg, Germany), according to kit instructions [20]. Monocytes and granulocytes were gated by side scatter and CD14 expression. Results are given as mean fluorescence intensity.

Statistical Analysis

All statistical calculations were done in Prism 5 (GraphPad, San Diego, California). A t test was used for the HEK-293E cell experiments. All whole-blood experiments were repeated with 6 different donors, unless otherwise stated. The LPS data were analyzed by repeated measures 1-way analysis of variance (ANOVA) of all 3 groups (LPS positive control, anti-CD14, and eritoran), with comparison of the latter 2 groups by post hoc Bonferroni correction for selected multiple testing. The E. coli and S. aureus data were analyzed by repeated measures 1-way ANOVA of the groups of interest (anti-CD14, eritoran, anti-CD14 plus compstatin, and eritoran plus compstatin). Comparisons of adjacent inhibitors were estimated by post hoc Bonferroni correction for selected multiple testing. A P value of <.05 was considered statistically significant. Compstatin Cp40 alone was included in the test panel to show the contribution of complement inhibition, but it was not included in the statistical analysis [13].

**Ethics**

Informed written consent was obtained from each blood donor. The local ethical committee approved the study.

**RESULTS**

**LPS-Induced Responses**

To demonstrate the inhibitory effects of eritoran and anti-CD14 on the LPS-induced inflammatory responses, inhibition of LPS-induced cytokine release and upregulation of CD11b and CD35 on leukocytes were tested in human whole-blood specimens (Figure 1). Both eritoran and anti-CD14 efficiently and significantly inhibited LPS-induced release of TNF, IL-1β, IL-6, and IL-8 (P <.01–P <.0001; Figure 1A). Eritoran showed equivalent (for TNF, IL-1β, and IL-6) or significantly more-effective (for IL-8) inhibition when compared to anti-CD14 (P <.05). Similarly, both inhibitors significantly attenuated the LPS-induced increase in monocye expression of CD11b (96% and 90% reduction, respectively; P <.01) and CD35 (95% and 94% reduction, respectively; P <.05; Figure 1B). Granulocytes were not activated under these conditions, as their LPS-responsiveness is much lower than seen for monocytes [22].

To investigate the inhibitory effects of eritoran on LPS-binding to CD14, HEK-293E cells transfected with human CD14 were used. The cells were preincubated with increasing doses of eritoran or anti-CD14 prior to LPS stimulation. Both eritoran and anti-CD14 inhibited LPS-binding to CD14 in a dose-dependent manner (Figure 2). However, anti-CD14 was significantly more effective (P <.05) when comparing concentrations sufficient to inhibit LPS-induced inflammatory responses in the whole-blood model (0.07 µM anti-CD14 and 1 µM eritoran).

The negative controls (IgG2/4 isotype control antibody and control peptide) did not inhibit any of the LPS readouts (data now shown).

**E. coli-Induced Inflammatory Responses**

To investigate the effects of eritoran alone, anti-CD14 alone, and the combination of each with the C3 inhibitor compstatin Cp40 in a more complex model of inflammation, E. coli was used to stimulate whole-blood specimens. Cytokine analysis showed that inhibition with anti-CD14 alone or eritoran alone markedly attenuated the release of TNF, IL-1β, IL-6, and IL-8, without significant differences between the 2 inhibitors (Figure 3). The combination of anti-CD14 and compstatin Cp40 inhibited the release of all cytokines down to background levels. For IL-6, this combination was significantly more efficient than the combination of eritoran and compstatin Cp40 (P <.05).

