

# Double Blockade of CD14 and Complement C5 Abolishes the Cytokine Storm and Improves Morbidity and Survival in Polymicrobial Sepsis in Mice

Markus Huber-Lang,\* Andreas Barratt-Due,<sup>†,‡</sup> Søren E. Pischke,<sup>§,¶</sup> Øystein Sandanger,<sup>||</sup> Per H. Nilsson,<sup>†</sup> Miles A. Nunn,<sup>#</sup> Stephanie Denk,\* Wilhelm Gaus,\*\* Terje Espevik,<sup>††</sup> and Tom E. Mollnes<sup>†,††,‡‡,§§</sup>

Sepsis and septic shock, caused by an excessive systemic host-inflammatory response, are associated with high morbidity and mortality. The complement system and TLRs provide important pattern recognition receptors initiating the cytokine storm by extensive cross-talk. We hypothesized that double blockade of complement C5 and the TLR coreceptor CD14 could improve survival of experimental polymicrobial sepsis. Mice undergoing cecal ligation and puncture (CLP)-induced sepsis were treated with neutralizing anti-CD14 Ab biG 53, complement C5 inhibitor coversin (*Ornithodoros moubata* C inhibitor), or a combination thereof. The inflammatory study (24-h observation) revealed statistically significant increases in 22 of 24 measured plasma biomarkers in the untreated CLP group, comprising 14 pro- and anti-inflammatory cytokines and 8 chemokines, growth factors, and granulocyte activation markers. Single CD14 or C5 blockade significantly inhibited 20 and 19 of the 22 biomarkers, respectively. Combined CD14 and C5 inhibition significantly reduced all 22 biomarkers (mean reduction 85%; range 54–95%) compared with the untreated CLP group. Double blockade was more potent than single treatment and was required to significantly inhibit IL-6 and CXCL1. Combined inhibition significantly reduced morbidity (motility and eyelid movement) and mortality measured over 10 d. In the positive control CLP group, median survival was 36 h (range 24–48 h). Combined treatment increased median survival to 96 h (range 24–240 h) ( $p = 0.001$ ), whereas survival in the single-treatment groups was not significantly increased (median and range for anti-CD14 and anti-C5 treatment were 36 h [24–48 h] and 48 h [24–96 h]). Combined with standard intervention therapy, specific blockade of CD14 and C5 might represent a promising new therapeutic strategy for treatment of polymicrobial sepsis. *The Journal of Immunology*, 2014, 192: 5324–5331.

**S**epsis and septic shock, caused by an excessive host inflammatory response to infections, burns, or trauma, are associated with high morbidity and mortality. Worldwide, millions of deaths each year are attributable to sepsis. The inci-

dence of sepsis is increasing, and it is one of the largest global health economic burdens (1). Early goal-directed resuscitation and administration of antibiotics have improved patient outcomes, but specific therapy has still not been developed (2, 3). Various therapeutic approaches, including attenuation of the detrimental host inflammatory response, have proven clinically ineffective (4). The sole exception, drotrecogin alfa activated protein C, which had Food and Drug Administration approval for treatment of sepsis, has recently been withdrawn, adding to the long list of unsuccessful interventions using single drugs (5).

Recently, issues related to management of sepsis were readdressed, identifying a pressing need to develop effective drugs and define new therapeutic approaches (6–9). In the current study, we demonstrate the efficacy of an intervention principle for sepsis based on the hypothesis that combined inhibition of key upstream sensor and effector systems of innate immunity will attenuate the initial development of uncontrolled systemic inflammation (10, 11). Specifically, double blockade of complement component C5 and the TLR family molecule CD14 was investigated in the clinically relevant cecal ligation and puncture (CLP) mouse model of sepsis (12).

CD14 is a promiscuous binding protein primarily known as an accessory molecule facilitating LPS transfer from LPS-binding protein to TLR4–MD2 complexes, thereby increasing sensitivity toward LPS, which is a major cell wall component of Gram-negative bacteria (13, 14). CD14 also recognizes a variety of other exogenous and endogenous molecular patterns and is involved in signaling through TLR2, TLR3, TLR7, and TLR9 (15), which are activated by a variety of ligands associated with Gram-positive bacteria, fungi, viruses, and damaged self. Thus, CD14

\*Department of Traumatology, Center of Surgery, University of Ulm, Ulm 89081, Germany; <sup>†</sup>Department of Immunology, Oslo University Hospital, Rikshospitalet, University of Oslo, Oslo N-0027, Norway; <sup>‡</sup>K.G. Jebsen Inflammation Research Centre, University of Oslo, Oslo N-0027, Norway; <sup>§</sup>The Interventional Centre, Oslo University Hospital, Oslo N-0027, Norway; <sup>¶</sup>Department of Anesthesiology, Oslo University Hospital, Oslo N-0027, Norway; <sup>||</sup>Research Institute of Internal Medicine, Oslo University Hospital, Oslo N-0027, Norway; <sup>#</sup>Centre for Ecology and Hydrology, Wallingford, Oxfordshire OX10 8BB, United Kingdom; <sup>\*\*</sup>Department of Epidemiology and Biostatistics, University of Ulm, Ulm 89081, Germany; <sup>††</sup>Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim 7491, Norway; <sup>‡‡</sup>Research Laboratory, Nordland Hospital Bodø, University of Tromsø, Tromsø 9019, Norway; and <sup>§§</sup>Faculty of Health Sciences, University of Tromsø, Tromsø 9019, Norway

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Address correspondence and reprint requests to Prof. Tom E. Mollnes, Department of Immunology, Oslo University Hospital, Rikshospitalet, Oslo N-0027, Norway. E-mail address: t.e.mollnes@medisin.uio.no

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Abbreviations used in this article: CLP, cecal ligation and puncture; DAMP, damage-associated molecular pattern; DPBS, Dulbecco's PBS; KO, knockout; MIP-1, macrophage inflammatory protein-1; MPO, myeloperoxidase; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; SIRS, systemic inflammatory response syndrome.

