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Combined Inhibition of Complement (C5) and CD14 Markedly Attenuates Inflammation, Thrombogenicity, and Hemodynamic Changes in Porcine Sepsis

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Complement and the TLR family constitute two important branches of innate immunity. We previously showed attenuating effects on inflammation and thrombogenicity by inhibiting the TLR coreceptor CD14 in porcine sepsis. In the present study, we explored the effect of the C5 and leukotriene B4 inhibitor *Ornithodoros moubata* complement inhibitor (OmCI; also known as coversin) alone and combined with anti-CD14 on the early inflammatory, hemostatic, and hemodynamic responses in porcine *Escherichia coli*-induced sepsis. Pigs were randomly allocated to negative controls (n = 6), positive controls (n = 8), intervention with OmCI (n = 8), or with OmCI and anti-CD14 (n = 8). OmCI ablated C5 activation and formation of the terminal complement complex and significantly decreased leukotriene B4 levels in septic pigs. Granulocyte tissue factor factor expression, formation of thrombin–anti-thrombin complexes (p < 0.001), and formation of TNF-α and IL-6 (p < 0.05) were efficiently inhibited by OmCI alone and abolished or strongly attenuated by the combination of OmCI and anti-CD14 (p < 0.001 for all). Additionally, the combined therapy attenuated the formation of plasminogen activator inhibitor-1 (p < 0.05), IL-1β, and IL-8, increased the formation of IL-10, and abolished the expression of wCD11R3 (CD11b) and the fall in neutrophil cell count (p < 0.001 for all). Finally, OmCI combined with anti-CD14 delayed increases in heart rate by 60 min (p < 0.05) and mean pulmonary artery pressure by 30 min (p < 0.01). Ex vivo studies confirmed the additional effect of combining anti-CD14 with OmCI. In conclusion, upstream inhibition of the key innate immunity molecules, C5 and CD14, is a potential broad-acting treatment regimen in sepsis as it efficiently attenuated inflammation and thrombogenicity and delayed hemodynamic changes. *The Journal of Immunology*, 2013, 191: 819–827.

Severe sepsis and septic shock are life-threatening conditions characterized by a whole-body inflammatory state and homeostatic imbalance (1, 2). Many immunopathologic alterations account for the morbidity and mortality, and despite advanced antimicrobial and adjunctive therapy, these conditions are still one of the leading causes of death among critically ill patients (3). Inhibition of downstream mediators of sepsis, such as TNF-α, IL-1β, and IL-6, has failed in clinical trials (1).

Abbreviations used in this article: DIC, disseminated intravascular coagulation; HR, heart rate; LT84, leukotriene B4; MAP, mean artery pressure; MPI, mean fluorescence intensity; MPAP, mean pulmonary artery pressure; OmCI, *Ornithodoros moubata* complement inhibitor; PAI-1, plasminogen activator inhibitor-1; PRR, pattern recognition receptor; SVV, stroke volume variation; TAT, thrombin–antithrombin; TCC, terminal complement complex; TF, tissue factor.

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reciprocal amplifications of the cascades serve host defense. However, uncontrolled systemic activation of innate immune responses as seen in sepsis may lead to hyperinflammatory and counterproductive reactions that are accompanied by disseminated intravascular coagulation (DIC) (18). As cardinal cellular effector cells of innate host response, the neutrophils play in particular an important role in this uncontrolled pathological condition (19).

Recently, we compared the effect of CD14 inhibition on whole bacteria *Escherichia coli*–induced sepsis with purely LPS-induced sepsis in pigs (20). Besides important inflammatory and hemostatic differences between the two models, the study documented that the *E. coli*–induced inflammation was relatively CD14-dependent. The significance of complement was recently shown in a model of *E. coli*–induced sepsis in baboons, as complement inhibition displayed broad anti-inflammatory effects, partially reversed microcirculatory dysfunction, and prevented systemic blood pressure from falling (21). Additionally, ex vivo whole blood studies on humans have shown that oxidative burst, upregulation of adhesion molecules, expression of TF, and granulocyte enzyme release are, to a large extent, complement-dependent in Gram-negative–induced inflammation (22, 23). Notably, the inhibitory effect on a broad spectrum of Gram-negative–induced inflammatory mediators is reinforced by a combined inhibition of complement and CD14 (24). We therefore hypothesized that inhibition of both of these upstream sensors of innate immunity might prove a useful therapeutic regimen in sepsis and other inflammatory conditions (25).

