

# Differential Effect of Inhibiting MD-2 and CD14 on LPS- Versus Whole *E. coli* Bacteria-Induced Cytokine Responses in Human Blood

D. Christiansen, O. L. Brekke, J. Stenvik, J. D. Lambris, T. Espevik and T. E. Mollnes

**Abstract** *Background:* Sepsis is a major world-wide medical problem with high morbidity and mortality. Gram-negative bacteria are among the most important pathogens of sepsis and their LPS content is regarded to be important for the systemic inflammatory reaction. The CD14/myeloid differentiation factor 2 (MD-2)/TLR4 complex plays a major role in the immune response to LPS. The aim of this study was to compare the effects of inhibiting MD-2 and CD14 on ultra-pure LPS-versus whole *E. coli* bacteria-induced responses. *Methods:* Fresh human whole blood was incubated with upLPS or whole *E. coli* bacteria in the presence of MD-2 or CD14 neutralizing monoclonal antibodies, or their respective controls, and/or the specific complement-inhibitor compstatin. Cytokines were measured by a multiplex (n=27) assay. NFκB activity was examined in cells transfected with CD14, MD-2 and/or Toll-like receptors. *Results:* LPS-induced cytokine response was efficiently and equally abolished by MD-2 and CD14 neutralization. In contrast, the response induced by whole *E. coli* bacteria was only modestly reduced by MD-2 neutralization, whereas CD14 neutralization was more efficient. Combination with compstatin enhanced the effect of MD-2 neutralization slightly. When compstatin was combined with CD14 neutralization, however, the response was virtually abolished for all cytokines, including IL-17, which was only inhibited by this combination. The MD-2-independent effect observed for CD14 could not be explained by TLR2 signaling. *Conclusion:* Inhibition of CD14 is more efficient than inhibition of MD-2 on whole *E. coli*-induced cytokine response, suggesting CD14 to be a better target for intervention in Gram-negative sepsis, in particular when combined with complement inhibition.

**Keywords** CD14 • MD-2 • Complement • Cytokines • LPS • TLR • *E. coli* • Sepsis • Inflammation

---

T. E. Mollnes (✉)  
Research Laboratory, Somatic Research Centre, Nordland Hospital,  
8092 Bodø, Norway  
e-mail: t.e.mollnes@medisin.uio.no

## 1 Introduction

Sepsis is a severe condition world-wide with high morbidity and if progressing to septic shock also with considerable mortality (Annane et al. 2005). Gram-negative bacteria account for approximately one-third of all cases with sepsis, *Escherichia coli* being the single most important pathogen (Martin et al. 2003). Thus, revealing the inflammatory mechanisms and development of new treatment strategies of Gram-negative sepsis is an important scientific and medical task.

The outer membrane of Gram-negative bacteria contains lipopolysaccharide (LPS) specific to each strain giving different reactions in various species. LPS-binding protein (LBP) enhances the binding of LPS to CD14, which exist in a soluble form (sCD14) and as a glycosyl-phosphatidylinositol-anchored membrane protein without transmembrane signaling properties. CD14 presents LPS to a complex of Toll-like Receptor 4 (TLR4) and myeloid differentiation factor 2 (MD-2) and further signaling is mediated via MyD88 and/or TRIF/TRAM giving cytokine production. In addition to being important for LPS recognition by TLR4, CD14 has also been shown to be implicated in TLR2 and TLR3 signaling (Miyake 2006; Akashi-Takamura and Miyake 2008; Nilsen et al. 2008; Janot et al. 2008). Recently it was even shown that CD14 is a co-receptor for TLR7 and TLR9 and is partly responsible for their inflammatory responses (Baumann et al. 2010). This is in accordance with the assumption that CD14 is a promiscuous upstream recognition molecule, reacting with a number of ligands with low affinity, transferring the ligand to receptors with higher degree of specificity and affinity (Antal-Szalmás 2000).

MD-2 was first described by Shimazu et al. (1999), documenting the need for assembling of MD-2 and TLR4 for cells to be LPS sensitive. Furthermore, translocation of TLR4 to the cell membrane was reported to depend on glycosylation, for which MD-2 was required (Ohnishi et al. 2001). MD-2 is found both as a membrane bound and soluble protein. In contrast to CD14, MD-2 is selectively associated with TLR4 and, in contrast to CD14, does not transmit signals from danger associated molecular patterns (Chun and Seong 2010).

MD-2 and TLR4 has over the past years become key target molecules for inhibiting innate immune responses induced both by endogenous and exogenous danger signals, including LPS and other potential ligands acting in Gram-negative sepsis. Notably, although LPS has been used in innumerable experimental studies, LPS is a surrogate marker of Gram-negative bacteria, which do not sufficiently reflect whole bacteria and only partly mimics sepsis in animal models. We have previously shown, using an *ex vivo* human whole blood model particularly designed for inflammatory crosstalk (Mollnes et al. 2002), that combined inhibition of CD14 and complement efficiently attenuates Gram-negative bacteria-induced inflammatory reaction (Brekke et al. 2007, 2008; Lappégard et al. 2009).

Thus, the aim of the present study was to compare the effects of inhibiting CD14 and MD-2 in the human whole blood model challenged with phenol-extracted ultra-

pure LPS (upLPS), highly specific for TLR4, or whole *E. coli* bacteria potentially recognized by numerous pattern recognition molecules. Since whole *E. coli* bacteria activates complement potently, in contrast to upLPS (Brekke et al. 2007), we included the specific complement inhibitor compstatin in whole bacteria experiments to reveal the relative role of complement, compared to CD14 and MD-2, in the cytokine response.

## 2 Materials and Methods

### 2.1 Bacterial Preparation

Non-opsonized smooth *E. coli*, strain LE392 (ATCC 33572), was obtained from American Type Culture Collection (Manassas, VA, USA). The bacteria was grown, heat-inactivated and washed nine times in PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Sigma, St. Louis, MO, USA) to remove extracellular LPS as previously described. A stock solution of  $1 \times 10^9$  bacteria/mL PBS was stored at  $+4^\circ\text{C}$ . The final concentration of *E. coli* used in the experiments was  $3 \times 10^6$ /mL.

