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***Ornithodoros moubata* complement inhibitor (OmCI) is an equally effective C5 inhibitor in pig and human¹**

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Abstract

Experimental evidence suggests that C inhibition and more particularly combined inhibition of C and the TLR co-receptor CD14 may be of therapeutic benefit in sepsis and other inflammatory conditions. A barrier to the testing and further development of many inhibitors is that their activity is species specific. Pig is a relevant species for experimental models of human disease, and this study undertakes a comprehensive comparison of the inhibitory efficacy of the C5 inhibitor *Ornithodoros moubata* C inhibitor (OmCI) in human and porcine whole blood *ex vivo* models of *Escherichia coli*-induced sepsis. The effect of OmCI on complement activity in pigs undergoing *E. coli* sepsis was also examined.

Porcine and human serum, and whole blood anticoagulated with lepirudin, was incubated with *E. coli* and the effect of OmCI investigated. The *ex vivo* results were virtually identical in pig and human. OmCI completely ablated the activity of all three C pathways at 0.64 μ M. *E. coli*-induced C activation and expression of CD11b (wCD11R3 in the pig), was abolished *ex vivo* at 0.32 μ M OmCI. Combining anti-CD14 and OmCI reduced the formation of IL-8 and TNF- α more potently than the single inhibitors. OmCI also efficiently bound *E. coli*-induced LTB4 in pig and human plasma. In support of our *ex vivo* findings, *in vivo* the activity of all C pathways was inhibited at 0.6 mg OmCI/kg pig. In conclusion, OmCI efficiently inhibited pig and human C activation, has accompanying anti-inflammatory effects, and is a promising candidate inhibitor for further *in vivo* studies of sepsis.

Introduction

C and the TLR family play essential parts in innate immune reactions. Upon pattern recognition these danger sensors identify and eliminate pathogens as well as endogenous danger motifs, thereby protecting the host and maintaining homeostasis (1,2).

The physiological effects of C are numerous and diverse (3). Activation is known to occur via three routes, the classical, the lectin and the alternative pathways, all converging and leading to the cleavage of the central C factor C3. The C3 fragments C3b and iC3b are important opsonins in the defense against bacteria. Activation of the terminal pathway with

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the cleavage of C5 is biologically highly potent. Release of the anaphylatoxin C5a is known to induce up-regulation of adhesion molecules, stimulate cytokine production, cause paralysis of neutrophils, increase vascular permeability and might lead to disseminated intravascular coagulation among other effects (4). C5b induces assembly of the terminal C5b-9 C complex (TCC), which can lyse certain pathogens and cells when incorporated into their lipid membranes (5).

The TLRs are an important class of pattern recognition receptors (PRR) (6). TLR4 is a PRR for LPS of Gram-negative bacteria, and is dependent on two important co-receptors, MD-2 and CD14 (7,8). TLR signaling induces NF- κ B, required for transcription of a wide variety of inflammatory and immune response genes (6).

Uncontrolled systemic activation of these upstream danger sensors may lead to a counterproductive response that endangers self as seen in septic shock (9,10). *Ex vivo* studies with human whole blood have demonstrated that *E. coli*-induced inflammation is strongly attenuated by a combined inhibition of C and CD14 (11,12). Consequently we hypothesised that combined inhibition of C and CD14 might prove a useful therapeutic regimen in sepsis and other inflammatory conditions (13). Notably, inhibition of the classical proinflammatory cytokines TNF- α and IL-1 β was shown not to have any impact on the *E. coli*-induced inflammatory reaction (14). We recently showed that inhibition of CD14 attenuated proinflammatory cytokines, granulocyte activation and hypercoagulation in *E. coli*-induced sepsis *in vivo* in pigs (15). Furthermore, selective inhibition of C improved organ function in a baboon model of sepsis (16). Combined inhibition of C and CD14 *in vivo* has yet to be tested.

Ticks produce a plethora of proteins that interfere with host immune and haemostatic defense responses (17). OmCI is a 16.8-kDa saliva protein from the soft tick *Ornithodoros moubata* (18). This small protein binds directly to C5, prevents cleavage by both the classical and the alternative C5 convertases and thus C5a and TCC is not formed (19). C5 inhibition by OmCI thus has vast anti-inflammatory potential, without affecting beneficial immunoprotective and immunoregulatory functions of upstream C activity (20). OmCI has cross-species C-inhibitory activity that has been demonstrated but not explicitly quantified in human, rat, mouse and guinea pig (18,21). The protein is protective in experimental passive and active autoimmune myasthenia gravis models in rats. OmCI-treated animals, exhibited significantly fewer symptoms and the inflammatory response was substantially attenuated (21,22). Like some of its closest known homologues (23), OmCI has bifunctional properties, capturing the inflammatory mediator leukotriene B4 (LTB4) in an internal binding pocket in addition to binding to C5 (M.A.N., personal observation). LTB4 is a potent chemotactic agent and activator of white cells in particular neutrophils (24). Despite its low molecular weight, the half-life of OmCI is approximately 30 h in rats due to stable binding to C5, making OmCI an interesting agent for use *in vivo* (21).

The aim of the present study was to compare the efficacy of OmCI in pigs and humans, using functional assays designed for these species. By using a unique whole blood *ex vivo* model for investigation of the crosstalk of the inflammatory network, C inhibitory efficacy as well as anti-inflammatory properties of OmCI was elucidated. In addition, the C inhibitory effect of OmCI was explored in pilot experiments using an *in vivo* porcine model of *E. coli*-induced sepsis.