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has broad upstream regulatory functions on the sensor systems of innate immunity.

Complement represents another important integral danger-sensing and effector arm of innate immunity, and uncontrolled activation involves all three complement pathways during sepsis (16). Such systemic activation of complement and accompanying release of anaphylatoxins (C3a and C5a) is potentially harmful (17). The most potent proinflammatory anaphylatoxin, C5a, is suggested to play a particularly important role in adverse clinical effects during sepsis (18, 19).

Although TLRs and complement are often considered discrete entities, an emerging body of evidence indicates that these key innate defense systems are interconnected by extensive cross-talk (20–23). The consequence of this interplay, which includes redundancy, synergism, and antagonism, suggests inhibiting only complement or TLRs may be insufficient to control inflammation. We have previously demonstrated broad anti-inflammatory effects by simultaneously inhibiting both CD14 and complement (24–29). In this study, we document the efficacy of C5 and CD14 inhibition on the systemic inflammatory response, morbidity, and survival of mice subject to polymicrobial sepsis.

## Materials and Methods

Ethics and study approval was obtained from the University of Ulm Committee on Use and Care of Animals, approval number 988.

### Coversin

Coversin, the recombinant *Ornithodoros moubata* C inhibitor, which prevents activation of C5, is a 16.8-kDa recombinant protein produced as described (30). Coversin was a kind gift from Volusion Immunopharmaceuticals. The dose-dependent *in vivo* effect of coversin was studied by giving mice a single *i.v.* or *i.p.* injection of coversin (0–100  $\mu\text{g}/\text{mouse}$ ) (Supplemental Fig. 1A). Blood was collected via cardiac puncture 1 h after injection and the serum tested in a hemolytic assay with sheep erythrocytes sensitized with mouse complement assay reagent (Comptech, Tyler, TX) according to the manufacturer's protocol. Briefly, serum was incubated with mouse complement assay reagent-sensitized erythrocytes for 30 min at 37°C. The degree of lysis, which is directly related to activation of C5, was determined at 405 nm and related to 100% lysis control. The results confirmed that the doses used in the present studies completely neutralized C5 and thus blocked complement-mediated C5a and C5b-9 generation.

### Anti-CD14

The anti-mouse CD14 Ab clone biG 53, produced in CD14 knockout (KO) mice, exists in different isotypes and inhibits binding of LPS to CD14. The IgG2a isotype was purchased from Biomedtec (Greifswald, Germany) and cleaved into F(ab')<sub>2</sub> (Genovis, Lund, Sweden). The F(ab')<sub>2</sub> was highly pure as determined by SDS-PAGE. The functional activity of biG 53 F(ab')<sub>2</sub> was tested *in vitro*. Immortalized mouse macrophages cultivated in DMEM (Invitrogen, Carlsbad, CA) with 10% FCS (Lonza, Basel, Switzerland) were incubated with a final concentration of 10  $\mu\text{g}/\text{ml}$  anti-CD14 F(ab')<sub>2</sub> for 10 min prior to incubation with ultrapure LPS (Invivogen, Toulouse, France). Twenty-four hours later, TNF was quantified in supernatants by ELISA (R&D Systems, Minneapolis, MN). The F(ab')<sub>2</sub> fragment efficiently abolished LPS-induced TNF production (Supplemental Fig. 1B). The dose needed for the *in vivo* experiments to attenuate LPS-induced cytokines was then tested and 100  $\mu\text{g}/\text{mouse}$  selected for the subsequent CLP experiments. A F(ab')<sub>2</sub> control fragment from an irrelevant control Ab was purchased from Diatec Monoclonals (Oslo, Norway).

### Analysis of inflammatory biomarkers

Twenty-three cytokines, including chemokines and growth factors, were measured by a multiplex assay from Bio-Rad (Hercules, CA). The assay included the following analytes: IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-17, eotaxin (CCL11), G-CSF, GM-CSF, IFN- $\gamma$ , keratinocyte chemoattractant (CXCL1), MCP-1 (CCL2), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ; CCL3), MIP-1 $\beta$  (CCL4), RANTES (CCL5), and TNF. The analysis was performed according to the manufacturer's instructions. Myeloperoxidase (MPO) was analyzed by ELISA (Hycult Biotech, Uden, The Netherlands).