Inhibition with anti-CD14 alone reduced the E. coli–induced upregulation of monocyte CD11b and CD35 by 82% and 83%, respectively, and was significantly more effective than inhibition with eritoran alone (33% reduction [P <.001] and 25%...
reduction \([P < .01]\), respectively; Figure 4). The combination of anti-CD14 and compstatin Cp40 reduced levels of both receptors to background levels, significantly more efficient than the combination of eritoran and compstatin Cp40 (45% and 34% reductions, respectively; \(P < .01\)). In contrast to monocytes, upregulation of granulocyte CD11b and CD35 was complement dependent (86% and 83% reductions by compstatin Cp40, respectively) and only moderately affected by inhibition with anti-CD14 alone (40% and 30% reductions, respectively) or eritoran alone (28% and 15% reductions, respectively; Figure 4). The combined treatments reduced the upregulation of both receptors down to background levels.

Figure 1. Effects of anti-CD14 and eritoran on lipopolysaccharide (LPS)–induced inflammation markers in human whole-blood specimens. A, Tumor necrosis factor (TNF), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8) release in response to incubating human whole-blood specimens with ultrapure LPS (10 ng/mL) for 120 minutes after preincubation with phosphate-buffered saline (PBS), anti-CD14, or eritoran. B, CD11b and CD35 expression on monocytes in response to stimulation of human whole-blood specimens with ultrapure LPS for 20 minutes after preincubation with PBS, anti-CD14, or eritoran. Data are presented as box plots, with upper and lower limits of the boxes representing interquartile ranges, whiskers representing 10th and 90th percentiles, horizontal lines within boxes representing median values, and vertical lines within boxes representing mean values; data are from 6 independent experiments, using 6 different donors. Statistical significance was estimated by using repeated measures 1-way analysis of variance (top line), and comparison between anti-CD14 and eritoran was estimated by post-hoc Bonferroni correction for selected multiple testing (closed line). *\(P < .05\), **\(P < .01\), ***\(P < .001\), and ****\(P < .0001\). Abbreviations: MFI, median fluorescence intensity; NS, nonsignificant.
Figure 2. Effects of anti-CD14 and eritoran on lipopolysaccharide (LPS)–binding to membrane-bound CD14. A, Human embryonic kidney 293E cells transfected with human CD14 were incubated with Cy5-labeled LPS after preincubation with increasing doses of anti-CD14 (aCD14), eritoran, or a control antibody. Data are shown as mean fluorescence intensity (MFI) and presented as mean ± standard error of the mean of 3 experiments. Statistical significance was estimated by a parametric t test between anti-CD14 0.07 µM and eritoran 1 µM data. *P < .05. B, Flow cytometry histograms showing data from one of the 3 virtually identical experiments presented in panel A.
The negative controls did not inhibit any of the *E. coli*–induced inflammatory readouts (data not shown).

**S. aureus–Induced Inflammatory Responses**

We next investigated whether eritoran alone, anti-CD14 alone, and the combination of each with the complement inhibitor Cp40 would have similar effects in a model involving gram-positive induction of inflammation. *S. aureus* was used in the same experimental design as described above for *E. coli*.

Cytokine analysis showed that inhibition of TNF, IL-1β, IL-6, and IL-8 was dependent on complement to a greater extent for *S. aureus* than for *E. coli*. The attenuating effect of single inhibition with anti-CD14 or eritoran was minimal, without significant differences between the 2 inhibitors (Figure 5). The combination of anti-CD14 and compstatin Cp40 reduced the cytokine release by 60%–75%, also showing a tendency of stronger attenuation than the combination of eritoran and compstatin Cp40, although these differences did not reach statistical significance.

Upregulation of monocyte CD11b and CD35 by *S. aureus* was reduced significantly more by the combination of anti-CD14 and compstatin Cp40 (70% and 75%, respectively) than by the combination of eritoran and compstatin Cp40 (47% and 34% reduction, respectively; *P* < .01; Figure 6). Inhibition with either anti-CD14 alone or eritoran alone showed no inhibitory effect. Upregulation of granulocyte CD11b and CD35 was mainly complement dependent and reduced by 92% and 78%, respectively, by compstatin Cp40 alone (Figure 6). No further effect was seen by the combined treatments, and again, single inhibition with either anti-CD14 or eritoran showed no inhibitory effect.