Material and methods

Reagents and equipment

Sterile PBS pH 7.2 was purchased from Sigma-Aldrich (St. Louis, MO), CryoTubes™ (polypropylene) from Nunc AS (Roskilde, Denmark), Lepirudin (Refludan®) from Pharmion (Hamburg, Germany) and 96 well plates (Costar® 3590) from Corning (New York, NY). Zymosan A (Z-4250) was purchased from Sigma (St. Louis, MO). Human gamma globulin (160 mg/ml, Beriglobin®) was purchased from Behring (Marburg, Germany) and converted to heat-aggregated immunoglobulin G (HAIGG) by heating a 10 mg/ml stock to 63 °C for 15 minutes. Albumin 200 mg/ml was purchased from Octapharma (Hurdal, Norway).

Bacteria

E. coli strain LE392 (ATCC 33572) from the American Type Culture Collection (Manassas, VA) was used in all experiments. *E. coli* was grown over night in LB-medium (tryptone 1 %, yeast extract 0.5 % and sodium chloride 0.9 %), then centrifuged and washed in PBS. After resuspension in PBS, *E. coli* was immediately heat-inactivated (1 h, 60 °C) for use in the *ex vivo* experiments. Growth plates confirmed that all bacteria were killed. Thereafter, the bacteria were washed nine times to remove extra-bacterial LPS and counted by flow cytometry. Live *E. coli* strain LE392 was used *in vivo*. Sepsis was induced with an increasing i.v. infusion of *E. coli*, as previously described (15). Each pig received a total of 1.075×10^8 *E. coli*/kg during the time course of the experiment, corresponding to 1.1×10^6 bacteria/ml blood.

Inhibitors

Recombinant bacterial OmCI (18), a 16.8 kDa protein, was produced in the laboratory of one of the authors (M.A.N). Before use it was stored in single use aliquots at -70 °C. The mouse anti-human CD14 (clone 18D11) F(ab')₂ and control F(ab')₂ (clone BH1), was purchased from Diatec Monoclonals AS (Oslo, Norway). Mouse anti-porcine CD14 monoclonal antibody clone MIL-2, isotype IgG2b, was purchased from Serotec (Oxford, UK). A mouse anti-human IgG2b (clone BH1, prod. no 2070), was purchased from Diatec Monoclonals AS and used as an isotype-matched control. The compstatin analog Ac-I[CV(1MeW)QDWGAHRC]T, which binds to and inhibits cleavage of C3, and the cyclic hexapeptide AcF[OPdChaWR], a C5a receptor antagonist (C5aRa) were produced as previously described (25,26). Eculizumab 10 mg/ml, (Soliris®), a humanized monoclonal IgG2/4k -antibody that binds and inhibits cleavage of C5 was from Alexion Pharmaceuticals (Cheshire, CT), kindly provided by dr. Yngvar Fløysand.

Ex vivo whole blood experiments

The whole blood model has previously been described in detail (27). Briefly, whole blood was collected from 15–60 kg pigs (*Sus scrofa domesticus*; Landrace; outbreed stock) and healthy human donors. Blood was drawn into tubes containing the anticoagulant lepirudin (Refludan®) at a final concentration of 50 µg/ml whole blood. The blood was immediately preincubated with inhibitors, controls or PBS for five minutes at 37 °C. PBS or *E. coli*, at a final concentration of 10⁵, 10⁶ or 10⁸ *E. coli*/ml whole blood, was then added and incubated for 2 h at 37 °C. After incubation, the tubes were put on ice and EDTA (final concentration 20 mM) was added to stop the C activation and the tubes were centrifuged for 15 minutes at 1400 × g at 4 °C. The plasma was stored at -70 °C until analysed.

In vivo experiments

Norwegian Landrace pigs ($n = 7$) of either sex, with a mean weight of 14.4 kg (range 14.0–16.0 kg) were used. Premedication, anesthesia, surgery, hemodynamic monitoring, induction of sepsis and euthanasia were performed as described (15). Pigs received OmCI i.v. as a bolus (22.5, 15, 7.5 and 3.75 mg) and thereafter as a continuous i.v. infusion (0.5 mg/h) for 4 h. The total amounts of OmCI given were 24.5 mg ($n = 1$), 17 mg ($n = 1$), 9.5 mg ($n = 2$) and 5.75 mg ($n = 1$). Two pigs served as positive controls, receiving saline only (9 mg/ml). Blood samples were drawn and physiological data registered after surgery at baseline (Tbasis), thereafter at 0 (T0), 30, 60, 120, 180, and 240 minutes.

Enzyme Immunoassays

The commercially available enzyme immune assay (EIA) (Wielisa, Wieslab, Lund, Sweden) was used to test functional activity of the classical, lectin and alternative C pathways. The test was designed for human C activity (28), but was later shown to cross-react with pig (29). Human C5a was analysed by a commercial EIA (BD Bioscience, San Jose, CA). Terminal C5b-9 C complex (TCC) was measured in an EIA, previously described in detail (30,31). Briefly, the monoclonal antibody aE11, specific for a C9 neopeptide in TCC, was used as capture antibody and a biotinylated monoclonal anti-C6 (Quidel Corporation, San Diego, CA) was used as detection antibody. Both antibodies cross-react with pig epitopes; thus the assay can be used to detect porcine as well as human TCC. The porcine cytokines TNF- α , IL-1 β , and IL-8 were analysed by Quantikine Porcine Immunoassay kits from R&D Systems (Minneapolis, MN). Human plasma samples were analysed using multiplex technology (Luminex 100; Bio-Rad Laboratories, Hercules, CA) – an immunoassay based on colored beads, each with a unique cytokine detection antibody which permits simultaneous measurement of a wide range of human biomarkers. A 27-plex kit purchased from Bio-Rad Laboratories (Hercules, CA) was used containing the following cytokines: IL-1ra, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, TNF- α , IFN- γ , eotaxin, FGF-basic, G-CSF, GM-CSF, IP-10, MCP-1, MIP-1 α , MIP-1 β , PDGF-BB, RANTES and VEGF. The analyses were performed according to the instructions from the manufacturer. Results are presented for the cytokines that exhibited responses to the inhibitors in comparison to the negative controls. LTB4 from pig and human plasma was measured using a competitive enzyme immunoassay from R&D Systems (Minneapolis, MN).