The complement inhibitor *Ornithodoros moubata* complement inhibitor (OmCI), which also binds leukotriene B4 (LTB4) (26), was recently demonstrated to be an equally effective C5 inhibitor in pigs and humans (27). In pigs, 0.6 mg OmCI/kg completely inhibited the activity of all three complement pathways. Thus, the aim of the present study was to examine the effect on inflammation, hemostasis, and hemodynamics of OmCI alone and OmCI combined with anti-CD14 in a well-described pig model of *E. coli*–induced sepsis.

### Materials and Methods

#### Bacteria and interventional drugs

*E. coli* strain LE392 (ATCC 33572) from the American Type Culture Collection (Manassas, VA) was used. Endotoxin-free recombiant bacterial OmCI (also known as coversin) (28), a 16.8-kDa protein, was provided by Varleigh Immunopharmacueticals (Jersey, Channel Islands). Mouse anti-porcine CD14 mAb clone MIL-2, isotype IgG2b, was purchased from MyBioSource (San Diego, CA). Mouse anti-porcine CD14 mAb clone 2F4/11 or the isotype-matched mouse IgG1-FITC conjugate (Sigma-Aldrich) for another 15 min and then washed, before flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ). The cells were washed with PBS/0.1% BSA (Biotest, Dreieich, Germany). The cells were lysed, and samples were centrifuged at 3000 rpm for 15 min. The samples were resuspended, and analyzed as described above. Data are given as mean fluorescence intensity (MFI).

#### Experimental design

Thirty pigs were randomly allocated to two arms (Fig. 1A). The pigs in the *E. coli* sepsis arm were further allocated to a positive control group (*n* = 8) or to one of two intervention groups: an OmCI group (*n* = 8) or a combined OmCI and anti-CD14 group (*n* = 8). The pigs in the negative control arm were allocated to two subgroups, saline only (*n* = 3) and OmCI and saline (*n* = 3), together referred to as the negative control group. The intervention groups received either a bolus of OmCI (1 mg/kg) or a bolus of OmCI (1 mg/kg) and anti-CD14 (5 mg/kg), administered 20–30 min before induction of sepsis. Excess OmCI in the bolus that is not bound to C5 is rapidly removed from circulation (30). Therefore, to bind C5 synthesized by pigs during the experiment, OmCI was infused at 0.5 mg/h throughout the time course (Fig. 1B). Sepsis was induced with an increasing i.v. infusion of *E. coli*, and each pig received a total of 1.075 × 10^9 *E. coli* per kg, corresponding to 1.1 × 10^9 bacteria/ml blood, as previously described (20). The positive control group received the same volume of saline (9 mg/ml) as the intervention groups. The negative control group received the same volume of saline only, or saline and OmCI delivered as described above. Blood samples were drawn and physiological data recorded after surgery at time point baseline, and thereafter at 0, 30, 60, 120, 180, and 240 min after induction of sepsis. Biopsies from the lung and liver were taken imme- diately after euthanasia. All animals received the same background infusion of Ringer’s acetate. Five hundred mll/h was given during the first 2 h to compensate for hemorrhagic needs and loss of sympathetic tone following induction of anesthesia. For the remainder of the experiment fluids were administered at 100 mll/h. Additional infusion of Ringer’s acetate was given based on stroke volume variation (SVV). If SVV > 12%, Ringer’s acetate was given until SVV < 7%. Norepinephrine was given only when refractory hypotension occurred. One pig in the OmCI group was excluded owing to an extreme need for norepinephrine reaching 7 SD above the mean of the group. All other animals completed the 4-h protocol. Norepinephrine was given to most of the pigs exposed to sepsis and the volume used for each pig was recorded.

#### Functional complement activity

A commercially available enzyme immune assay (Complement System Scramble, Wieslab; Euro Diagnostica, Malmö, Sweden) was used to test functional activity of the classical, lectin, and alternative complement pathways.