### Induction of polymicrobial sepsis by CLP

Specific pathogen-free adult male mice, strain C57BL/6 (20–25 g in body weight; Charles River Laboratories, Munich, Germany), were used. Experimental sepsis was induced by the CLP procedure as previously described (12). In brief, mice were anesthetized with a 2.5% sevoflurane (Sevorane; Abbott, Wiesbaden, Germany) and 97.5% oxygen mixture under a continuous flow of 0.5 l/min at a fraction of inspired oxygen of 1.0 via an inhalation mask. The mice were placed in supine position and their abdomens shaved. Before surgery, mice received buprenorphine 0.01 mg/kg body weight *s.c.* for adequate analgesia and every 12 h in the follow-up. An abdominal midline incision of 1 cm was made to expose the cecum. The cecum was ligated by 75% and punctured twice with a 21-gauge (0.723-mm) needle to induce a high-grade sepsis in accordance with Rittirsch et al. (12). The abdominal incision was closed in layers. In sham-operated controls, laparotomy was performed in a similar fashion, but the cecum was neither ligated nor punctured. Two separate studies were performed to measure the inflammatory response (24 h) and the survival (10 d), respectively.

### Inflammatory study

Mice were allocated to five groups ( $n = 8/\text{group}$ ): 1) the coversin-treated group received 300  $\mu\text{g}$  coversin/mouse at CLP and 6 h after; 2) the anti-CD14-treated group received 100  $\mu\text{g}$  anti-CD14 F(ab')<sub>2</sub>/mouse; 3) the combined coversin and anti-CD14 group received the same doses as the individual groups; 4) the positive control CLP group; and 5) the sham group received Dulbecco's PBS (DPBS). All groups received the same volume (*i.e.*, 200  $\mu\text{l}$  *i.v.* at CLP and at 6 h). After sacrifice (24 h after CLP), blood was obtained by heart puncture and serum separated at 4°C and then stored at –80°C until analyzed in one batch.

### Survival study

Twelve animals were allocated to each of five groups: 1) the coversin-treated group received 100  $\mu\text{g}$  coversin in 300  $\mu\text{l}$  DPBS *i.v.* directly after CLP, and, in addition, 200  $\mu\text{g}$  coversin (in 300  $\mu\text{l}$  DPBS) was given *i.p.* 24, 48, 72, 96, 120, and 144 h after CLP; and 2) the anti-CD14 group received 100  $\mu\text{g}$  anti-CD14 F(ab')<sub>2</sub> (in 300  $\mu\text{l}$  DPBS) *i.v.* as a single shot immediately after CLP and thereafter *i.p.* every 24 h for 6 d. Thereafter, 300  $\mu\text{l}$  DPBS was given *i.p.* every 24 h after CLP to ensure equal volume loading to the groups. 3) The combined coversin and anti-CD14 group underwent the coversin protocol with the addition of 100  $\mu\text{g}$  anti-CD14 F(ab')<sub>2</sub> in the first injection; 4) the vehicle group (positive CLP control group) received 100  $\mu\text{g}$  control F(ab')<sub>2</sub> fragment (in 300  $\mu\text{l}$  DPBS) *i.v.* immediately after CLP, followed by the 300  $\mu\text{l}$  DPBS *i.p.* applications at the given time points; and 5) sham animals (negative CLP control group) received only injections with 300  $\mu\text{l}$  DPBS at each given time point. Mice were not given antibiotics or specific fluid resuscitation. Survival rates were determined over a 10-d (240-h) period, with clinical assessment (weight, mobility, and eyelid motility) every 8 h for the first 48 h and every 12 h thereafter to day 10. All mice had unrestricted access to food and water.

### Statistics

Cytokine readouts in the inflammatory study (Figs. 1–3) were compared using one-way ANOVA with post hoc Dunnett's correction for multiple testing. In the survival study (Fig. 4), median survival time after surgery was calculated by Hodges-Lehmann estimator. Survival times between intervention groups were compared using the log-rank test. The overall hypothesis "survival is the same in all four groups" was tested as the first level of an a priori-ordered hypotheses. This hypothesis was rejected ( $p = 0.0001$ ). The second level of the a priori-ordered hypotheses had three specific hypotheses. On this second level,  $p$  values were adjusted with the Bonferroni-Holm procedure for multiple testing. The three specific hypotheses were differences among: 1) active therapy groups versus the untreated positive control group; 2) monotherapies of coversin or anti-CD14 versus combination thereof; and 3) monotherapy of coversin versus monotherapy of anti-CD14. These three specific comparisons are independent; each of them deliver truly new information. Morbidity scores in the survival analysis (Table I) were compared using a linear mixed-effects model with treatment and interaction of treatment and time as fixed effects and subject number as a random effect. All pairwise comparison among groups was corrected for multiple testing using the Bonferroni-Holm procedure.

## Results

### Activity of coversin and anti-CD14 F(ab')<sub>2</sub>

Coversin and the anti-CD14 Ab (clone biG 53) are known to neutralize mouse C5 and CD14, respectively. For the purpose of

this study, the dose of coversin needed to completely neutralize C5 when administered i.v. and i.p. was examined in vivo (Supplemental Fig. 1A). The dose used in the subsequent CLP experimental protocols was in sufficient excess to completely prevent complement-mediated hemolysis. To avoid adverse effects of whole IgG, we used F(ab')<sub>2</sub> fragments of anti-CD14. To quality control this preparation, we examined the functional activity by testing the inhibitory effect on LPS-induced TNF production by mouse macrophages (Supplemental Fig. 1B). The functional effect was confirmed to be preserved in the F(ab')<sub>2</sub> fragment.

#### *In vivo studies*

We first aimed to investigate the initial cytokine storm induced by CLP polymicrobial sepsis. This study included a broad panel of inflammatory biomarkers and was designed as a separate protocol to ensure sufficient serum for analysis of the initial (24 h) inflammatory response and not interfere with the results of a survival study due to complications during blood sampling and loss of circulating blood volume.