### 2.2 Inhibitors

The anti-MD-2 monoclonal antibody (mAb) (clone 5D7) was a kind gift from Dr. Greg Elson, NovImmune SA, Geneva, Switzerland, while the corresponding IgG2b isotype control (clone TEN/0) was purchased from Dako (Glostrup, Denmark) and dialyzed to remove azide. The anti-CD14  $\text{F(ab')}_2$  mAb (clone 18D11) and its corresponding control (clone BH1) was obtained from Diatec (Oslo, Norway). The compstatin analog Ac-I[CV(1MeW)QDWGAHRC]T, which binds to and inhibits cleavage of C3 was produced as previously described (Katragadda et al. 2006). The inactive peptide Ac-IAVVQDWGHHRAT was used as control. Compstatin and anti-CD14 have previously been titrated and found to have maximum effects at 25  $\mu\text{M}$  and 10  $\mu\text{g}/\text{mL}$  (not published), respectively. Anti-MD-2 mAb was titrated five-fold from 25  $\mu\text{g}/\text{mL}$  in initial experiments in the present study to obtain a working dose for the further experiments.

### 2.3 Whole Blood Model of Sepsis

The study was approved by the regional ethics committee. Human whole blood was drawn from healthy individuals after they gave their written consent. The whole blood model of sepsis has been described in detail previously (Mollnes et al. 2002).

Briefly, blood was drawn into Nunc tubes (Roskilde, Denmark) containing lepirudin (Refludan<sup>®</sup>, Pharmion, Copenhagen, Denmark) to a final concentration of 50 µg/mL. Inhibitors, controls or PBS with Ca<sup>2+</sup> and Mg<sup>2+</sup> were administered to Nunc tubes prior to blood sampling and immediately after the sample was obtained, blood was added to the respective tubes. Tubes were preincubated at 37°C for minimum four minutes and then further incubated with 3 × 10<sup>6</sup>/mL *E. coli* or 100 ng/mL phenol-extracted upLPS from *E. coli* (InvivoGen, San Diego, CA, USA). Samples were rotated in an incubator for two hours, then put on ice and ethylenediaminetetraacetic acid (EDTA) (Sigma) was added to a final concentration of 10 mM. Samples were centrifuged at 3220 × g, plasma was obtained and stored at -80°C until further analysis.

## 2.4 Cytokine and TCC Measurements

Cytokines in plasma was analyzed using a multiplex cytokine kit from BioRad (Hercules, CA, USA). The assay of 27 interleukins, chemokines and growth-factors, was performed according to instructions from the manufacturer and comprised the following analytes: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, eotaxin, basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF. Complement activation was measured as generation of the soluble terminal sC5b-9 complement complex (TCC) using an ELISA kit from Becton Dickinson (C5b-9 BD OptEIA<sup>™</sup>, San Diego, CA, USA). The assay was performed according to instructions from the manufacturer.

## 2.5 Transient Transfection and NF-κB Luciferase Assay

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) from Euroclone (Siziano, Italy), L-glutamine and 10-µg/mL ciprofloxacin (Bayer AG, Leverkusen, Germany) at 37°C and 8% CO<sub>2</sub>. Transient transfection was done using GeneJuice<sup>™</sup> transfection reagent (Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. In short, cells were plated at a cell density of 1 × 10<sup>4</sup> cells/well in 96-well dishes and grown to 50% confluency. Plasmids used were human CD14 in pcDNA3 kindly provided by Dr. D. Golenbock (University of Massachusetts Medical School), human MD-2 in pEFBOS kindly provided by Dr. K. Miyake (University of Tokyo), human TLR4 in pcDNA3 kindly provided by Dr. R. Medzhitov and Dr. C. Janeway (Yale University, New Haven, CT), and human TLR2 in pRK7 kindly provided by Dr. C. Kirschning (Technical University of Munich). As reporter we used the NF-κB dependent luciferase plasmid pELAM-luc (Chow et al. 1999). The pRL-TK vector

(Promega, Madison, WI, USA) encoding the sea pansy (*Renilla reniformis*) luciferase was co-transfected and served as endogenous control with a low constitutive expression. Each plasmid was transfected at a dosage of 25 ng/well and pcDNA3 (Invitrogen, Carlsbad, CA, USA) was used to adjust the total amount of plasmid to 100 ng/well. All plasmids were isolated using the EndoFree plasmid kit (Qiagen, Hilden, Germany). After incubation for 24 h, the cells were stimulated for 18 h. Cytoplasmic extracts were prepared and the Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega) was used to measure sequentially the firefly and sea pansy luciferase activities with the Victor<sup>3</sup><sup>™</sup> 1420 multilabel counter (Perkin Elmer, Waltham, Massachusetts, USA). Results from triplicate wells are given as fold induction relative to cells transfected with reporter and pcDNA3 plasmids only. Positive controls for TLR2 activation were the synthetic compounds fibroblast stimulating lipopeptide (FLS-1), Pam<sub>3</sub>Cys-Ser-Lys<sub>4</sub> (P3CYS), and macrophage-activating lipopeptide 2 kD (MALP-2) from EMC microcollections GmbH (Tuebingen, Germany), and standard *S. aureus* lipoteichoic acid (LTA) and upLPS 0111:B4 from Invivogen (San Diego, CA, USA).