#### Soluble C5b-9 (TCC)

Soluble C5b-9 (TCC) was measured using multiplex xMAP technology (Bio-Plex) as previously described (31). Briefly, mouse anti C5b-9 Ab (clone aE11; Diotec, Oslo, Norway) was coupled to carboxylated nonmagnetic beads (Bio-Rad, Hercules, CA) using a Bio-Plex amine coupling kit (Bio-Rad). Coupled beads were then incubated with samples, followed by bio- tinylated anti-C6 mAb (Quidel, San Diego, CA) and streptavidin–R–PE (Qiagen, Hilden, Germany). These Abs are known to react with porcine C5b-9. Soluble human C5b-9 protein (Complement Technology, Tyler, TX) was used as standard. Measurement and data analysis were performed with the Bio-Plex 100 system and the Bio-Plex Manager software version 6.1.

#### LTB4

LTB4 from serum was measured using a competitive enzyme immunoassay from R&D Systems (Minneapolis, MN).

#### Flow cytometry

In pig whole blood, neutrophils are clearly discriminated from mononuclear cells, but lymphocytes and monocytes cannot be separated by forward/side scatter dot plots. For measurements of wCD11R3 on neutrophils (the pig ortholog to human CD11b), blood was collected at baseline, 0 min, and 180 min, anticoagulated with EDTA, and stained with mouse anti-porcine wCD11R3 FITC clone 2F4/11 or the isotype-matched mouse IgG1-FITC negative control Ab (AbD Serotec). For measurements of TF on granulocytes, blood was collected at baseline, 0 min, and 180 min, anti-coagulated with citrate, and stained with sheep anti-human TF (Affinity Biologicals, Ancaster, ON, canada) or control sheep IgG (Sigma-Aldrich, Saint Louis, MO). All samples were incubated for 15 min in the dark, red cells were lysed, and samples were centrifuged at 300 × g for 5 min at 4°C. The cells were washed with PBS/0.1% BSA (Biotest, Dreieich, Germany). Samples for wCD11R3 analysis were resuspended in PBS/0.1% BSA before flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ). Samples for TF analysis were further stained with donkey anti-sheep IgG-FITC conjugate (Sigma-Aldrich) for another 15 min and then washed, resuspended, and analyzed as described above. Data are given as mean fluorescence intensity (MFI).

#### Hematology, coagulation, and fibrinolysis

Hematological parameters were obtained from the Oslo University Hospital Diagnostic Hematology Laboratory (Cell-Dyn 4000; Abbott Diagnostics, Abbott Park, IL). The leukocyte differential count fully discriminated neutrophils in porcine blood; however, porcine monocytes and lymphocytes cannot be separated by this instrument. Thrombin activation was
measured in citrate plasma using a human thrombin–antithrombin (TAT) immunoassay kit (Dade Behring, Marburg, Germany) that works for porcine plasma (32). Plasminogen activator inhibitor-1 (PAI-1) was measured in citrate plasma by a porcine PAI-1 kit (Molecular Innovations, Novi, MI).

Inflammatory mediators

The cytokines TNF-α, IL-1β, IL-6, and IL-8 were analyzed by Quantikine porcine immunoassay kits from R&D Systems (Minneapolis, MN). IL-10 was analyzed by a porcine kit from BioSource/Invitrogen (Carlsbad, CA).

Histopathological evaluation

Two-micrometer-thick sections from formalin-fixed lung and liver specimens were deparaffinized and stained by H&E and safron. The lung biopsies were evaluated by morphometry to estimate the volume density of interstitial and alveolar inflammatory changes. In the liver biopsies granulocyte infiltration in the portal tracts was evaluated at ×145 magnification and edema in the portal tracts at ×58 magnification.

Ex vivo experiments with pig whole blood

The ex vivo whole blood model has previously been described in detail (33). Briefly, whole blood was collected from healthy pigs and drawn into tubes containing the anticoagulant lepirudin (Refludan, Pharmion, Copenhagen, Denmark) at a final concentration of 50 μg/ml whole blood. The blood was preincubated with inhibitors, controls, or PBS for 5 min at 37°C. The blood was then either incubated with 10^6 E. coli/ml for 2 h for the measurement of IL-8, with 5 × 10^6 E. coli/ml for 3 h for the measurement of TF, or with PBS. After incubation the tubes were placed on crushed ice and EDTA or citrate was added to stop the activation of IL-8 and TF, respectively.