#### *Effects of C5 and CD14 blockade on the early cytokine storm*

The four groups of animals undergoing CLP and the sham group comprised eight animals each. Within 24 h, defined as the end of the experiment and reflecting the initial inflammatory cytokine storm, three mice died in the positive control group, two in the anti-CD14, and none in the sham, coversin, or combined groups. Thus, the number of animals included in the serum measurements for these groups was five, six, eight, eight, and eight, respectively.

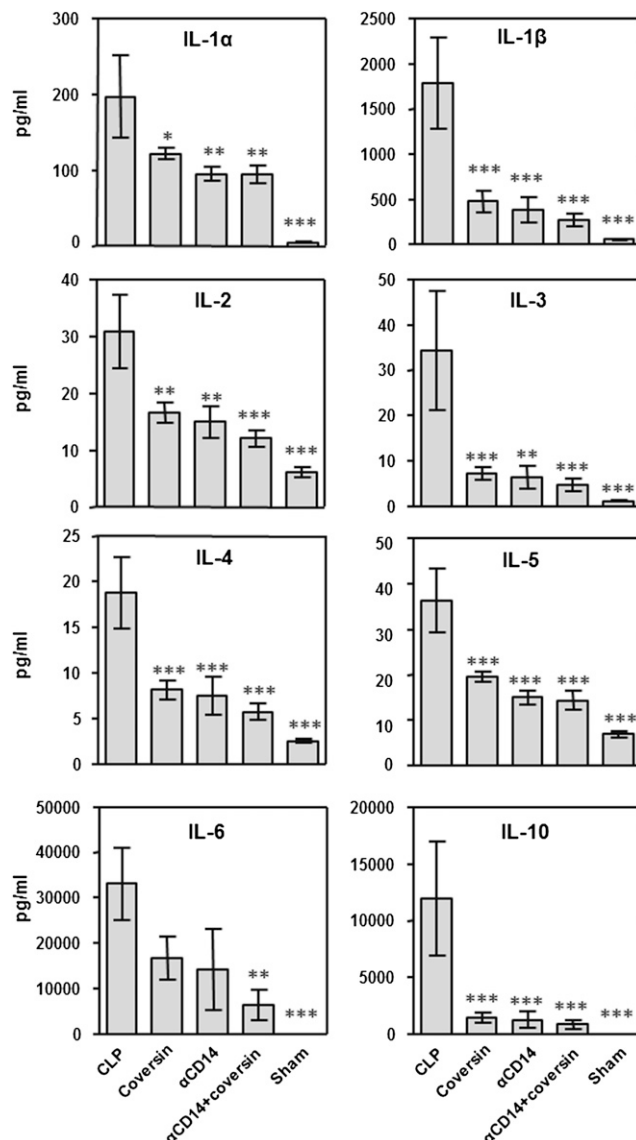
We examined 24 inflammatory markers using an assay for the granulocyte activation marker MPO and a multiplex kit to assay 23 selected cytokines including ILs, chemokines, and growth factors. The majority of the biomarkers (22 of 24) increased substantially and significantly ( $p < 0.05$  to  $< 0.001$ ) in the CLP-positive control group compared with the sham group (Figs. 1, 2, 3). Two inflammatory markers (IL-9 and G-CSF) were not altered and excluded from further analysis.

We then tested the inhibitory effect of coversin, anti-CD14, and the combination thereof on the 22 biomarkers that increased in the CLP-positive group (Figs. 1–3). The quantitative inhibition was substantial for both single and combined regimens (Supplemental Table I). Single inhibition with coversin significantly inhibited 19 of 22 (not IL-6, CXCL1, and MPO), and single inhibition with anti-CD14 significantly inhibited 20 of 22 markers (not IL-6 and CXCL1). Notably, IL-6 (Fig. 1) and CXCL1 (Fig. 3), which were not significantly inhibited by single treatment, were significantly inhibited by the combined treatment. All 22 inflammatory markers were significantly inhibited by the double blockade; inhibition of CCL2 and CCL4 was particularly pronounced, and essentially ablated, compared with single blockade (Fig. 2).

#### *Effects of C5 and CD14 blockade on mortality*

The positive results of the inflammatory study prompted us to design a survival study with a 10-d observation period. The same treatment groups were included, and the numbers were increased from 8 to 12 mice in each group. Furthermore, the positive control CLP group received a control F(ab')<sub>2</sub> fragment instead of buffer control to exclude any nonspecific effects of anti-CD14 F(ab')<sub>2</sub> on survival. Animals were given negative control F(ab')<sub>2</sub> or coversin and/or anti-CD14 F(ab')<sub>2</sub> i.v. immediately after CLP and thereafter i.p. every 24 h for 6 d. Mice did not receive antibiotics or fluid resuscitation.