Flow cytometry

For analyses of CD11b (human) or wCD11R3 (pig orthologue) whole blood was preincubated with inhibitor, control or PBS for seven minutes at 37 °C. PBS or *E. coli* at a final concentration of 10^8 *E. coli*/ml whole blood, was added and incubated for 10 minutes before the cells were fixed with 0.5% (v/v) paraformaldehyde for 4 minutes at 37 °C. Human fixed samples were stained with PE conjugated anti human CD11b (catalog no. 333142) or an isotype-matched PE conjugated IgG2a control antibody (catalog no. 349053) (both BD Biosciences Pharmingen, San Jose, CA). Pig fixed samples and EDTA anticoagulated blood from the *in vivo* experiments were stained with FITC conjugated anti porcine wCD11R3 (catalog no. MCA2309F) or an isotype-matched FITC conjugated IgG1 control antibody (catalog no. MCA928F) (both AbD Serotec, Oxford, UK), and incubated for 15 minutes at room temperature in the dark. The red blood cells were then lysed for 7 minutes thereafter centrifuged and washed before being resuspended in PBS containing 1% albumin. Samples were analysed by FACS (FACScan, Becton Dickinson, Franklin Lakes, NJ). Granulocytes were gated in a FSC/SSC dot plot and CD11b and wCD11R3 expression were given as median fluorescence intensity (MFI).

Statistics

GraphPad Prism version 5 (GraphPad Software, San Diego, CA) was used for the statistical analysis. The data were analysed by one-way ANOVA, followed by Dunnett's Multiple Comparison Test. A two-tailed *p*-value below 0.05 was considered statistically significant.

Ethics

Informed written consent was obtained from each blood donor, and the study was approved by the local Ethical Committee. The animals used were treated in adherence to the Norwegian laboratory animal regulations, and the study was approved by the Norwegian Animal Research Authority.

Results

In vitro

The effect of OmCI on the different complement pathways—In serum OmCI dose-dependently inhibited all three C activation pathways similarly, and the inhibition was complete at a dose of 0.64 μM OmCI in both pig and human (Figure 1). Concentrations between 1.28 μM and 20 μM were also completely inhibitory (data not shown).

The effect of OmCI on the formation of C5a and fluid-phase TCC—Pig and human whole blood was incubated with 10^8 *E. coli*/ml for 30 minutes. OmCI dose-dependently inhibited human C5a formation (Figure 2, left panel). At concentrations of OmCI equal to or higher than 0.32 μM , the formation of human C5a was completely ablated. OmCI also dose-dependently inhibited TCC formation in both pig and human whole blood (Figure 2, middle panels). At concentrations of OmCI equal to or higher than 0.32 μM , the formation of TCC was completely inhibited in both species. When serum was activated with either HAIGG or zymosan, which are potent activators of the classical and lectin/alternative C pathway respectively, TCC formation was completely inhibited by 0.64 μM OmCI in both species (Figure 2, right panels).

The effect of OmCI on *E. coli*-induced CD11b/wCD11R3 up-regulation on granulocytes—Whole blood was incubated with 10^8 *E. coli*/ml for 10 minutes and the up-regulation of CD11b (human) and wCD11R3 (pig orthologue) on granulocytes was examined. OmCI dose-dependently inhibited the expression of the cell surface marker in both species (Figure 3). In human granulocytes the CD11b up-regulation was reduced by more than 60 % and in pigs the wCD11R3 up-regulation was completely abolished at 0.32 μM OmCI.

The effect of OmCI, anti-CD14, and the combination thereof, on *E. coli*-induced cytokine formation in porcine and human whole blood—In pig, OmCI significantly reduced the formation of IL-8 compared to the positive control, while TNF- α and IL-1 β were non-significantly reduced (Figure 4, upper panels). Inhibition of CD14 significantly reduced the formation of pig TNF- α and IL-1 β , whereas combined inhibition by anti-CD14 and OmCI had no additional effect (Figure 4, upper panels). In human, OmCI significantly reduced the formation of IL-1 β and IL-8 while TNF- α was non-significantly reduced (Figure 4, lower panels). Inhibition of CD14 significantly reduced the formation of human TNF- α , IL-1 β and IL-8 (Figure 4, lower panels). In contrast to pig, combined inhibition in human by anti-CD14 and OmCI appeared to enhance the inhibitory effect observed for cytokines (Figure 4, lower panels). In human, the combined effect of OmCI and anti-CD14 (i.e near complete ablation of TNF- α , IL-1 β and IL-8) was similar to that of

compstatin (C3 inhibitor) or C5 receptor antagonist combined with anti-CD14 (data not shown).