IL-8 was analyzed by Quantikine porcine immunoassay kits from R&D Systems. For measurements of TF on granulocytes, one sample was split into two tubes and stained with sheep anti-human TF (Affinity Biologicals) and control sheep IgG (Sigma-Aldrich), respectively. All samples were incubated for 30 min at 4°C, and red cells were lysed and thereafter centrifuged at 300 × g for 5 min at 4°C. The cells were washed with PBS with 0.1% BSA (Bioteck). Samples were further stained with rabbit anti-sheep IgG (Santa Cruz Biotechnology, Dallas, TX) for another 30 min at 4°C and then washed twice as described above. The cells were resuspended in PBS with 0.1% BSA before they were analyzed by the flow cytometer (FACSCalibur). Granulocytes were gated in forward scatter/side scatter dot plots, and TF expression was given as MFI.

Ethics

The animals were treated in accordance with the Norwegian Laboratory Animal Regulations, and the study was approved by the Norwegian Animal Research Authority.

Data presentation and statistics

The negative subgroups (the saline plus OmCI and saline group) were merged into one negative control group because OmCI by itself had no effect on the readouts measured, except for the functional complement activity. GraphPad Prism version 5 (GraphPad Software, San Diego, CA) was used for statistical analyses. Data were examined with a repeated-measures two-way ANOVA followed by Bonferroni’s correction for multiple tests. The difference in LB4 formation between the positive control group and both intervention groups was analyzed using an unpaired t test. Ex vivo data were examined with a one-way ANOVA, followed by Bonferroni’s multiple comparison test. A p value <0.05 was considered statistically significant.

Results

Effect of OmCI on complement activity, formation of soluble TCC, and LTb4

The experimental design of the study is shown in Fig 1. To ensure OmCI was being used at a high enough dose to completely inhibit complement, we measured complement activity in serum. The activity was measured in serum from three pigs from the positive control group, from the OmCI group and the combined OmCI and anti-CD14 group, as well as from two negative saline controls at baseline, 0 min, and 60 min (Fig. 2, upper panels). Complement activity was also measured from all pigs at 180 min. OmCI completely ablated the generation of C5b-9 by the classical, lectin, and alternative pathways immediately after administration and remained effective throughout the experiment. Complement activity in animals not treated with OmCI (positive and saline controls) remained at 100% throughout the time course.

The formation of the soluble terminal TCC was measured in plasma from all animals at baseline and 240 min. Mean concentration at baseline was 12 ng/ml, and no differences were observed between the different groups (data not shown). TCC was substantially increased in the positive control group (Fig. 2, lower left panel). OmCI alone and OmCI combined with anti-CD14 abolished the formation of TCC at 240 min (p < 0.01). At 240 min, mean concentrations of TCC in both intervention groups were comparable to what was observed in the negative control group.

LTB4 was measured in serum samples from all animals. Compared to baseline, the average ratio of LTB4 formation more than tripled in the positive control group and was virtually unchanged in the negative control and intervention groups from 0 to 180 min (Fig. 2, lower middle panel). Combining data from both intervention groups, the concentration of LTB4 was 68% lower in the intervention groups compared with the positive control group at 180 min (p = 0.001) (Fig. 2, lower right panel).

Hemostatic parameters

OmCI alone attenuated the expression of TF on blood granulocytes by reducing the MFI signal detected by flow cytometry by 46% (p < 0.001) at 180 min compared with the positive control group (Fig. 3, left panel). Combined OmCI and anti-CD14 completely abolished TF MFI at 180 min (p < 0.001 as compared with both the positive control group and the OmCI group). TAT increased markedly by 120 min to 240 min in the positive control group (Fig. 3, middle panel). OmCI alone significantly reduced TAT formation by 51 and 42% at 180 and 240 min, respectively (p < 0.001). Combined OmCI and anti-CD14 virtually abolished TAT formation at 180 min and reduced it by 68% at 240 min (p < 0.001). The combination of OmCI and anti-CD14 inhibited TAT significantly more than OmCI alone at 240 min (p < 0.05). PAI-1 increased markedly from 120 to 240 min in the positive control group (Fig. 3, right panel). Only combined OmCI and anti-CD14 reduced PAI-1 formation. At 240 min the reduction was 32% (p < 0.05). All hemostatic parameters remained at baseline in the negative control group.