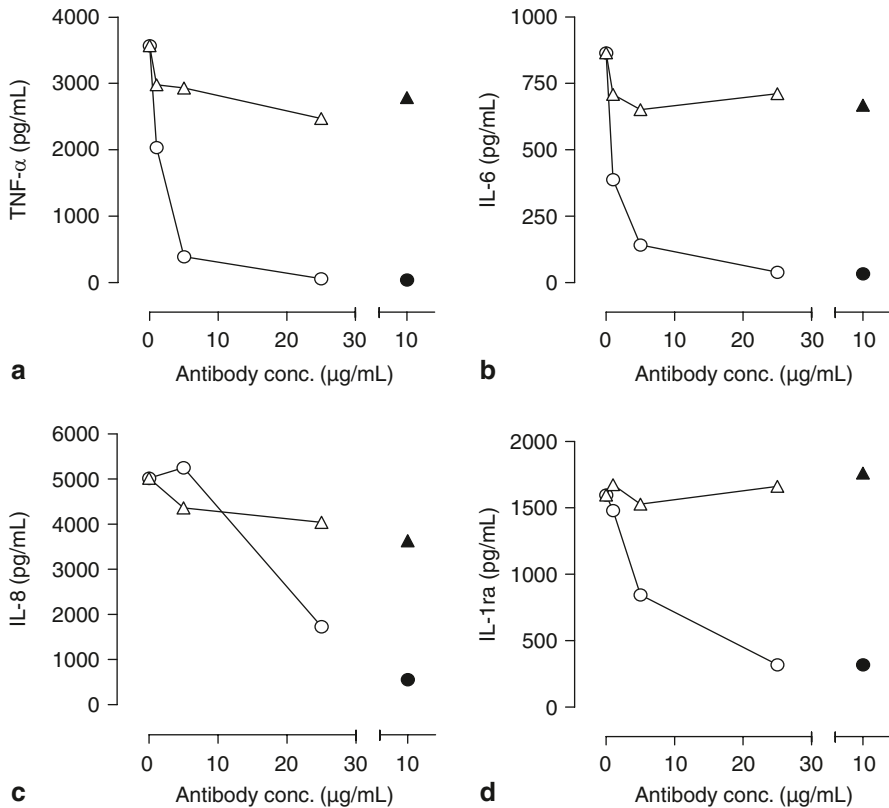
## 2.6 Statistics

Statistics were calculated with one-way ANOVA with Dunnett's multiple comparison post-test using GraphPad Prizm for Windows. P<0.05 was considered significant.

## 3 Results

### 3.1 Effect of MD-2- and CD14-Inhibition on *E. coli* UpLPS-Induced Cytokine Response in Whole Blood

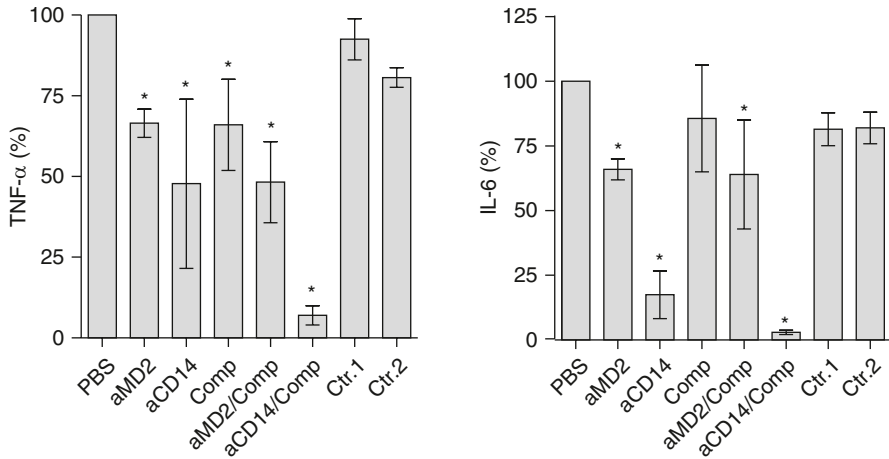
We first studied the effect of neutralizing MD-2 and CD14 on the cytokine response to phenol extracted highly purified LPS (upLPS). The anti-MD-2 mAb dose-dependently and efficiently inhibited a broad panel of the cytokines studied, including TNF- $\alpha$ , IL-6, IL-8 and IL-1ra (Fig. 1). Anti-MD-2 mAb at 25  $\mu$ g/mL abolished the effect of upLPS and was equal to the effect of the anti-CD14 F(ab')<sub>2</sub> mAb, suggesting that the anti-MD-2 mAb and the anti-CD14 mAb had equal effects on upLPS-induced cytokine release. The release of IL-8 (Fig. 1, panel C) was, however, not completely inhibited by the anti-MD-2 mAb, in contrast to the effect observed with the anti-CD14 F(ab')<sub>2</sub> mAb. This finding is in accordance with the fact that anti-MD-2 mAb is whole IgG and activated complement in the system (see below), an event that is known to stimulate IL-8 production.



**Fig. 1** Effect of MD-2- and CD14-inhibition on cytokine response to *E. coli* upLPS. To compare the effects of MD-2- and CD14-inhibition we titrated anti-MD-2 mAb and the isotype matched (IgG2b) control antibody five-fold from 25 µg/mL to 1 µg/mL, while anti-CD14 F(ab')<sub>2</sub> and control F(ab')<sub>2</sub> antibody were used at a fixed dose (10 µg/mL), known from previous studies to be inhibiting in this model. Whole blood was challenged with 100 ng/mL *E. coli* upLPS for 2 hours at 37°C. Open circles; anti-MD-2 mAb, open triangles; IgG2b-control mAb, closed circles; anti-CD14 F(ab')<sub>2</sub>, closed triangles; F(ab')<sub>2</sub> control antibody. Panel A; TNF-α, panel B; IL-6, panel C; IL-8, panel D; IL-1ra. The figure displays one representative out of three experiments

### 3.2 Effect of MD-2-, CD14- and Complement-Inhibition on Whole *E. coli* Bacteria-Induced Cytokine Response in Whole Blood

We then studied the effect of neutralizing MD-2 and CD14 mAbs on whole *E. coli* bacteria-induced cytokine response, since whole bacteria are more clinically relevant than LPS and they are known to activate complement more efficiently compared to soluble LPS. Thus, we also included the complement inhibitor compstatin in these experiments.