The effect of OmCI on LTB4—Pig blood was preincubated with OmCI and human blood was preincubated with OmCI and the human specific complement inhibitors, eculizumab and compstatin, then activated with 10^6 *E. coli*/ml and incubated for 2 h at 37 °C. OmCI, as well as eculizumab and compstatin, significantly reduced the formation of LTB4 (Figure 5, left panels). The data from the specific complement inhibitors indicates that LTB4 formation is C dependent. To demonstrate direct binding of OmCI to LTB4, pig and human whole blood were incubated with *E. coli* to induce LTB4-enriched plasma, and then equimolar doses of OmCI were added. By sequestering the LTB4 within the β -barrel of the lipocalin structure (23), OmCI dose-dependently decreased the signal in the LTB4 assay by preventing the leukotriene interacting with the capture antibody (Figure 5, right panels). As expected, eculizumab did not have any neutralizing effect on LTB4.

In Vivo

The effect of OmCI on complement activity in an experimental sepsis model in pigs—To investigate the effect of OmCI on functional C activity *in vivo*, we used our established pig model of Gram-negative sepsis. The effect of different bolus doses of OmCI with uniform continuous infusion of the protein (0.5 mg/h) was assessed in five 15 kg pigs. No pathological side effects were observed when administering OmCI. Two control animals received *E. coli* only. The three largest bolus doses of OmCI (22.5 mg, 15 mg and 7.5 mg) completely ablated the classical, lectin and alternative pathways immediately after administration and remained effective throughout the experiment (Figure 6). The lowest bolus dose of OmCI (3.75 mg) did not totally ablate C activity at the beginning of the experiment but ablation was almost complete by the end of the experiment.

Discussion

In this study we show for the first time that OmCI is an excellent C inhibitor of humans and is as least as effective in pigs. Additionally, we demonstrated that OmCI attenuated C induced proinflammatory mediators in pig and human.

In the Wielisa analysis OmCI was an equally potent inhibitor of the catalytic activity of the classical/lectin (C4bC3bC2a), and alternative (C3bBb3b) C5 convertases of humans and pigs. These effects were confirmed by dose-dependent inhibition of C5a and TCC formation in whole blood incubated with *E. coli*. The potency of OmCI as a complement inhibitor was supported by total abrogation of TCC formation in serum activated with HAIGG or zymosan, which are very strong activators of the classical and alternative/lectin pathways, respectively. OmCI is known to bind directly and tightly to the C5 alpha chain in the vicinity of the C5-C345C domain (18,20,32). OmCI binding stabilizes the overall conformation of C5 and inhibits the effect of the C5 convertases without directly blocking the cleavage site on C5 (19). At present, we lack detailed information on the precise locality and binding details of OmCI to C5, so the reason why OmCI is an equally potent inhibitor of both human and pig C5 is unknown.

OmCI significantly decreased the expression of the human cell surface marker CD11b and the pig orthologue wCD11R3, which together with CD18 constitute phagocyte C receptor 3. Since OmCI blocks the formation of C5a (18), this result is consistent with previous work showing that up-regulation of this important inflammatory receptor is mainly dependent on the formation of C5a (33).

E. coli induced cytokine formation was more dependent on CD14 than on C. CD14 is a well known recognition molecule for LPS, co-operating with MD-2 in TLR4 signaling, but CD14 has been implicated in TLR2 and TLR3 signaling (8,34,35) and even acts as co-receptor for TLR7 and TLR9 (36). Thus, CD14 appears to be a promiscuous upstream recognition molecule, reacting with a number of ligands with low affinity, and transferring the ligand to receptors with a higher degree of specificity and affinity (37). The experiments showed that OmCI differentially attenuated *E. coli*-induced cytokine formation in pig and human, with a significant effect on IL-8 in both species, and on IL-1 β in humans. This is consistent with previous findings showing that these cytokines are relatively more dependent on C in Gram-negative-induced inflammation (33). Interestingly, combined inhibition using OmCI and anti-CD14 enhanced the attenuating effect on cytokine formation in human, compared to the individual inhibition by these two upstream recognition molecules of innate immunity. This effect was similar to that of compstatin or C5a receptor antagonist combined with anti-CD14, and fully agrees with previous results (12).

The leukotrienes are important multifunctional mediators of inflammation. LTB₄ promotes neutrophil chemotaxis, increases adherence of neutrophils to capillary walls and is a potent inducer of chemokinesis and neutrophil infiltration (38). In the present study we showed that inhibition of C profoundly attenuated the expression of *E. coli*-induced LTB₄. This finding supports existing evidence that LTB₄-synthesis is C5a dependent (39). However, because OmCI also captures LTB₄ in an internal hydrophobic binding pocket it is difficult to attribute the inhibitory effect of OmCI on the formation of LTB₄ that is due to C inhibition or due to direct binding to the leukotriene. OmCI's dual inhibitory activity may provide anti-inflammatory advantages as recently shown in an experimental model of immune complex alveolitis (M.A.N. unpublished). There is evidence that the level of LTB₄ significantly increases as an endotoxemic response in pigs (40), and our data indicates that OmCI will inhibit LTB₄-induced inflammatory responses.