All animals in the sham group were alive at the end of the study (240 h; Fig. 4). In the positive control CLP group, all animals died within 42 h (median survival 36 h). The three treatment regimens, anti-CD14, coversin, and the combination thereof, considered



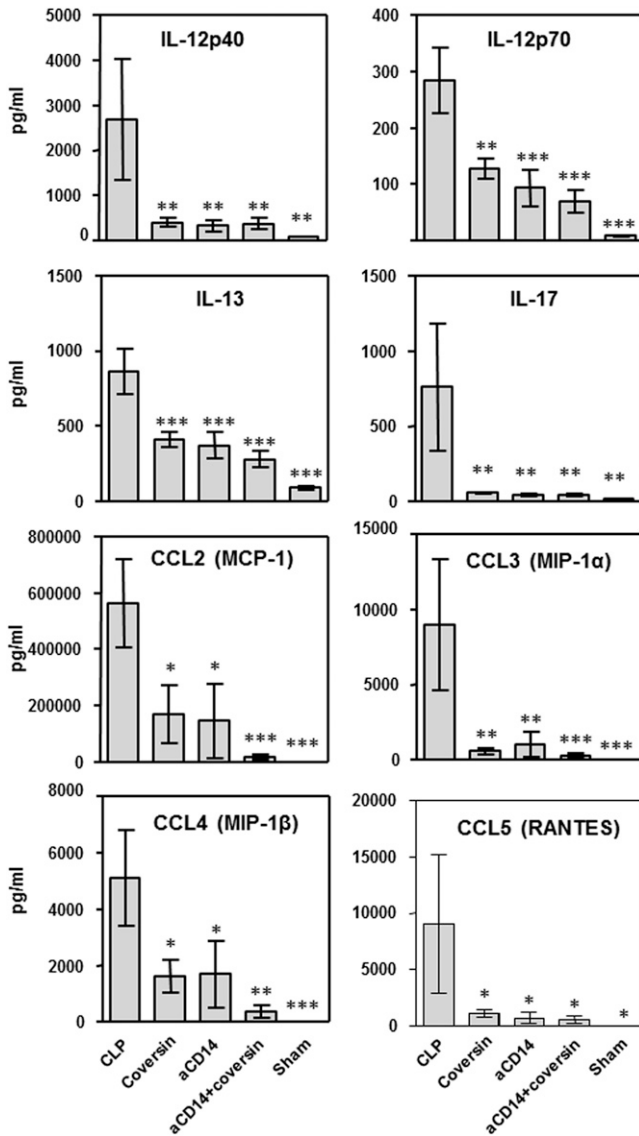
**FIGURE 1.** Inflammatory markers I. Serum IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, and IL-10 increased significantly after 24 h in untreated CLP mice ( $n = 5$ ) as compared with sham mice ( $n = 8$ ). Single treatment with coversin ( $n = 8$ ) or anti-CD14 ( $n = 6$ ) significantly inhibited all biomarkers except IL-6. Combined treatment ( $n = 8$ ) significantly inhibited all biomarkers. Significance compared with CLP positive control: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .  $\alpha$ CD14, anti-CD14.

together increased survival significantly (median survival 36, 48, and 96 h, respectively;  $p = 0.0106$ ). Effect of single inhibitions did not differ significantly from each other ( $p = 0.16$ ), whereas combined inhibition lead to a significant increase in survival in comparison with single intervention ( $p = 0.0012$ ).

#### *Effects of C5 and CD14 blockade on morbidity*

In addition to survival, the following clinical signs were recorded: weight, mobility, and eyelid motility. Mean weight (gram) decreased modestly from the start of the experiment to the death of the last animal in each of the CLP groups (positive control CLP group: 23.4–21.7; anti-CD14 group: 24.1–22.3; coversin group: 24.2–20.3; and the combined group: 23.9–22.3), but not in the sham group (23.0–23.2). Mobility and eyelid motility were scored, and all data are presented in Table I. Full mobility and eyelid motility (score 2) persisted throughout the experiment in the sham group. Both single inhibitions showed a delay in clinical impairment



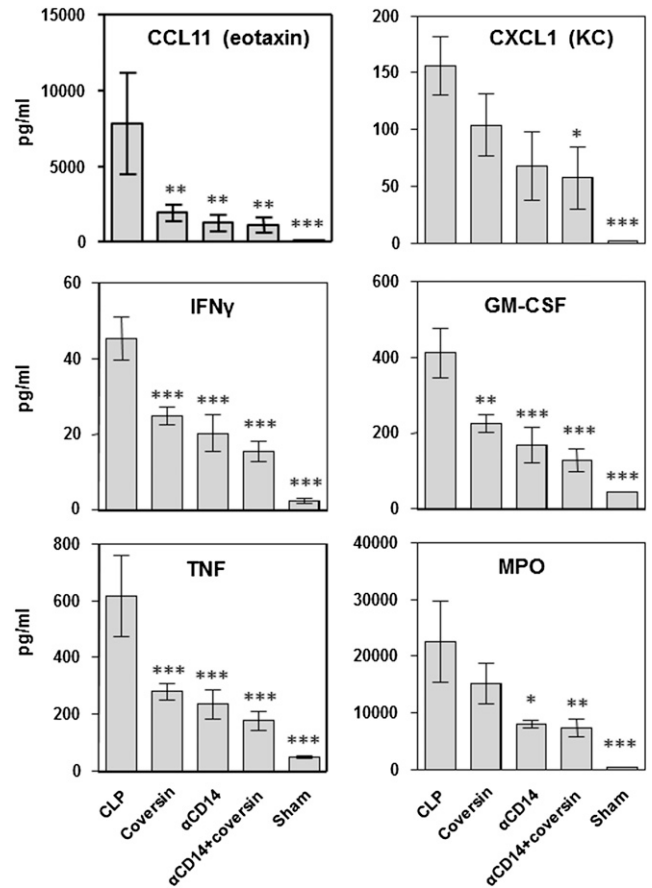


**FIGURE 2.** Inflammatory markers II. Serum IL-12p40, IL-12p70, IL-13, IL-17, CCL2 (MCP-1), CCL3 (MIP-1 $\alpha$ ), CCL4 (MIP-1 $\beta$ ), and CCL5 (RANTES) increased significantly after 24 h in the untreated CLP mice ( $n = 5$ ) as compared with the sham mice ( $n = 8$ ). Single treatment with coversin ( $n = 8$ ) or anti-CD14 ( $n = 6$ ), as well as combined treatment ( $n = 8$ ) significantly inhibited all biomarkers. Significance compared with CLP positive control: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .  $\alpha$ CD14, anti-CD14.