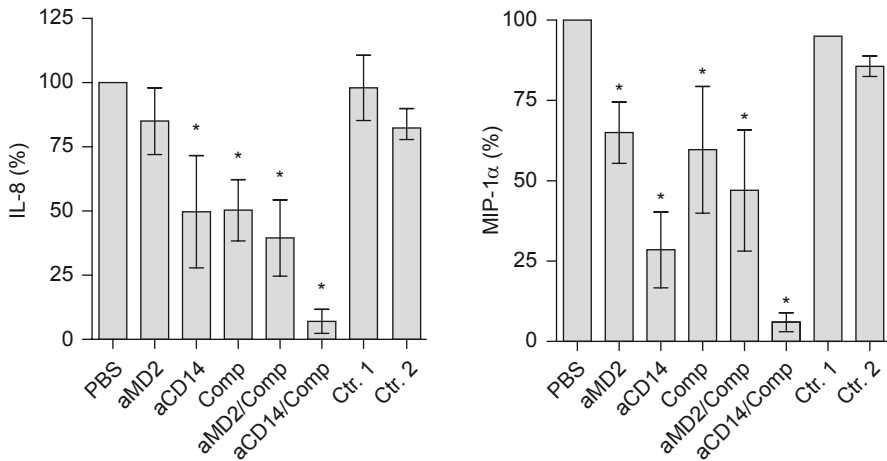


**Fig. 2 Effect of MD-2-, CD14- and complement-inhibition on proinflammatory cytokine response to whole *E. coli* bacteria.** Anti-MD-2 mAb (aMD-2) in the highest concentration used in the initial titration experiments (25 µg/mL) was compared to anti-CD14 F(ab')<sub>2</sub> (aCD14) (10 µg/mL), the complement C3 inhibitor compstatin (Comp) (25 µM), and combinations thereof. The first control (Ctr. 1) is the isotype matched control to anti-MD-2 mAb (IgG2b) in combination with the control peptide to compstatin. The second control (Ctr. 2) is the control antibody to anti-CD14 F(ab')<sub>2</sub> in combination with the control peptide to compstatin. Whole blood was challenged with 3 × 10<sup>6</sup> *E. coli*/mL for 2 hours at 37°C. Left panel; TNF-α, right panel; IL-6. Data (mean ± SD) are from experiments (n=3–4) performed on separate days (n=2 for Ctr. 1) and are given as % of the positive control (PBS), due to inter-individual variations. \* = p < 0.01 compared to the positive control

The *E. coli*-induced increase in the proinflammatory cytokines TNF-α and IL-6 was partially and significantly (p < 0.01) inhibited by anti-MD-2 mAb and anti-CD14 F(ab')<sub>2</sub>, anti-CD14 F(ab')<sub>2</sub> being the most efficient (Fig. 2). TNF-α, but not IL-6, was significantly (p < 0.01), though modestly, inhibited by compstatin alone. The combination of anti-CD14 F(ab')<sub>2</sub> and compstatin inhibited the TNF-α release by 93% and IL-6 by 97% (p < 0.01 for both). In comparison the combination of anti-MD-2 mAb and compstatin inhibited the formation of each cytokine by 52% and 36%, respectively (p < 0.01 for both). The less efficient effect of neutralizing MD-2 compared with CD14 in the combination with compstatin could not be explained by the complement-activation potential of the anti-MD-2 mAb since the presence of compstatin completely inhibited complement activation (see below).

### 3.3 Effect of MD-2-, CD14- and Complement-Inhibition on Whole *E. coli*-Induced Chemokine Response in Whole Blood

The chemokines IL-8 and MIP-1α were partially and significantly (p < 0.01) inhibited by anti-CD14 F(ab')<sub>2</sub> (Fig. 3). The effect of anti-MD-2 mAb was less pronounced

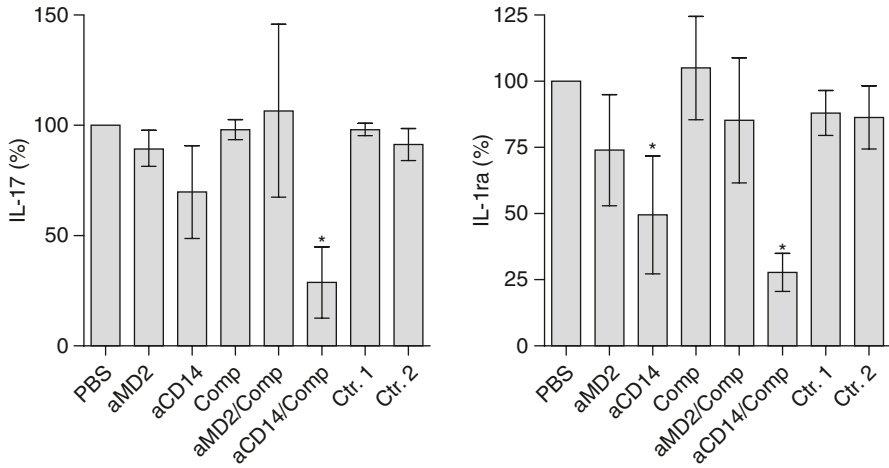


**Fig. 3** Effect of MD-2-, CD14- and complement-inhibition on chemokine response to whole *E. coli* bacteria. Anti-MD-2 mAb (aMD-2), anti-CD14 F(ab')<sub>2</sub> (aCD14), compstatin (Comp), combinations thereof, and controls, were used exactly as described in legend to Fig. 2. Whole blood was challenged with  $3 \times 10^6$  *E. coli*/mL for 2 hours at 37°C. Left panel; IL-8, right panel; MIP-1 $\alpha$ . Data (means  $\pm$ SD) are presented as described in the legend to Fig. 2. \*= $p < 0.01$  compared to the positive control

and significant ( $p < 0.01$ ) only for MIP-1 $\alpha$  formation. Compstatin alone significantly ( $p < 0.01$ ) inhibited both chemokines, most pronounced for IL-8, which was inhibited to the same degree by anti-CD14 F(ab')<sub>2</sub> and compstatin (50% for each of them). The combination with compstatin markedly enhanced the effect of both mAbs. IL-8 was reduced 93% by compstatin and anti-CD14 F(ab')<sub>2</sub> and 60% by compstatin and anti-MD-2 mAb, whereas the corresponding reduction in MIP-1 $\alpha$  was 95% and 53%, respectively ( $p < 0.01$  for all).

### 3.4 Effect of MD-2-, CD14- and Complement-Inhibition on Whole *E. coli*-Induced IL-17 and IL-1ra Response in Whole Blood

The proinflammatory cytokine IL-17 and the anti-inflammatory mediator IL-1ra were then investigated (Fig. 4). IL-17 was significantly (71%,  $p < 0.01$ ) inhibited only by the combination of compstatin and anti-CD14 F(ab')<sub>2</sub>. IL-1ra was inhibited by both antibodies, but significantly ( $p < 0.01$ ) only for anti-CD14 F(ab')<sub>2</sub>. Compstatin alone did not inhibit IL-1ra, but enhanced the effect of anti-CD14 F(ab')<sub>2</sub> when these were combined (72%,  $p < 0.01$ ).