Data from the experiments on pigs, convincingly demonstrate that OmCI is an efficient inhibitor of C *in vivo*. The bolus doses that were used, gave estimated blood-concentrations of OmCI (roughly 0.31 – 1.83 μ M) corresponding to the ranges that were used in the *ex vivo* experiments, where 0.64 μ M OmCI in undiluted serum completely inhibited C and 0.32 μ M OmCI in whole blood ablated TCC formation in response to *E. coli*. This enables comparison between the *ex vivo* and the *in vivo* data. Using the three highest bolus doses of OmCI (roughly 0.61, 1.22 and 1.83 μ M) C was completely inhibited *in vivo*. Whereas, using the smallest bolus dose of OmCI (roughly 0.31 μ M in blood) we observed incomplete inhibition of all three C pathways at the beginning of the experiment, but complete inhibition by the end of the experiment. The initial bolus dose was too low to ablate the activity of the standing pool of C5 completely, whereas the continuous infusion gradually blocked more C5. Continuous infusion of OmCI is needed to inhibit newly synthesized C5, since the unexpectedly long half-life of OmCI is dependent on stable binding to C5, thus, surplus OmCI is rapidly cleared from plasma (21).

In conclusion, the present data documents that OmCI is an effective inhibitor of C activation with anti-inflammatory properties in pig and human. The comparable potency of OmCI in the two species and correspondence between the amount of OmCI needed to inhibit C *ex vivo* and *in vivo* opens the door to further testing of OmCI in pig models and will facilitate progression towards clinical testing. This is of substantial interest as the list of conditions where C is involved in the disease pathology is extensive and increasing. Given the mortality and morbidity rates associated with sepsis and the existing data on the potential efficacy of combined inhibition of C and CD14 (12,14,33,41) it seems imperative to proceed with animal studies of sepsis combining inhibition of these two important upstream danger sensors of innate immunity.

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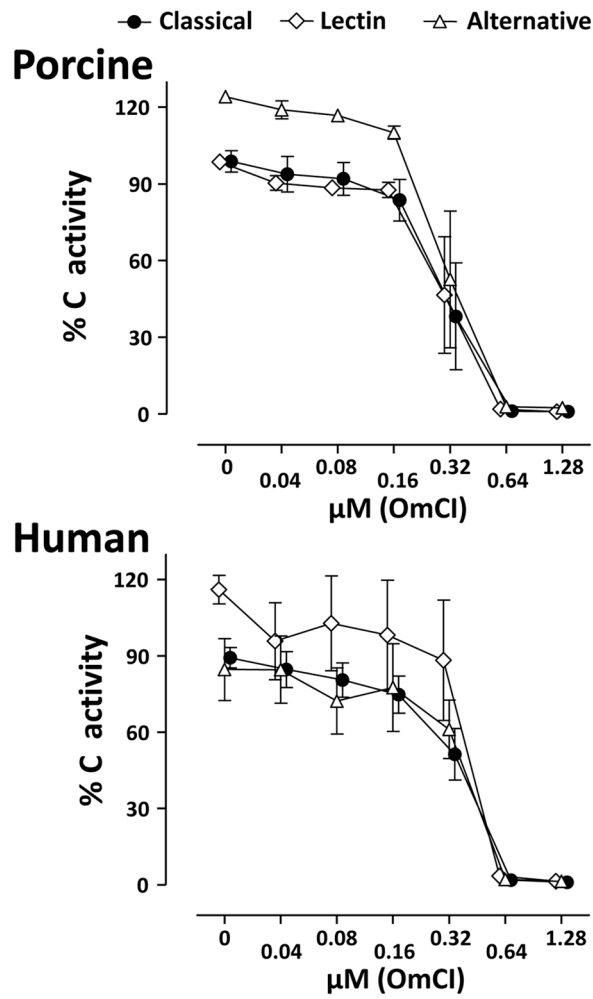


Figure 1. Effect of OmCI on complement functional activity

The effect of OmCI on the functional activity of the classical, lectin and alternative pathways of C were examined in serum from humans and pigs using the Wielisa assay. The data are expressed as % of a standard defined as 100% activity and are presented as mean \pm SEM ($n = 3$).