during the first 24 h, but declined afterward and were thus not significantly different to untreated controls over the whole observation period. The animals subjected to double blockade showed significantly better mobility ( $p < 0.001$  in comparison with positive control and single anti-CD14 treatment and  $p = 0.027$  in comparison with single coversin treatment) and eyelid motility ( $p < 0.001$  in comparison with positive control and single anti-CD14 treatment and  $p = 0.012$  in comparison with single coversin treatment) throughout the whole observation period (Table I).

**Discussion**

The present study was designed to examine the efficacy of the combined C5 and CD14 blockade regimen in a murine model of CLP-induced sepsis, which better mimics human sepsis compared with models using i.v. infusion of whole bacteria or LPS (31). We demonstrate that combined inhibition of these key molecules, belonging to two main pattern recognition receptor (PRR) sys-

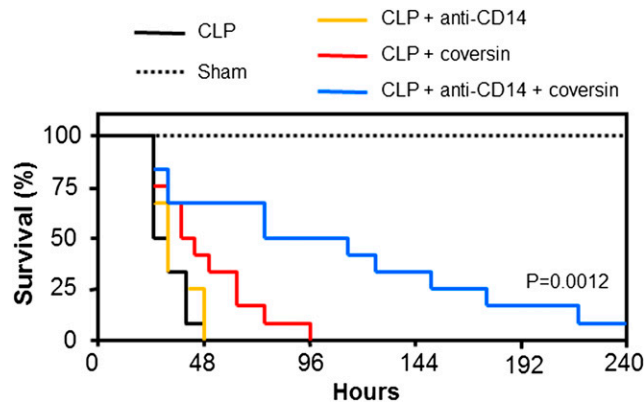


**FIGURE 3.** Inflammatory markers III. Serum CCL11 (eotaxin), CXCL1 (keratinocyte chemoattractant [KC]), IFN- $\gamma$ , GM-CSF, TNF, and MPO increased significantly after 24 h in the untreated CLP mice ( $n = 5$ ) as compared with the sham mice ( $n = 8$ ). Coversin ( $n = 8$ ) significantly inhibited all biomarkers except CXCL1 and MPO. Anti-CD14 ( $n = 6$ ) significantly inhibited all biomarkers except CXCL1. Combined coversin and anti-CD14 significantly inhibited all biomarkers. Significance compared with CLP positive control: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .  $\alpha$ CD14, anti-CD14.

tems, exerts a profound and broad-acting anti-inflammatory effect on numerous biomarkers generated during polymicrobial sepsis. Importantly, double blockade also significantly increased the survival and clinical score of septic animals compared with single inhibition of either C5 or CD14, which on their own had no effect on survival in this severe model. This new treatment regimen, targeting upstream recognition molecules of innate immunity that are triggered by infection, appears promising when compared with earlier work specifically targeting downstream mediators of inflammation, such as the cytokines TNF and IL-1 $\beta$  (32, 33).

The innate immune system serves as a first line of defense against invading pathogens and contains various PRRs, which recognize evolutionarily conserved structures on pathogens, the pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which are released from self-cells during sepsis. The TLRs and the complement system are among the first PRR systems to be activated (34–36). Upon activation, the TLR4/CD14/MD2 complex triggers the production of several proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, IL-8, TNF, MIP-1 $\alpha$ , and MIP-1 $\beta$  (37), and even causes the release of neutrophil extracellular traps (38).

CD14 is a particularly important molecule for LPS-induced inflammation. Studies have shown that absence or blocking of CD14 protected mice, rabbits, and monkeys from organ damage and death after LPS infusion (39–41). By contrast, mice over-



**FIGURE 4.** Survival study. Twelve animals were allocated to each of the following groups and observed for 10 d (240 h): CLP with positive control F(ab')<sub>2</sub> (black line), sham (dotted line), anti-CD14 (yellow line), coversin (red line), and combined coversin and anti-CD14 (blue line). Kaplan-Meier plot shows survival in each of the groups. Overall survival in all treatment groups combined compared with the CLP positive control was significant ( $p = 0.016$ ), and survival in the combined group was significantly increased compared with the single therapies considered together ( $p = 0.0012$ ), which were not significantly different from each other ( $p = 0.16$ ).