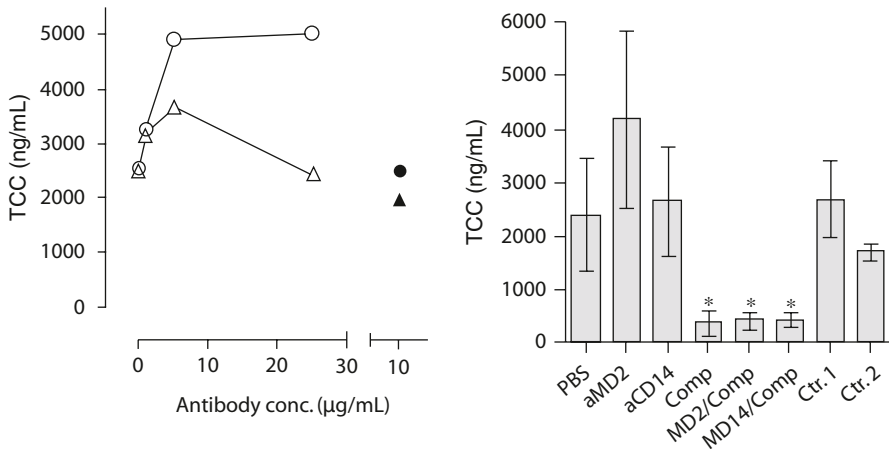
**Fig. 4 Effect of MD-2-, CD14- and complement-inhibition on IL-17 and IL-1ra response to whole *E. coli* bacteria.** Anti-MD-2 mAb (aMD-2), anti-CD14 F(ab')<sub>2</sub> (aCD14), compstatin (Comp), combinations thereof, and controls, were used exactly as described in the legend to Fig. 2. Whole blood was challenged with 3 × 10<sup>6</sup> *E. coli*/mL for 2 hours at 37°C. *Left panel*; IL-17, *right panel*; IL-1ra. Data (means ± SD) are presented as described in Fig. 2. \* = p < 0.01 compared to the positive control

### 3.5 Effect of MD-2-, CD14- and Complement-Inhibition on *E. coli* UpLPS and Whole Bacteria Induced Complement Activation in Whole Blood

Complement activation was measured as formation of TCC. In the experiments described above using upLPS (presented in Fig. 1.), there was a dose-dependent increase in TCC with the anti-MD-2 mAb which was not seen for the anti-CD14 F(ab')<sub>2</sub> (Fig. 5, left panel). In the experiments described above using whole *E. coli* bacteria (presented in Figs. 2, 3, 4), compstatin abolished TCC formation, either alone or in combination with the two antibodies (Fig. 5, right panel), confirming that compstatin completely inhibited complement activation in these experiments. TCC formation was not influenced by anti-CD14 F(ab')<sub>2</sub>, but a modest increase in TCC was observed when anti-MD-2 mAb alone was used.

### 3.6 Effect of Whole *E. coli* Bacteria and TLR Ligands on NFκB Activity

HEK-cells transfected with TLR2 or TLR4/MD-2 in combination with or without CD14 were used to investigate the effect of whole *E. coli* bacteria and TLR4 and