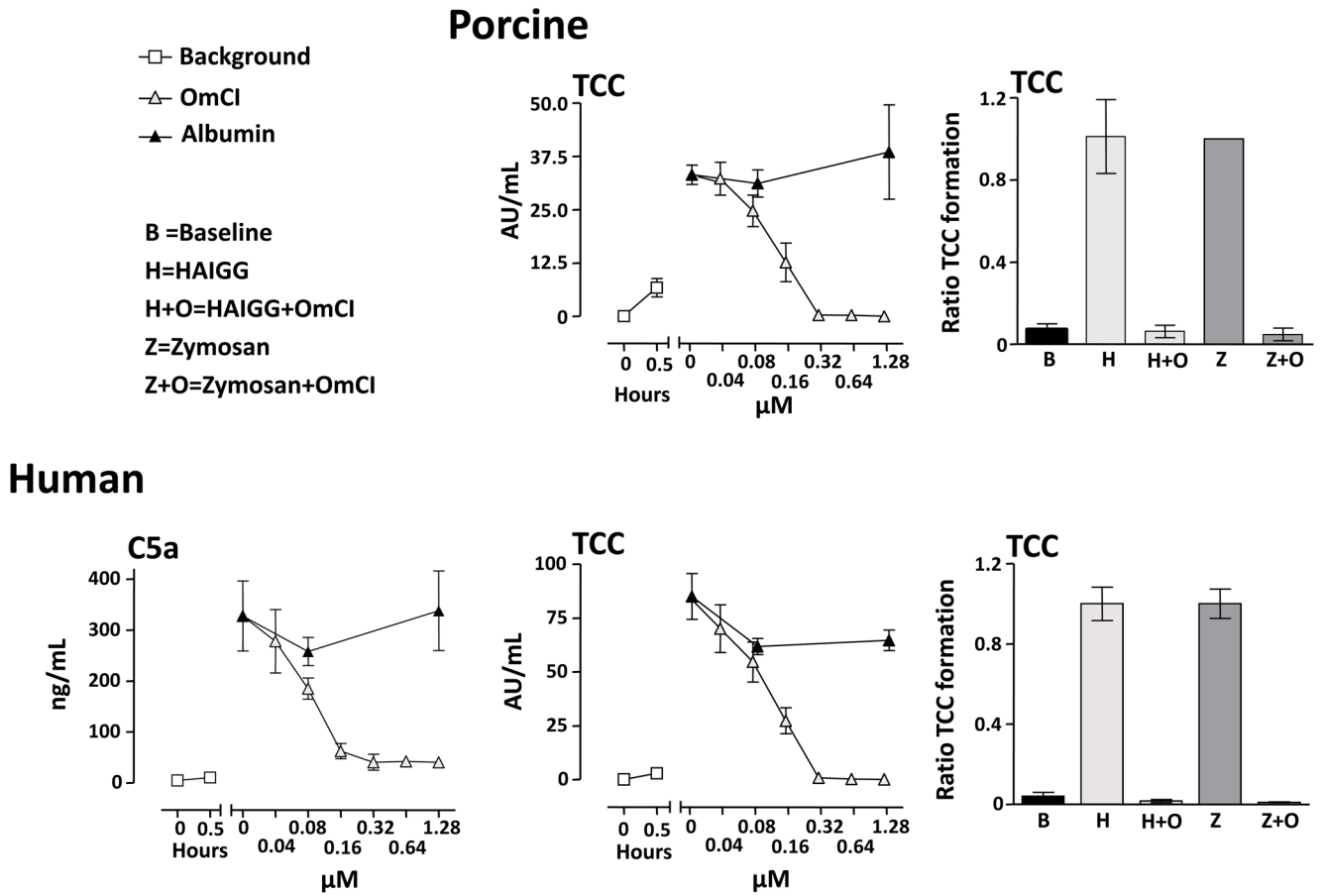


Figure 2. Effect of OmCI on complement induced formation of C5a and TCC in vitro
Left panel: Human whole blood preincubated with OmCI or equimolar amounts of albumin was incubated with 10^8 *E. coli*/ml for 30 minutes. Data are expressed as ng/ml and presented as mean \pm SEM ($n = 3$).
Middle panels: Human and pig whole blood preincubated with OmCI or equimolar amounts of albumin was incubated with 10^8 *E. coli*/ml for 30 minutes at 37 °C. Data are expressed as AU/ml and presented as mean \pm SEM ($n = 3$ for human and $n = 4$ for pig).
Right panels: Human and pig serum preincubated with 0.64 μ M OmCI or albumin was activated with heat aggregated IgG (HAIGG) or zymosan, both at a final concentration of 1 mg/ml. In all experiments, zymosan induced TCC formation in pig whole blood was higher than the upper standard so these are set to 1.0. Data are expressed as TCC ratio, where 1.0 is defined as the amount of TCC formation induced by HAIGG and zymosan, respectively. The data are presented as mean \pm SD ($n = 5$ for human and $n = 3$ for pig).

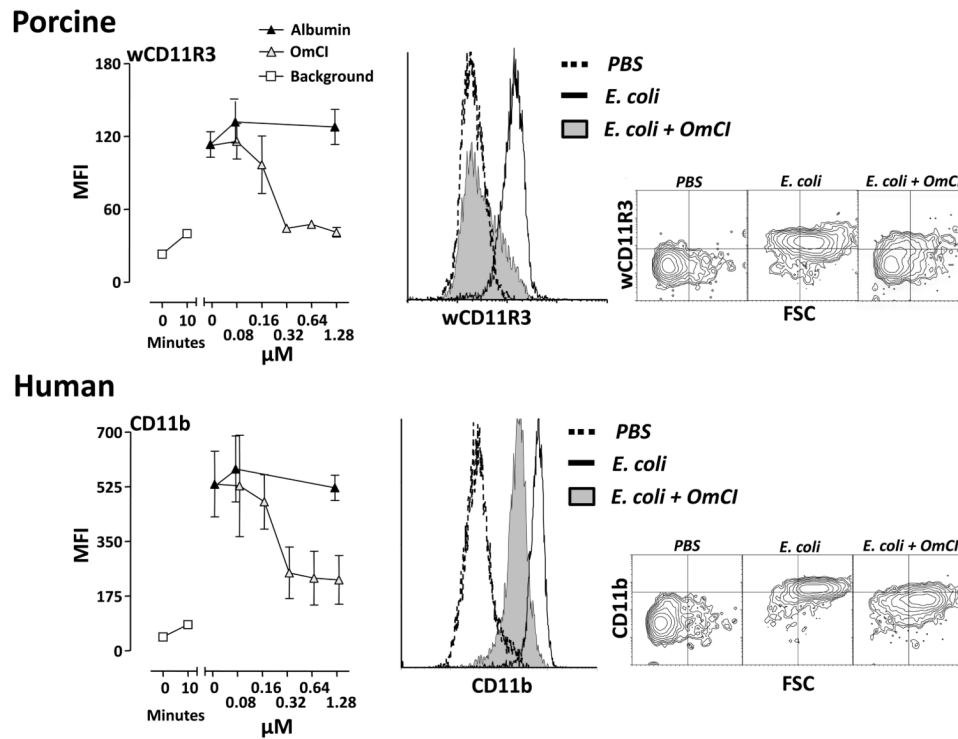


Figure 3. Effect of OmCI on *E. coli*-induced expression of CD11b and wCD11R3

Human and pig whole blood was preincubated with OmCI or equimolar amounts of albumin and then incubated with 10^8 *E. coli*/ml for 10 minutes at 37 °C. Expression of human CD11b and pig wCD11R3 were measured using flow cytometry. Median fluorescence intensity (MFI) is expressed as mean \pm SD ($n = 3$ for humans and $n = 4$ for pigs). To the right, histograms and contour plots of the effect of 1.28 μ M OmCI for pig and human.