expressing CD14 were more sensitive to LPS-induced shock than normal animals (42). Furthermore, blockade of CD14 in humans almost completely inhibited the proinflammatory cytokine release induced by i.v. injection of LPS (43). This is in line with our previous study in a pig model of Gram-negative sepsis, which showed that inhibition of CD14 efficiently attenuated the proinflammatory cytokine response and granulocyte activation (44). The *Escherichia coli*-induced cytokine response in human whole blood was also greatly abrogated by a CD14-neutralizing Ab (26). In the current study of polymicrobial sepsis, inhibition of CD14 significantly inhibited the CLP-induced release of various inflammatory markers indicative for a robust systemic and CD14-dependent inflammatory response. This underscores the broad upstream regulatory function of CD14 and implies mechanisms of action not exclusively limited to TLR4 and LPS. However, the overall survival rate was not affected by single CD14 inhibition, suggesting that harmful physiological effects resulting from the polymicrobial challenge were insufficiently negated by CD14 blocking strategies alone. Ebong et al. (45) also found that CD14 KO mice displayed a 2–4-fold downregulation of pro- and anti-inflammatory cytokines in response to CLP. However, CD14 KO and control animals did not show differences in activity levels, temperature, body weight, or survival rate after CLP. This might be due to the fact that the majority of microorganisms released by CLP are Gram-negative *Bacteroides* and some minor populations of Gram-positive bacteria (46) in which LPS might play a less predominant role (47).

The complement system represents an ancient PAMP- and DAMP-sensing and transmission system that translates various danger signals to an early fluid-phase and cellular response, clinically evident as systemic inflammatory response syndrome (SIRS). During development of SIRS and sepsis, there is an early activation of complement via different pathways and perhaps via activated coagulation and fibrinolysis factors (48, 49), leading to generation of complement activation products, circulating C5a receptors, complement consumption, and development of complementopathy (50), similar to the development of sepsis-induced coagulopathy. In the experimental setting of both mono- and polymicrobial sepsis, there are multiple reports that control of complement at the level of C3 (e.g., by compstatin) or C5 (e.g., by

anti-C5a Abs) results in improved molecular and cellular functions, amelioration of the classical signs of coagulopathy, immune and organ dysfunction, and improved survival (18, 51, 52).

The crucial involvement of C5 in human inflammation has been closely studied using whole blood from C5-deficient humans (28). Several interactions exist between the complement and the coagulation cascades, favoring their reciprocal activation (49). Targeting the terminal complement pathway, C5 or its receptor(s), does not affect immunoprotective and immunoregulatory functions of upstream C3 activity but does offer significant anti-inflammatory potential due to the many important biological roles of C5a (53). We previously demonstrated that coversin is a potent C5 inhibitor in pigs as well as in humans, decreasing *E. coli*-induced cytokine release in whole blood (54). Furthermore, in a porcine model of *E. coli*-induced sepsis, coversin attenuated central proinflammatory cytokines such as TNF and IL-6 and efficiently reduced thrombogenicity by reducing the expression of tissue factor (29). The present study demonstrates that coversin led to a significant decrease of almost all tested inflammatory markers. However, as seen for single CD14 blockade, in this severe septic challenge, survival rate was not improved by complement inhibition alone. This is consistent with the broad-spectrum PAMPs and DAMPs that are released and cause immune activation during polymicrobial sepsis (12). When comparing cytokine inhibition with mortality rate, it should be noted that the blood samples for cytokine analysis were obtained at a single time point (24 h). In this study, it was not possible to follow cytokine levels throughout the survival experiment, and it is possible that cytokine patterns after >24 h might have better reflected the differential mortality observed in the different treatment groups. It is also worth noting that the concentration of inflammatory mediators in plasma mirrors the inflammatory condition without revealing the local inflammatory state. Thus, the increased survival obtained by the combined inhibition may be caused by a differential activity at the effector sites, for example within specific organs, as recently shown with anti-CD14 in a model of *E. coli*-induced sepsis in pigs (55).

Although TLRs and the complement cascade are coactivated upon pathogen invasion, they were for a long time considered distinct components of the innate immune system. However, recent studies indicate considerable cross-talk between complement and TLRs, revealing that they can compensate, synergize, or antagonize each other. TLR activation and pre-exposure to TLR agonists can increase cell sensitivity toward C5a both in vitro and ex vivo (22) and augment the C5a-mediated proinflammatory responses (23). Other studies revealed that TLR activation by LPS could additionally enhance complement protein synthesis (e.g., factor B) and effector functions (21, 56). Bidirectionality of the TLR–C5aR interaction is supported by the finding that complement activation in DAF<sup>-/-</sup> mice, which exhibit enhanced levels of complement deposition on cell surfaces, markedly increased TLR4-induced cytokine production. This effect was mainly C5aR mediated and involved the MAPKs ERK1/2 and JNK, possibly representing key connecting molecules between the complement and TLR pathways (57).

The synergistic interactions between C5 and TLRs may help to combat infections, but may also lead to an excessive proinflammatory response (20). Our current data do not identify what are the harmful responses that are better controlled by the combined therapy. We note, however, that only the dual- and not single-blockade treatments significantly inhibited formation of IL-6, which previously has been reported to be negatively associated with sepsis, both in the murine CLP model and human sepsis (58–60).