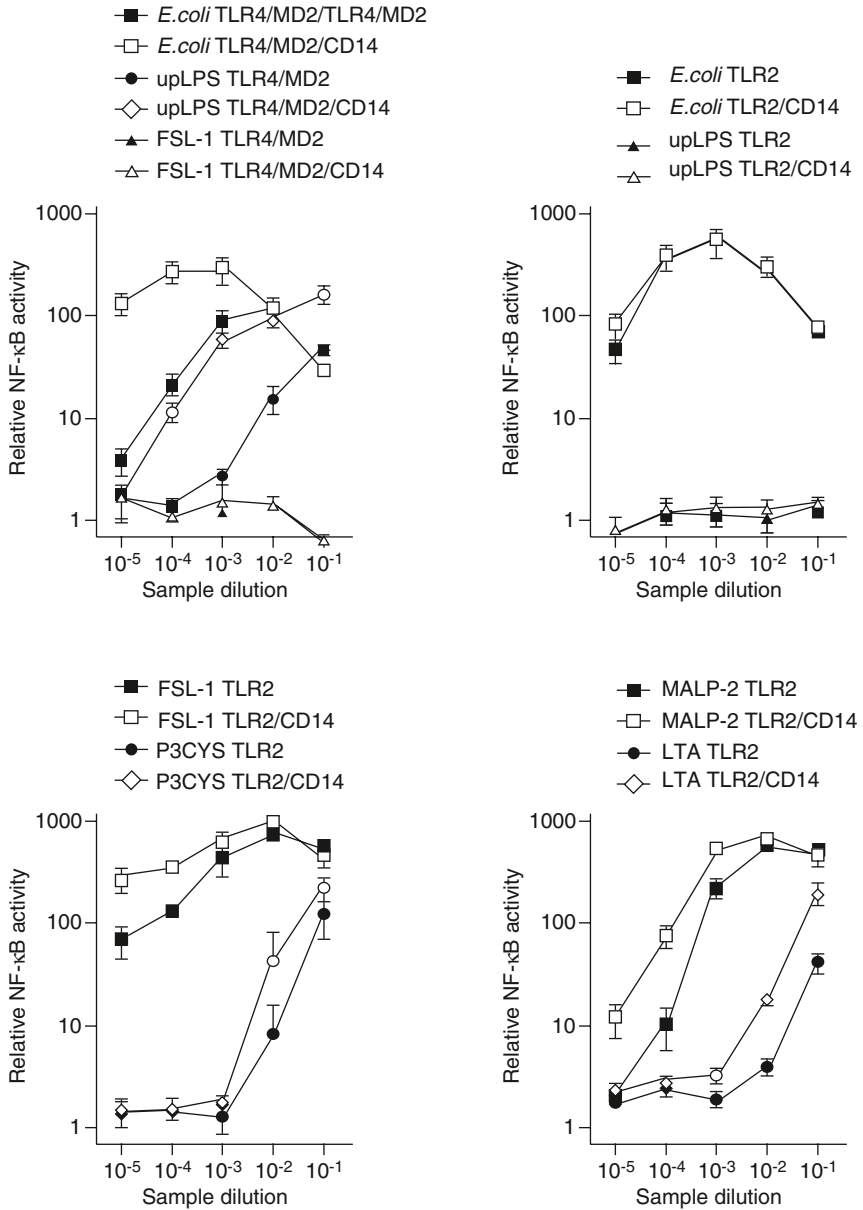


**Fig. 5 Effect of MD-2-, CD14- and complement-inhibition on upLPS and *E. coli*-induced complement activation induced by upLPS and whole *E. coli* bacteria.** Complement activation was measured as formation of the terminal complement complex (TCC). *Left panel:* TCC formation was examined in the experiment described in Fig. 1 (upLPS). Anti-MD-2 mAb (*open circles*) and the isotype matched (IgG2b) control mAb (*open triangles*) were titrated from 25 µg/mL to 1-µg/mL while anti-CD14 F(ab')<sub>2</sub> (*closed circle*) and control F(ab')<sub>2</sub> antibody (*closed triangle*) was used at a fixed dose (10 µg/mL). *Right panel:* TCC formation was examined in the experiment described in Fig. 2. Anti-MD-2 mAb (aMD-2), anti-CD14 F(ab')<sub>2</sub> (aCD14), compstatin (Comp), combinations thereof, and controls, were as described in the legend to Fig. 2. Data (means ±SD) are presented as ng/mL. Statistics was calculated between the positive control and the groups containing the complement inhibitor. \* =  $p < 0.01$  compared to the positive control

TLR2 ligands on NFκB activity (Fig. 6). The TLR4 effect of upLPS was largely dependent on CD14 (Fig. 6, upper left). Whole *E. coli* bacteria induced a significant response in the absence of CD14, compared with upLPS, but the signal was markedly enhanced in the presence of CD14. The TLR4 effect of FLS-1 and TLR2 effect of upLPS was nil, both in the presence and absence of CD14 (Fig. 6, upper left and right). CD14 did not influence the TLR2 response to whole *E. coli* bacteria (Fig. 6, upper right). In contrast, the TLR2/1 ligand P3CYS and the TLR2/6 ligands (FSL-1, MALP-2, and LTA) were enhanced by CD14 (Fig. 6, lower panels), although not as much as the TLR4-response toward *E. coli* and upLPS.

## 4 Discussion

This study is the first to show the different effect of specific inhibition of two key TLR-related molecules, CD14 and MD-2, on upLPS- versus whole *E. coli* bacteria-induced inflammatory response. The experiments were performed in an established ex vivo human model designed for inflammatory crosstalk (Mollnes et al. 2002) and are thus regarded to be relevant for human sepsis. The data emphasize that



**Fig. 6** Effect of CD14 on TLR4 and TLR2 mediated NF-κB activation by whole *E. coli* bacteria, upLPS and TLR2 ligands (MALP2, FSL-1, P3CYS). Relative induction (means ± SD) of NF-κB in HEK293 cells transiently transfected with NF-κB luciferase and TLR4/MD-2 alone or in combination with CD14 (*upper left panel*). NFκB induction through TLR2 with or without CD14 (*upper right and bottom panels*). The highest sample concentration during treatment ( $10^{-1}$  dilution) was  $1 \times 10^8$  bacteria/mL, 5 μg/mL LTA and 1 μg/mL of the other ligands

the multifunctional CD14 molecule, acting very upstream by being co-receptors for several TLRs, is a more promising target to attenuate whole Gram-negative bacteria-induced inflammation, than the more specific LPS-binding molecule MD-2 (Antal-Szalmas 2000).

LPS from Gram-negative bacteria is regarded as the main inducer of the host inflammatory response to these bacteria. It should be noted, however, that although LPS is definitely of importance, the bacteria contain a number of other molecules recognized by the human innate immune system. Notably, CD14 is a promiscuous recognition molecule reacting with numerous ligands of both Gram-negative and Gram-positive bacteria, as well as fungi (Pugin et al. 1994) and dozens of specific ligands have been identified (Antal-Szalmas 2000). This is in contrast to MD-2, which seems to be specific for LPS in the interaction with TLR4. Our data are in accordance with this concept. Inhibition of MD-2 efficiently abolished upLPS-induced cytokine release, whereas inhibition of CD14 proved much more efficient in inhibiting the whole bacteria response. Our findings support the importance of using whole bacteria instead of LPS in experimental models of Gram-negative sepsis, as detailed in a recent experimental study in pigs (Thorgersen et al. 2009). The assumption of using whole bacteria instead of LPS in experimental models is further supported by the fact that neutralizing LPS has not proven effective in treatment of sepsis (Nahra and Dellinger 2008).

CD14 is known to play a role not only in TLR4, but also TLR2 and TLR3 signaling (Miyake 2006; Akashi-Takamura and Miyake 2008; Nilsen et al. 2008; Janot et al. 2008). We therefore investigated whether the MD-2-independent CD14 effect could be explained by TLR2/CD14. Our data using transfected HEK cells do not support this assumption, as *E. coli* activated TLR2 in a CD14 independent fashion. Still, CD14 increased signaling of TLR2 in response to low levels of TLR2/1 and TLR2/6 ligands, as has also been shown by others (Nakata et al. 2006; Erridge et al. 2008). The reason for this difference between whole *E. coli* and soluble TLR2 ligands is not known. It is likely that the TLR2 ligands in *E. coli* are immobilized and activate TLR2 by aggregating the receptor and that this activation process is unaffected by CD14. In any case, our data, consistently observed with all inflammatory readouts, collectively point to CD14 as a key target molecule for inhibition of whole *E. coli* bacteria-induced inflammation.