Porcine

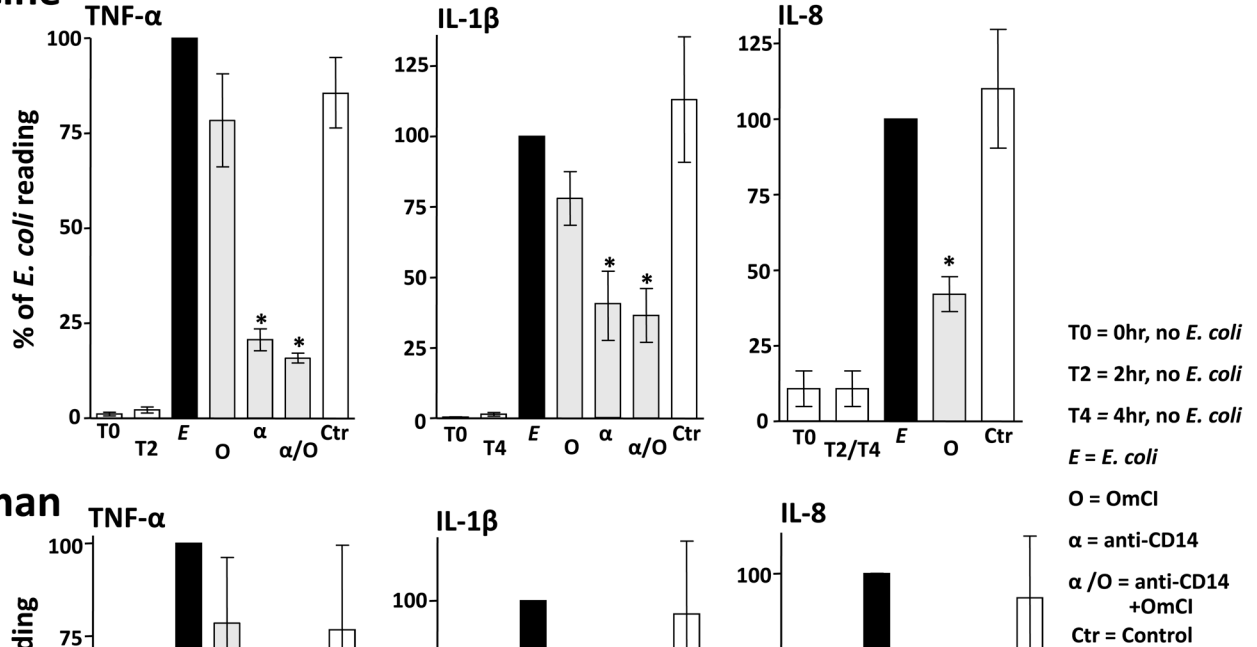


Figure 4. Effect of inhibiting complement, CD14, and a combination thereof, on *E. coli*-induced cytokine release

Upper panels: Pig whole blood was preincubated with 0.64 μ M OmCI (O), 25 μ g/ml anti-CD14 (whole IgG) (α), and the combination of both and then incubated with 10^6 *E. coli*/ml for 2 h (TNF- α and IL-8) and/or 10^5 bacteria/ml for 4 h (IL-1 β and IL-8). The effect of anti-CD14 on pig IL-8 could not be measured because of assay interference. Albumin and an isotype-matched control Ab (IgG2b) were used as controls (Ctr) when analyzing TNF- α and IL-1 β , whereas albumin only was used as control when analyzing IL-8. For IL-8, 2 and 4 hour sample data were pooled. Data are normalized to the *E. coli* group which is defined as 100%. Data are presented as mean \pm SEM ($n = 3, 4$ and 5 for TNF- α , IL-1 β and IL-8, respectively).

Lower panels: Human whole blood was preincubated with 0.64 μ M OmCI, 10 μ g/mL anti-CD14 (F(ab')₂), combinations thereof, and controls for 5 minutes and then incubated for 2 h with 10^6 *E. coli*/ml at 37 $^{\circ}$ C. As control (Ctr) albumin was used in combination with a control F(ab')₂, both in equimolar amounts to OmCI and anti-CD14, respectively. Data are normalized to the *E. coli* group which is defined as 100% and presented as mean \pm SEM ($n=3$). Statistical comparisons were performed between the effect of OmCI, anti-CD14 and the combinations of both versus no inhibition, * $p < 0.05$.