Cytokines

OmCI alone attenuated the formation of TNF-α by 51% at 120 min (p < 0.01), and that of IL-6 by 46 and 26% at 120 and 180 min, respectively (p < 0.05), compared with the positive control group (Fig. 4, upper panels). OmCI alone nonsignificantly attenuated the formation of IL-1β and IL-8 by 20 and 29%, respectively. The formation of IL-10 was not influenced by OmCI alone.

Combined OmCI and anti-CD14 essentially abolished the formation of TNF-α at 120 min (Fig. 4, upper panel). IL-6 formation was abolished at 120 min, and reduced by 83 and 80% at 180 and 240 min, respectively. IL-1β formation was abrogated at 180 min and reduced by 75% at 240 min. IL-8 formation was decreased by 88% at 180 min. In contrast, formation of the anti-inflammatory cytokine IL-10 increased by 215% at 180 min. All of these changes seen for combined OmCI and anti-CD14 were statistically significant (p < 0.001) compared with the positive control group, and they were significantly greater (p < 0.05) than the changes seen with OmCI alone. All cytokines remained close to baseline in the negative control group.

Neutrophil activation

The number of neutrophils steadily fell from 6.9 × 10^9/l at baseline to 1.9 × 10^9/l at 240 min in the positive control group (Fig. 4, left panel). When compared to baseline, the average ratio of neutrophil activation more than tripled in the positive control group and was virtually unchanged in the negative control and intervention groups from 0 to 180 min (Fig. 4, lower panel). Combining data from both intervention groups, the concentration of neutrophil activation was 68% lower in the intervention groups compared with the positive control group at 180 min (p = 0.001) (Fig. 4, lower right panel).
In the OmCI group, neutrophil number remained stable from baseline to 120 min, although the difference was not statistically different from the positive control group, and thereafter fell steeply. In the combined OmCI and anti-CD14 group, the number of neutrophils remained preserved after induction of sepsis and was higher than the positive control group from 60 min ($p < 0.05$) until the end of the experiment ($p < 0.001$). A higher neutrophil count was also observed in the combined group at 180 and 240 min compared with the OmCI group ($p < 0.05$). In the negative control group, a modest decrease was observed in the first 60 min, and thereafter the number of neutrophils increased and remained above baseline.

**FIGURE 1.** Experimental design. (A) Thirty pigs were randomly allocated to a positive control group ($n = 8$), two intervention groups receiving either OmCI alone ($n = 8$) or OmCI combined with anti-CD14 ($n = 8$), and a negative control group. (B) Schematic illustration of E. coli–induced sepsis and delivery of drugs. Baseline represents the time after surgery and stabilization followed by induction of sepsis at time 0 and successive registration time points in minutes. The infused volume of OmCI and anti-CD14 was substituted with saline in the positive control and negative control groups. Bacterial infusion was substituted with saline in the negative control group.

**FIGURE 2.** Effect of OmCI on functional complement activity and on the expression of LTB4. **Upper panels,** The effects of OmCI on the functional activity of the three complement pathways. Complement activity was measured in selected pigs from the positive control group ($n = 3$), the OmCI group ($n = 3$), and the combined OmCI and anti-CD14 group ($n = 3$) at baseline, 0 min, and 60 min. Complement activity was also measured in all pigs included in the study ($n = 30$) at 180 min. The data are given as percentages of a standard defined as 100% activity and expressed as mean ± SEM. **Lower left panel,** Quantification of TCC at 240 min. The data are presented as mean ± SEM. Statistical comparisons were performed between the positive control group and both intervention groups. **Lower middle panel,** Time course showing average ratio of LTB4 formation in serum compared with baseline (defined as 1). **Lower right panel,** Quantification of LTB4 at 180 min. Data for both intervention groups were combined (cf. **lower left panel**) to include all OmCI-treated animals. The data are presented as means ± SEM. Statistical comparisons were performed between all OmCI-treated pigs versus the positive control group. **$p = 0.001.$** Bas, Baseline; Neg, negative; Pos, positive.
The cell surface marker wCD11R3 (the pig ortholog to human CD11b) increased in the positive control group and in the OmCI group from 0 to 180 min, whereas wCD11R3 MFI was reduced by 71% at 180 min by combined OmCI and anti-CD14 ($p < 0.001$) (Fig. 4, lower right panel). wCD11R3 remained at baseline in the negative control group.