The negative controls did not inhibit any of the *S. aureus*–induced inflammatory readouts (data not shown).

**Bacterial Phagocytosis**

**E. coli**

Monocyte phagocytosis of *E. coli* was reduced by 24% by inhibition with anti-CD14 alone and was significantly more...
effective than inhibition with eritoran alone (no reduction; \( P < .01 \); Figure 7A). The combination of anti-CD14 and compstatin Cp40 reduced the phagocytosis by 79%, which was not significantly different from the combination of eritoran and compstatin Cp40 (57% reduction). Granulocyte phagocytosis was only minimally affected by inhibition with anti-CD14 alone or eritoran alone (11% and 6% reduction, respectively; Figure 7A). The combination of anti-CD14 and compstatin Cp40 reduced phagocytosis by 90%, similar to the combination of eritoran and compstatin Cp40 (87% reduction).

**S. aureus**

Monocyte phagocytosis of *S. aureus* was complement dependent (75% reduction by compstatin Cp40; Figure 7B). Single inhibition with anti-CD14 showed a 20% reduction and did not significantly differ from eritoran (7% reduction). The combination of anti-CD14 and compstatin Cp40 (93% reduction) showed a minor additional inhibitory effect, although not statistically significant, compared to the combination of eritoran and compstatin Cp40 (76% reduction). Granulocyte phagocytosis was completely complement dependent, being reduced to background levels by compstatin Cp40 alone, without any effect of single inhibition of anti-CD14 or eritoran (Figure 7B).

**Eritoran Versus the Combined Inhibition of CD14 and Complement**

Since eritoran was used to treat sepsis but failed, we compared the effects of eritoran alone with that of combined anti-CD14 and complement inhibition, which we propose as an alternative treatment approach [15, 17]. Noteworthy, for 18 of 20 readouts reported for the bacteria-induced inflammatory responses (Figures 3–7), the combination of anti-CD14 and compstatin Cp40 was substantially more efficient than inhibition with eritoran alone (\( P < .05 \)– \( P < .0001 \); mean \( P < .0001 \)).

**DISCUSSION**

In the present study, we have demonstrated that, although eritoran was an effective inhibitor of LPS-induced inflammation, it showed no effect on *E. coli*–induced leukocyte activation markers or inflammation induced by *S. aureus*. Anti-CD14 showed a broader inhibitory effect than eritoran, as it also efficiently
inhibited monocyte activation by E. coli. The addition of a complement inhibitor was necessary to inhibit granulocyte activation and S. aureus inflammatory responses. The combined inhibition of CD14 and complement was significantly more efficient than the combination of eritoran and complement inhibition, especially in terms of monocyte activation.

In addition to blocking the TLR4-MD2 complex, eritoran has also been reported to bind to soluble CD14, preventing further ligand binding to MD2 [10]. We here show that even though eritoran attenuated LPS-binding to membrane-bound CD14 dose dependently, anti-CD14 inhibited the LPS-binding much more effectively.

Both anti-CD14 and eritoran showed efficient attenuating effects on E. coli–induced cytokine responses, in line with the LPS-driven activation of TLR4. However, adding a complement inhibitor increased the effect. Moreover, there was a trend indicating that the combination of anti-CD14 and compstatin Cp40 was more effective than the combination of eritoran and compstatin Cp40, with the difference reaching significance for IL-6. Eritoran had minimal effect on S. aureus–induced cytokine responses. The different effects of eritoran on E. coli– and S. aureus–induced inflammation are not surprising, since S. aureus does not contain LPS and is thought to activate the inflammatory system mainly through TLRs other than TLR4, particularly TLR2 and TLR8, and the complement system [14,23–25]. The addition of a complement inhibitor was necessary to efficiently inhibit S. aureus–induced cytokine release.