Complement activation can take place in blood when antibodies bind to their epitope. The anti-MD-2 antibody is IgG2b with an intact Fc-region. In contrast, the anti-CD14 antibody used was a well established F(ab')<sub>2</sub>. As documented in the present study, anti-MD-2 activated the complement system, revealed by increase in TCC. Thus, the results obtained with anti-MD-2 alone should be interpreted with caution. In our experiments, however, we always included the complement inhibitor compstatin in parallel, both in order to neutralize the complement activation potential of the antibody, and to reveal the effect of complement activation by the bacteria. The results unequivocally documented the effect of the anti-MD-2 antibody *per se*, that is, in the presence of a complement inhibitor. Unfortunately we did not have access to large scale anti-MD-2 mAb to produce F(ab')<sub>2</sub> fragments. By using the complement inhibitor, however, we circumvented this problem. Our data highlight

the importance of using inhibitory agents that do not activate complement, unless a combined treatment with a complement inhibitor can be used.

The present study included a number of inflammatory read-outs including the biomarkers in the 27-plex cytokine assay. Principally, all these mediators responded similarly to the combined inhibition of CD14 and complement. Of particular interest is IL-17, which recently has been reported to play a critical role in protection against polymicrobial sepsis (Freitas et al. 2009). In contrast, Flierl et al. (2008) reported increased survival when blocking IL-17A, reported IL-17A to be responsible for the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in a cecal ligation and puncture model and, thus, postulated IL-17 to be a novel target for treatment of sepsis. Recently it was shown that C5a was responsible for the increase in IL-17 in this model (Xu et al. 2010). In our study we showed that combination of anti-CD14 and compstatin effectively reduces the formation of IL-17, consistent with a previous observation in our laboratory (Brekke et al. 2008). In the present study we show that inhibition of MD-2 had no effect on *E. coli*-induced IL-17. We therefore suggest that the combined upstream treatment regimen targeted at CD14 and complement attenuates a number of mediators potentially contributing to *E. coli*-induced systemic inflammatory response, including IL-17 release. Although in our model we found inhibition of CD14 to be generally more efficient than inhibition of MD-2 with respect to the cytokine response, others have documented an MD-2-dependent, CD14-independent, LPS-mediated influx of neutrophils into the lung alveoli during experimental pneumonia in mice (Cai et al. 2008). Thus, it cannot be excluded that MD-2 may be a target for inhibition also in humans under conditions where signaling is CD14-independent and specific targeting of MD-2 is desired.

The effect of blocking CD14 and complement in the case of Gram-positive bacteria or other Gram-negative bacteria including rough strain, still needs to be investigated. Due to the diversity of potential invaders, a therapeutic for sepsis may well consist of a cocktail of inhibitors targeting essential upstream recognition molecules. In our opinion, targeting downstream single mediators of inflammation is not a rational strategy for treatment of sepsis. Accordingly, it was recently shown in a phase II clinical trial that treatment with eritoran, an LPS antagonist interacting with the CD14/MD-2/TLR4 might improve the clinical condition in sepsis (Tidswell et al. 2010), in contrast to the disappointing results observed in a number of clinical studies targeting single cytokines like TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Remick 2007). We recently documented a profound effect on *E. coli*-induced inflammation in a pig model of sepsis by blocking CD14 (Thorgersen et al. 2009). Furthermore, we have recently shown in a baboon model of sepsis that complement-inhibition substantially improved the disturbed pathophysiology, including the fall in systemic blood pressure (Silasi-Mansat et al. 2010). It remains to be shown whether combination of CD14- and complement-inhibition will improve the outcome in experimental and clinical sepsis studies.

In conclusion, inhibition of CD14 and MD-2 was equally effective in attenuating upLPS-induced cytokine response, whereas inhibition of CD14 was substantially more effective in attenuating the inflammatory response to whole *E. coli* bacteria. This effect was further markedly enhanced by combining CD14- and complement-

inhibition, which virtually abolished the whole cytokine response. The marked effect of inhibiting CD14 is consistent with its broad pattern recognition of a number of danger ligands, and its position as co-receptor for numerous TRLs as well.

**Acknowledgments** The authors thank Judith Krey Ludviksen and Anne Pharo for the excellent performing of cytokine and TCC measurements. The authors acknowledge support by **The Odd Fellow Foundation (TEM)** and US Public Health Service Grants GM062134, AI030040, AI072106, AI068730 (JDL).

## References

- Akashi-Takamura,S., Miyake,K. (2008). TLR accessory molecules. *Curr. Opin. Immunol.* 20, 420-425.
- Annane,D., Bellissant,E., Cavailon,J.M. (2005). Septic shock. *Lancet* 365, 63-78.
- Antal-Szalmas,P. (2000). Evaluation of CD14 in host defence. *Eur. J Clin. Invest* 30, 167-179.
- Baumann,C.L., Aspalter,I.M., Sharif,O., Pichlmair,A., Bluml,S., Grebien,F., Bruckner,M., Pasierbek,P., Aumayr,K., Planyavsky,M., Bennett,K.L., Colinge,J., Knapp,S., Superti-Furga,G. (2010). CD14 is a coreceptor of Toll-like receptors 7 and 9. *J Exp. Med.* 207, 2689-2701.
- Brekke,O.L., Christiansen,D., Fure,H., Fung,M., Mollnes,T.E. (2007). The role of complement C3 opsonization, C5a receptor, and CD14 in E. coli-induced up-regulation of granulocyte and monocyte CD11b/CD18 (CR3), phagocytosis, and oxidative burst in human whole blood. *J Leukoc. Biol.* 81, 1404-1413.
- Brekke,O.L., Christiansen,D., Fure,H., Pharo,A., Fung,M., Riesenfeld,J., Mollnes,T.E. (2008). Combined inhibition of complement and CD14 abolish E. coli-induced cytokine-, chemokine- and growth factor-synthesis in human whole blood. *Mol. Immunol* 45, 3804-3813.
- Cai,S., Zemans,R.L., Young,S.K., Worthen,G.S., Jeyaseelan,S. (2008). MD-2-Dependent and -Independent Neutrophil Accumulation During Escherichia coli Pneumonia. *Am. J Respir. Cell Mol. Biol.* 40, 701-709.
- Chow,J.C., Young,D.W., Golenbock,D.T., Christ,W.J., Gusovsky,F. (1999). Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol. Chem.* 274, 10689-10692.
- Chun,K.H., Seong,S.Y. (2010). CD14 but not MD2 transmit signals from DAMP. *Int. Immunopharmacol.* 10, 98-106.
- Erridge,C., Kennedy,S., Spickett,C.M., Webb,D.J. (2008). Oxidised phospholipid inhibition of toll-like receptor (TLR) signalling is restricted to TLR2 and TLR4 - roles for CD14, LPS-binding protein and MD2 as targets for specificity of inhibition. *J Biol. Chem.* 283, 24748-24759.
- Flierl,M.A., Rittirsch,D., Gao,H., Hoesel,L.M., Nadeau,B.A., Day,D.E., Zetoune,F.S., Sarma,J.V., Huber-Lang,M.S., Ferrara,J.L., Ward,P.A. (2008). Adverse functions of IL-17A in experimental sepsis. *FASEB J* 22, 2198-2205.
- Freitas,A., ves-Filho,J.C., Victoni,T., Secher,T., Lemos,H.P., Sonogo,F., Cunha,F.Q., Ryffel,B. (2009). IL-17 receptor signaling is required to control polymicrobial sepsis. *J Immunol* 182, 7846-7854.
- Janot,L., Secher,T., Torres,D., Maillet,I., Pfeilschifter,J., Quesniaux,V.F., Landmann,R., Ryffel,B., Erard,F. (2008). CD14 Works with Toll-Like Receptor 2 to Contribute to Recognition and Control of Listeria monocytogenes Infection. *J Infect. Dis.* 198, 115-124.
- Katragadda,M., Magotti,P., Sfyroera,G., Lambris,J.D. (2006). Hydrophobic effect and hydrogen bonds account for the improved activity of a complement inhibitor, compstatin. *J Med Chem.* 49, 4616-4622.
- Lappegard,K.T., Christiansen,D., Pharo,A., Thorgersen,E.B., Hellerud,B.C., Lindstad,J., Nielsen,E.W., Bergseth,G., Fadnes,D., Abrahamsen,T.G., Høyby,E.A., Schejbel,L., Garred,P.,