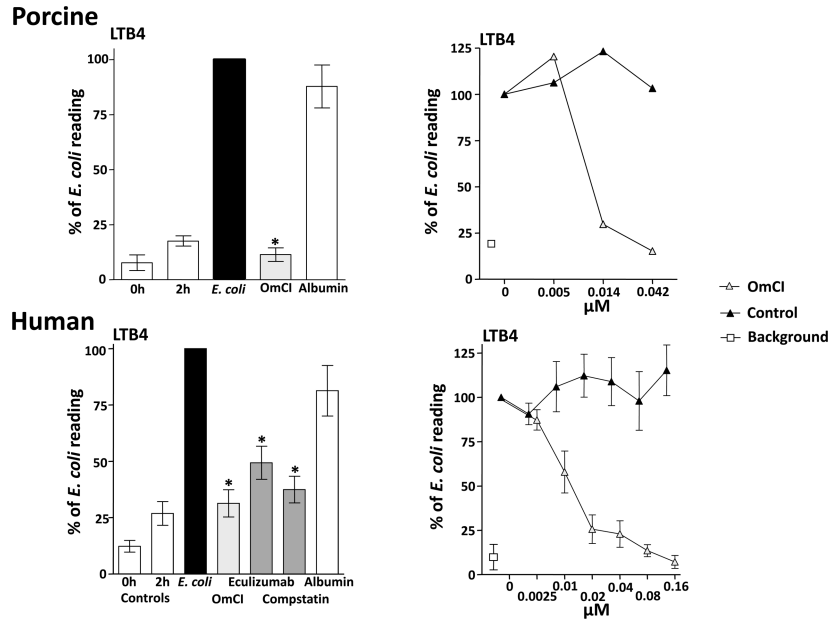


Figure 5. Complement-dependent LTB4 release and binding of LTB4 to OmCI
Left panels: Pig and human whole blood was preincubated with 0.64 μM OmCI or albumin and human whole blood was also preincubated with the human specific C inhibitors compstatin (25 μM) and eculizumab (0.67 μM), thereafter incubated with 10^6 *E. coli*/ml for 120 minutes at 37 °C. Data are presented as mean \pm SEM ($n = 3$ for pig and $n = 7$ for human). Statistics were performed on the effect of the C inhibitors versus the effect of *E. coli* only, $*p < 0.05$.
Right panels: LTB4-enriched plasma was generated by incubating pig and human whole blood with 10^6 *E. coli*/ml for 120 minutes at 37 °C. Thereafter OmCI was added to the activated plasma and incubated for 15 minutes at 37 °C. A control peptide or eculizumab were used as controls for pig and human respectively. Samples were then analysed for LTB4. Data are normalized to LTB4 in *E. coli* activated plasma (defined as 100%). Data are presented as mean \pm SEM for human ($n = 4$), and as mean for pig ($n = 2$).

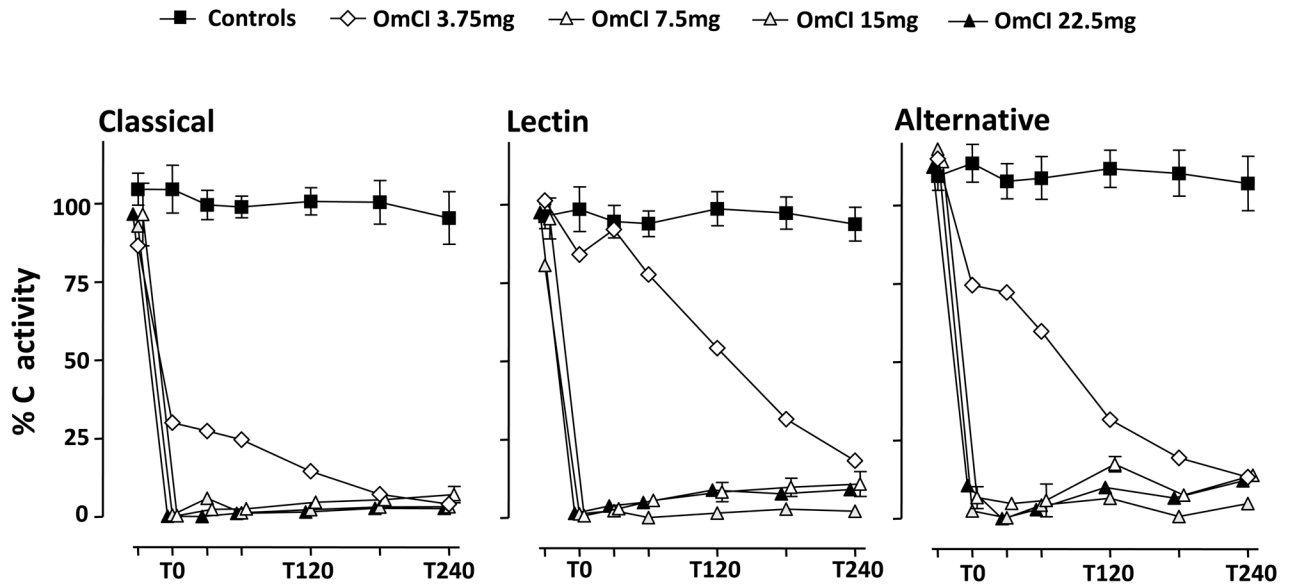


Figure 6. In vivo effects of OmCI in pigs

Different amounts of OmCI were tested in a pig model of *E. coli*-induced sepsis. Five 15 kg pigs received the following boluses of OmCI: 22.5 mg ($n = 1$), 15 mg ($n = 1$), 7.5 mg ($n = 2$) or 3.75 mg ($n = 1$) prior to the induction of sepsis, and thereafter a continuous infusion of 0.5 mg OmCI/h. Two animals served as positive controls, receiving *E. coli* and saline (9 mg/ml). Blood samples were drawn at baseline (Tbasis), and then at 0 (T0), 30, 60, 120, 180 and 240 minutes. The effect of OmCI on the functional activity of the three C pathways is shown using the Wielisa assay. The data are expressed in % of a standard defined as 100% activity. Data points are given as single values or as mean with range for 7.5 mg dose and the *E. coli* positive control.