Leukocyte activation is a hallmark of bacterial infection. CD11b and CD35 have been shown to increase significantly on monocytes and neutrophils in patients with bacterial infections and sepsis, compared with healthy controls [26,27]. Monocyte activation by gram-negative bacteria has previously been shown to be largely CD14 dependent [28]. This observation has been attributed to LPS-driven activation of TLR4.

In the current study, eritoran completely abolished LPS-induced expression of the 2 leukocyte activation markers CD11b and CD35 on monocytes but, surprisingly, had almost no effect on the E. coli–induced expression. In contrast to eritoran, inhibition with anti-CD14 alone significantly and efficiently reduced the expression of these activation markers. This suggests that...
redundant pathways of CD11b and CD35 upregulation that are independent of soluble LPS and TLR4-MD2 but dependent on CD14 may be activated by whole bacteria.

Similar results were obtained for *S. aureus*-induced expression of CD11b and CD35 on monocytes, where combined inhibition of CD14 and complement clearly outperformed the combination of compstatin Cp40 with eritoran, again indicating that anti-CD14 mediates effects beyond TLR4-MD2 inhibition. However, inhibition with anti-CD14 alone did not show an inhibitory effect under these conditions.

Granulocyte activation and phagocytosis of *E. coli* and *S. aureus* by monocytes and granulocytes were largely complement dependent, consistent with earlier observations [20, 28]. Interestingly, inhibition of CD14 alone was more efficient than eritoran alone in reducing monocyte phagocytosis of *E. coli*. In addition to phagocytosis, a possible effect of the various inhibitors on plasma bacterial lysis could be of importance for the effect observed on the inflammatory reaction. Working with heat-inactivated bacteria precludes use of colony-forming units as a readout. However, we have previously shown that inhibition of CD14 did not reduce the killing of live *E. coli* in porcine whole-blood specimens, whereas inhibition of C3 reduced but did not abolish bacterial killing [29]. This effect of complement inhibition might reduce the amount of plasma LPS and thus attenuate the cytokine storm.

There was much optimism expressed surrounding eritoran and its potential to reduce mortality in sepsis. Yet, again, when the phase 3 trial was concluded, no improvement was seen in 28-day mortality [11]. Savva et al pointed out that merely inhibiting a single pattern-recognition receptor could be insufficient, since other receptors might fill in for TLR4, particularly when gram-positive bacteria are involved, thus overriding the inhibitory effect [30]. We have hypothesized that a combined inhibitory approach is necessary in sepsis. Indeed, when comparing combined CD14 and complement inhibition to inhibition by eritoran alone, as tested in clinical trials, the combined approach was vastly more efficient in our model. Furthermore, our data indicate that complement and CD14 inhibition combined would be more efficient than combining a complement inhibitor with eritoran. This warrants further validation in animal studies.

Development of new treatments for sepsis has proven difficult, demonstrating the discrepancies between preclinical
models and clinical outcomes. There are obvious shortcomings in our model, particularly the pretreatment approach, because most patients would not receive medications at such an early stage. However, finding complex human systems that can better reflect the clinical effects of treatments is important to improve the selection of therapies for clinical trials. Here we demonstrate important shortcomings of TLR4-MD2 inhibition alone, particularly in gram-positive inflammation; this conclusion corresponds to clinical findings. Thus, the whole-blood model could be a promising tool for identifying potential therapeutic approaches to complex inflammatory diseases.

Although *E. coli* and *S. aureus* are the leading causative agents in sepsis, a long list of other microbial pathogens can be found, including the setting of polymicrobial infections, underscoring the need for targeting broad-acting pattern-recognition receptors and upstream in the recognition systems. We have recently demonstrated that the importance of combined inhibition, as compared to inhibition by anti-CD14 alone, also increases with incremental doses of *E. coli* [31]. Since septic patients have an overwhelming dysregulation of the inflammatory system, one might suspect that the benefits of combined inhibition instead of single-agent inhibition would be even more pronounced in that setting. Thus, in conclusion, we have shown that a combined inhibition strategy against CD14 and complement might be a promising treatment for human sepsis.

**Notes**

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