Hemodynamic parameters and fluid balance

An abrupt increase in heart rate (HR) and mean pulmonary artery pressure (MPAP) was observed in the positive control group after 60 min. The increased HR and MPAP were delayed by 60 and 30 min, respectively, in the combined OmCI and anti-CD14 group, being significantly lower than the positive control group at 120 ($p < 0.05$) and 90 min ($p < 0.001$) (Fig. 5, upper and middle panels). Mean artery pressure (MAP) and the systemic vascular resistance index decreased and the cardiac index increased similarly in all sepsis groups (data not shown). The MPAP/MAP ratio was significantly lower at 90 min in the combined group compared with the positive control group ($p < 0.001$) (Fig. 5, lower panel).

The amount of norepinephrine used did not differ significantly between the positive control group and the interventions groups. In the negative control group, all hemodynamic parameters remained unchanged and only a modest increase in HR was observed.

The requirement for extra fluid was guided by the stroke volume variation (SVV). The mean accumulated amounts of fluid given in the positive control group, the OmCI group, and the combined OmCI and anti-CD14 group (minus urinary output) were 2433 ± 942, 2357 ± 534, and 2170 ± 647 ml (all 95% CI), respectively (data not shown). The 10% lower requirement in the combined group compared with the positive control group was not statistically significant. All sepsis groups required significantly more extra fluid than did the negative control group, which received the background infusion only.

Histopathological evaluation of lung and liver

Lung. In the positive control group, a variable fractions of the alveolar septa were swollen and filled with inflammatory cells dominated by granulocytes (Supplemental Fig. 1). Foci with ex...
expression close to background activity (\(p < 0.01\)), whereas anti-CD14 alone reduced the formation by 35% \((p < 0.01)\) (Fig. 6, left panel). Combined inhibition with OmCl and anti-CD14 reduced IL-8 formation by 79% \((p < 0.01)\). The effect of the combined inhibition was significantly more pronounced as compared with both single inhibitors \((p < 0.01)\).

**Tissue factor expression.** Both OmCI and anti-CD14 reduced the granulocyte expression of TF MFI, but not to a significant level. Combined inhibition with OmCI and anti-CD14 abolished the expression close to background activity \((p < 0.05)\) (Fig. 6, right panel).

**Discussion**

To our knowledge, this study demonstrates for the first time that combined inhibition of C5 and CD14 efficiently attenuated a broad panel of proinflammatory mediators, enhanced IL-10, reduced thrombogenicity, and delayed hemodynamic changes induced by *E. coli* sepsis in pigs. Additionally, the study documents that C5 inhibition alone counteracts the procoagulant state by attenuating the expression of TF, TAT, and release of PAI-1, and it exerts attenuating effects on important proinflammatory cytokines such as TNF-\(\alpha\) and IL-6.

In sepsis, increased blood thrombogenicity and eventually DIC occur concurrently with the overwhelming systemic inflammatory response. The crosstalk and mutual interaction between complement-induced inflammation and coagulation may amplify these detrimental reactions. TF is a transmembrane glycoprotein that, in vivo, is regarded as the key initiator of coagulation (34). TF is expressed by subendothelial cells that are not normally exposed to the circulating blood, but disruption of vascular integrity exposes TF and initiates the coagulation cascade (35). Inflammatory mediators and LPS induce the expression of TF on endothelial and polymorphonuclear cells and platelets, thereby increasing blood thrombogenicity (36, 37).

In this study, the expression of TF was totally ablated by the combined inhibition of C5 and CD14 and was markedly and significantly reduced by C5 inhibition alone. This highlights the close relationship between inflammation and coagulation and shows a potent and remarkable effect of upstream inhibition of PRRs. OmCI binds directly to C5 and prevents cleavage by the C5 convertases, thus preventing formation of C5a and TCC (28, 38). The attenuating effect on TF is in accordance with previous reports describing C5a-dependent TF expression in both neutrophils and endothelial cells (15, 16). Recently it was shown that *E. coli*-induced TF expression on human monocytes and TF activity in plasma microparticles was C5a-dependent (39). This may be of great importance, as the formation of microparticles seems to reflect the severity of DIC (40, 41). There is evidence that expression of TF is also dependent on TLR4/MD-2 signal transduction (42). Anti-CD14 may have prevented CD14 transferring LPS to the receptor complex TLR4/MD-2, thereby preventing intracellular signaling (10, 11) and subsequent induction of TF. In an earlier study (20), anti-CD14 alone significantly reduced the concentration of surrogate marker for thrombin generation, TAT, and the major inhibitor of fibrinolysis, PAI-1. In the present study, TAT and PAI-1 increased in the positive control group and were suppressed by the combined therapy, whereas TAT was suppressed also by OmCI alone. As compared with TAT, which is immediately formed from precursors, PAI-1 needs to be synthesized and the time frame for the current model might have limited this. Furthermore, the regulation of PAI-1 is complex and subject to a wide range of different mediators, such as hormones, cytokines, and growth factors (43), which may explain why PAI-1 was less affected by the combined regimen.