- Harboe,M., Mollnes,T.E. (2009). Human genetic deficiencies reveal the roles of complement in the inflammatory network: lessons from nature. *Proc Natl Acad Sci U S A* 106, 15861-15866.
- Martin,G.S., Mannino,D.M., Eaton,S., Moss,M. (2003). The epidemiology of sepsis in the United States from 1979 through 2000. *N. Engl. J Med.* 348, 1546-1554.
- Miyake,K. (2006). Roles for accessory molecules in microbial recognition by Toll-like receptors. *J. Endotoxin. Res.* 12, 195-204.
- Mollnes,T.E., Brekke,O.L., Fung,M., Fure,H., Christiansen,D., Bergseth,G., Videm,V., Lappégard,K.T., Kohl,J., Lambris,J.D. (2002). Essential role of the C5a receptor in *E coli*-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* 100, 1869-1877.
- Nahra,R., Dellinger,R.P. (2008). Targeting the lipopolysaccharides: still a matter of debate? *Curr. Opin. Anaesthesiol.* 21, 98-104.
- Nakata,T., Yasuda,M., Fujita,M., Kataoka,H., Kiura,K., Sano,H., Shibata,K. (2006). CD14 directly binds to triacylated lipopeptides and facilitates recognition of the lipopeptides by the receptor complex of Toll-like receptors 2 and 1 without binding to the complex. *Cell Microbiol.* 8, 1899-1909.
- Nilsen,N.J., Deininger,S., Nonstad,U., Skjeldal,F., Husebye,H., Rodionov,D., von,A.S., Hartung,T., Lien,E., Bakke,O., Espevik,T. (2008). Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling; role of CD14 and CD36. *J Leukoc. Biol.* 84, 280-291.
- Ohnishi,T., Muroi,M., Tanamoto,K. (2001). N-linked glycosylations at Asn(26) and Asn(114) of human MD-2 are required for toll-like receptor 4-mediated activation of NF-kappaB by lipopolysaccharide. *J Immunol* 167, 3354-3359.
- Pugin,J., Heumann,I.D., Tomasz,A., Kravchenko,V.V., Akamatsu,Y., Nishijima,M., Glauser,M.P., Tobias,P.S., Ulevitch,R.J. (1994). CD14 is a pattern recognition receptor. *Immunity.* 1, 509-516.
- Remick,D.G. (2007). Pathophysiology of sepsis. *Am. J Pathol.* 170, 1435-1444.
- Shimazu,R., Akashi,S., Ogata,H., Nagai,Y., Fukudome,K., Miyake,K., Kimoto,M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp. Med* 189, 1777-1782.
- Silasi-Mansat,R., Zhu,H., Popescu,N.I., Peer,G., Sfyroera,G., Magotti,P., Ivanciu,L., Lupu,C., Mollnes,T.E., Taylor,F.B., Kinasewitz,G., Lambris,J.D., Lupu,F. (2010). Complement inhibition decreases the procoagulant response and confers organ protection in a baboon model of *E. coli* sepsis. *Blood* 116, 1002-1010.
- Thorgersen,E.B., Hellerud,B.C., Nielsen,E.W., Barratt-Due,A., Fure,H., Lindstad,J.K., Pharo,A., Fosse,E., Tonnessen,T.I., Johansen,H.T., Castellheim,A., Mollnes,T.E. (2009). CD14 inhibition efficiently attenuates early inflammatory and hemostatic responses in *Escherichia coli* sepsis in pigs. *FASEB J* 24, 712-722.
- Tidswell,M., Tillis,W., Larosa,S.P., Lynn,M., Wittek,A.E., Kao,R., Wheeler,J., Gogate,J., Opal,S.M. (2010). Phase 2 trial of eritoran tetrasodium (E5564), a Toll-like receptor 4 antagonist, in patients with severe sepsis. *Crit Care Med.* 38, 306-308.
- Xu,R., Wang,R., Han,G., Wang,J., Chen,G., Wang,L., Li,X., Guo,R., Shen,B., Li,Y. (2010). Complement C5a regulates Interleukine-17 by affecting the crosstalk between DCs and gammadelta T cells in CLP-induced sepsis. *Eur. J Immunol* 40, 1079-1088.