The beneficial effect of the double blockade in the current study is in line with previous results from our group demonstrating

Table I. Clinical data (mobility and eyelid movement scores) from the survival CLP study

	Hours						Days									
	0	8	16	24	32	40	2	2 to 3	3 to 4	4 to 5	5 to 6	6 to 7	7 to 8	8 to 9	10	
<b>Mobility<sup>a</sup></b>																
<b>Sham</b>																
2	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<b>Positive control</b>																
2	12	12	4	0	0	— <sup>b</sup>										
1	0	0	8	2	0	—										
0	0	0	0	4	1	—										
<b>Anti-CD14</b>																
2	12	12	12	4	2	2	—									
1	0	0	0	5	2	1	—									
0	0	0	0	0	0	0	—									
<b>Coversin</b>																
2	12	12	12	7	4	4	2	1	1	—						
1	0	0	0	2	3	3	1	1	0	—						
0	0	0	0	0	0	0	1	0	0	—						
<b>Combined</b>																
2	12	12	12	7	7	7	8	6	4	4	0	0	0	0	0	0
1	0	0	0	4	1	1	0	2	2	1	4	3	2	2	1	1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<b>Eyelids<sup>c</sup></b>																
<b>Sham</b>																
2	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<b>Positive control</b>																
2	12	0	0	1	0	—										
1	0	12	8	2	0	—										
0	0	0	4	3	1	—										
<b>Anti-CD14</b>																
2	12	12	12	4	1	1	—									
1	0	0	0	4	2	2	—									
0	0	0	0	1	1	0	—									
<b>Coversin</b>																
2	12	12	12	7	4	4	2	1	1	—						
1	0	0	0	2	3	3	1	1	0	—						
0	0	0	0	0	0	0	1	0	0	—						
<b>Combined</b>																
2	12	12	12	8	8	8	8	8	6	4	4	3	2	2	1	1
1	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0

Number of animals for each score in each group are indicated over time. Study groups ( $n = 12/\text{group}$ ): sham (operation without CLP), positive control (CLP with isotype control Ab), anti-CD14 (anti-CD14 treatment), coversin (coversin treatment), and combined (anti-CD14 and coversin treatment).

<sup>a</sup>Mobility score: 2, spontaneous mobility; 1, provoked mobility; 0, no mobility.

<sup>b</sup>Deaths (—): all animals in the actual group were dead at this time point.

<sup>c</sup>Eyelid motility score: 2, open; 1, open by touching; 0, closed.

a significantly more pronounced effect of double blockade compared with single blockade of CD14 and complement, even when the inflammation is predominantly LPS and therefore CD14 dependent (61). In the earlier study using a porcine model of *E. coli*-induced sepsis, inhibition of C5 and CD14 attenuated inflammation and thrombogenicity and delayed hemodynamic changes (29). In the current study, it is tempting to suggest that the combined inhibition may have had similar effects on thrombogenic and hemodynamic parameters, which may explain the beneficial effect of combined inhibition on survival.

A limitation of the current study is the use of mice. This CLP model is, however, generally accepted to represent a clinically relevant model to investigate SIRS and organ dysfunction caused by polymicrobial sepsis because it combines tissue trauma caused by the laparotomy, cell necrosis due to cecum ligation, and infection from leakage of endogenous intestinal microbial flora into the peritoneum (12). Translocation of enteric bacteria into the circulation triggers SIRS and leads to profound upregulation of

various inflammatory mediators. Studies of the inflammatory response and bacterial load in the organs would provide important information of the pathophysiology and the response to the double blockade, and we intend to investigate these aspects in future studies.

Simultaneous suppression of pro- and anti-inflammatory mediators could have a negative effect on outcome of sepsis; however, synchronous downregulation of anti- (e.g., IL-10) and proinflammatory (e.g., IL-6) cytokines caused by double inhibition of complement and CD14 not only improved clinical parameters but also survival rate. This supports the new paradigm that a diminished magnitude and duration of both the pro- and anti-inflammatory genetic storm can beneficially influence the clinical course of pathologies, as recently shown in neutrophils during endotoxemia and after tissue trauma in humans (62).

In a clinical setting, many septic patients are admitted to the hospital at a stage of the disease at which it is difficult to reverse the pathological progress, even after intensive resuscitation, including

antibiotic therapy and early goal-intensive care treatment. In our model, we did not include any rescue treatment, because the aim of the study was to investigate the effect of complement and CD14 inhibition, as a proof of concept, not being interfered by rescue therapy. Because we documented a significant effect on both morbidity and mortality in this severe model, we will aim to study this therapeutic regimen in a CLP rescue model (63) in future experiments. It is tempting to speculate that double blockade combined with antibiotic and intensive resuscitation would improve survival even further.

Complement inhibitors are already in clinical use for certain rare diseases. A number of diseases are on the list of possible future candidates for complement therapeutics and several target molecules are candidates for inhibition (64). The complement inhibitor of choice will depend on the pathophysiology of the disease. Some inhibitors work in both animal models and humans, like the coversin used in this study. In contrast, the anti-CD14 Abs are frequently species specific. Thus, we recently produced a recombinant human-specific anti-CD14 IgG2/4 chimeric Ab that showed no Fc-mediated effector function, but efficiently neutralized CD14 (65).

In conclusion, the present in vivo study demonstrates that the combined C5 and CD14 inhibition significantly improves systemic inflammation, clinical signs, and survival rate in a clinically relevant model of polymicrobial sepsis. Thus, the combined inhibition of the complement and TLR pathway represents a most promising therapeutic approach to improve outcomes for patients with polymicrobial sepsis.

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## Disclosures

M.A.N. undertakes paid consultancy for Volution Immuno-Pharmaceuticals, which is developing coversin for clinical use as a drug to treat complement-mediated disorders, though not sepsis.

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