C5 inhibition alone attenuated central proinflammatory cytokines such as TNF-\(\alpha\) and IL-6, consistent with reduced formation of C5a. The importance of C5a has been broadly uncovered in polymicrobial (cecal ligation and puncture)-induced sepsis in rodents (8). Inhibition of C5a or one or both of its receptors (C5aR/C5L2) are all treatment strategies that have demonstrated reduced inflammation and increased survival, notably, also when given after induction of sepsis (44–46). Recently, the significance of complement was clearly revealed in a baboon model of *E. coli*-induced sepsis (21). C3 inhibition partially reversed DIC and the inflammatory response, and it protected against endothelial dysfunction and hypotension. In the present study, the complement inhibition was at the C5 level, leaving C3 activation open for microbial defense, including C3b opsonization and release of C3a. This might be a beneficial approach in the treatment of sepsis, although activation at the C3 level may contribute to the inflam-
matory response. Still, we argue that inhibition of C5 attenuates the detrimental effects induced by complement activation substantially. In this study we documented that the whole complement cascade was activated in vivo, as detected by an increase in plasma TCC. This increase was completely blocked by OmCI in both intervention groups, confirming sufficient dose of the inhibitor.

Neutrophil-mediated tissue injury with release of cytokines, reactive oxygen species, proteinases, and other cell-derived content is an important feature in the pathogenesis of sepsis (19, 47). Owing to impaired migration, sequestration of neutrophils also strikes organs not affected by infection (48). The lungs are particularly susceptible, and impaired lung function correlates with the intensity of neutrophil infiltrates (49, 50). As previously shown, C5a and sublytic doses of TCC can stimulate production of LTB4 (27, 51). Furthermore, OmCI has been proven to capture LTB4 within and sublytic doses of TCC can stimulate production of LTB4 (27, 51). Furthermore, OmCI has been proven to capture LTB4 within an internal binding pocket and also prevent alveolar inflammation induced by direct installation of LTB4 to the murine lung (26). The LTB4 is tightly bound to OmCI, with an estimated $K_d$ of 1 nm (52, 53), similar to the $K_d$ for the LTB4 receptor binding (54). OmCI will remove LTB4 from circulation, preventing its measurement by ELISA, and it will prevent LTB4 from interacting with its receptors. Thus, the overall reduction in LTB4 levels shown in Fig. 2 (lower panel) may be due to both complement inhibition and direct binding to LTB4. OmCI has the potential to reduce the migration of neutrophils, as LTB4 promotes neutrophil chemotaxis, increases adherence to capillary walls, and is a potent inducer of chemokinesis, neutrophil infiltration, and degranulation (55). Furthermore, this might have been reflected in the histopathological lung changes, with the presence of less swollen septa and fewer foci filled with inflammatory cells in the OmCI-treated group compared with positive control group.

The number of neutrophils in blood in the combined OmCI and anti-CD14 group was preserved or even partially increased during the course, in contrast to the positive control group, where a marked decrease was observed, consistent with leukopenia normally observed during sepsis. The preservation of leukocytes in the combined group may be partially explained by the inhibited expression of wCD11R3 (the pig homolog of CD11b/CD18), which is an important adhesion molecule leading to binding of neutrophils to the endothelium and plays a critical role in sequestering neutrophils from the circulation (56). It also mediates phagocytosis and recognizes a broad range of different microbial molecules (57). In humans, upregulation of CD11b on monocytes and granulocytes is differentially dependent on CD14 and C5a, but combined inhibition of both pathways is required for sufficient ablation in both cell types (22, 23). In pilot experiments, we have observed a pronounced upregulation of wCD11R3 within 60 min after induction of E. coli sepsis in the current model. Thus, the preserved level of neutrophils may be due to the combined treatment with OmCI and anti-CD14.

