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References

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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 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).

Complement and the TLR family constitute two important upstream sensor and effector systems of innate immunity. Their pattern recognition receptors (PRRs) identify and eliminate pathogens as well as endogenous danger motifs, thereby protecting the host and maintaining homeostasis (4, 5). The physiological effects of complement are diverse (6). Activation of complement occurs via three routes—the classical, lectin, and the alternative pathways—all converging at and leading to the cleavage of the central complement factor C3. Activation of the terminal pathway with the cleavage of C5 releases C5b and the biologically potent anaphylatoxin C5a. C5b induces assembly of the terminal C5b–9 complement complex (TCC), which can lyse certain pathogens and cells when incorporated into their lipid membranes (7). C5a is known to induce upregulation of adhesion molecules, stimulate cytokine production, cause paralysis of neutrophils, and increase vascular permeability among other effects (8). The TLRs are an important class of PRRs (9). TLR4 is a PRR for LPS of Gram-negative bacteria that signals most effectively in concert with coreceptors MD-2 and CD14 (10, 11). CD14/MD2/TLR4 signaling induces NF-κB and other transcription factors such IFN regulatory factor 3, IFN regulatory factor 5, and AP-1, required for transcription of a wide variety of inflammatory and immune response genes (9, 12).

The coagulation system is intimately connected to host defense and can both activate complement and be stimulated by activated complement (13, 14). For instance, C5a is known to induce upregulation of tissue factor (TF) on endothelial and white cells (15, 16), whereas thrombin is able to cleave C5 (17). Under physiological conditions both systems act locally, and the interplay and
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 effectors 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-ependent 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 CD5 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
Live E. coli strain LE392 (ATCC 33572) from the American Type Culture Collection (Manassas, VA) was used. Endotoxin-free recombinant bacterial OmCI (also known as coversin) (28), a 16.8-kDa protein, was provided by Varlegh Immuno-Pharmaceuticals (Jersey, Channel Islands). Mouse anti-porcine CD14 mAb clone LTB4 (26) 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.

Hematology, coagulation, and fibrinolysis
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 eA11; Diotec, Oslo, Norway) was coupled to carboxylate 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.

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, anticoagulated 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 (Biotein, Dreieich, Germany). Samples for wCD11R3 analysis were resuspended in PBS/0.1% BSA before flow cytometry (FACSCalibur; 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) immunnoassay 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 saffron. 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 either incubated with 10^6 E. coli/ml for 2 h for the measurement of IL-8, with 5 x 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 x g for 5 min at 4°C. The blood was then washed with PBS with 0.1% BSA (Biotest). Samples were further stained with rabbit anti-TF-FITC conjugate (Santa Cruz Biotechnology, Dallas, TX) for an additional 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 (FACS Calibur). 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 LTB4 formation between the positive control group and both intervention groups was compared 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 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 from 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 highly 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 x 10^7/l at baseline to 1.9 x 10^7/l at 240 min in the positive control group (Fig. 4,
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.

![Experimental design](image-url)

**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.

![Effect of OmCI on functional complement activity and on the expression of LTB4](image-url)

**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 $2433 \pm 942$, $2357 \pm 534$, and $2170 \pm 647$ 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...
Combined inhibition with OmCl and anti-CD14 abolished the ex-granulocyte expression of TF MFI, but not to a significant level. The effect of the combined inhibition was significantly more pronounced as compared with both single inhibitors.

IL-8. OmCl alone attenuated the formation of IL-8 in plasma by 48% (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 OmCl and anti-CD14 reduced the granulocyte expression of TF MFI, but not to a significant level. Combined inhibition with OmCl 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-α 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. OmCl binds directly to C5 and prevents cleavage by the C5 convertases, thus preventing formation of 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 OmCl 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-α 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/CL5L2) 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 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 leucopenia 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 leukocytes 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-
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References


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phonuclear leukocyte receptors for leukotriene B4. Identification of a subset of
high affinity receptors that transduce the chemotactic response. J. Exp. Med.
159: 1027–1041.

55. Yoshikai, Y. 2001. Roles of prostaglandins and leukotrienes in acute inflam-


61. Lee, C. C., A. M. Avalos, and H. L. Ploegh. 2012. Accessory molecules for Toll-

mediated ischemia-reperfusion injury: lessons learned from animal and clini-