In this forceful model of acute septic shock, capillary leak was present in all sepsis groups after 120 min. A fluid requirement up to 17% of body weight was documented objectively using SVV, which is a dynamic variable of fluid responsiveness and currently one of the best hypovolemia predictors (58). The necessity of fluid resuscitation together with decreased MAP reflects a hyperdynamic low vascular state that would have been even more pronounced without the use of norepinephrine. Pigs have resident macrophages in the lungs closely resembling human Kupffer cells (59), and they are extremely vulnerable to pulmonary vasoconstriction (60). Increased pulmonary artery pressure is a typical, early feature of porcine sepsis that is probably due to local release of a broad spectrum of endogenous metabolites (60). It is therefore an important observation that both the increase in HR and MPAP was delayed by the combined inhibition of C5 and CD14. Furthermore, the MPAP/MAP ratio was similarly delayed, emphasizing a more stable hemodynamic state in the combined treatment group. Thus, it is tempting to suggest that the beneficial hemodynamic effects obtained by the combined treatment regimen would have been more pronounced in a less forceful model, although this remains unanswered and a subject of speculation.

The complex cross-talk between TLRs and complement (5, 25) might explain the efficacy of the combined treatment regimen. Both pathways are part of the redundant host defense system and can either compensate, synergize, or antagonize each other, depending on the activation mechanism. The central role of CD14 as an accessory molecule in the TLR system is illustrated by its known interactions with TLR2, TLR3, TLR4, TLR7, and TLR9 (61). Thus, CD14 appears to be a promiscuous upstream recognition molecule, binding to a number of ligands with low affinity and transferring the ligand to receptors with a higher degree of specificity and affinity. The present sepsis model reveals strong dependency on CD14. Despite this, the importance of complement appears clear and significant. The latter is highly underscored by the ex vivo experiments, demonstrating an unquestionable superior effect obtained by the combined regimen versus single inhibition with either OmCI or anti-CD14. Furthermore, the efficacy of the combined inhibition on IL-8 and TF clearly illustrates this regimen’s ability to cope with host redundancy. In conditions where complement is relatively more important than in the present CD14-

**Figure 6.** Ex vivo effects of inhibiting C5, CD14, and the combination thereof on E. coli-induced IL-8 formation and expression of TF on granulocytes. Pig whole blood was preincubated with OmCI (0.32 μM), anti-CD14 (25 μg/ml), and the combination thereof and then incubated with either $10^6$ E. coli/ml for 2 h (IL-8) or $5 \times 10^6$ E. coli/ml for 3 h (tissue factor). For IL-8, data are normalized to the E. coli group (positive control), defined as 100%. Data are presented as means ± SEM ($n = 6$). Statistical comparisons were performed between the effect of OmCI, anti-CD14, and the combination thereof, versus positive control, and between single inhibition with either OmCI or anti-CD14 versus combined inhibition. *$p < 0.05$, **$p < 0.01$. aCD14, Anti-CD14; BG, background activity; Pos ctrl, positive control.
driven model, such as polymicrobial sepsis (8) and ischemic/ reperfusion injury (62), the contribution of complement inhibition in the combined treatment regimen is likely to be more pronounced. This hypothesis should be tested using relevant models.

In conclusion, the present data suggest that combined inhibition of complement and CD14 efficiently attenuate the inflammatory response, thrombogenicity, and hemodynamic disturbances in E. coli–induced sepsis.

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Disclosures

The authors have no financial conflicts of interest.

References


Suppl. Figure 1

Histopathology of lung and liver

The biopsies shown were taken from one representative pig from the OmCI group and positive- and negative control groups. *Left panel, lung:* Inflamed and swollen alveolar septa were observed in the positive control pig. A variable extent of inflammation was seen in the OmCI treated pig. No inflammation was observed in the negative control pig. Original magnification 7.2 x. *Right panel, liver:* Edema and increased amount of inflammatory cells in the portal tract were observed in the positive control pig and the OmCI treated pig. No inflammation was observed in the negative control pig. Original magnification 58 x. The biopsies are stained with Hematoxylin (blue) and Eosin (red).