

Systemic CD14 Inhibition Attenuates Organ Inflammation in Porcine *Escherichia coli* Sepsis

Ebbe Billmann Thorgersen,^{a,b} Søren Erik Pischke,^{a,c} Andreas Barratt-Due,^{a,c} Hilde Fure,^d Julie Katrine Lindstad,^a Anne Pharo,^a Bernt Christian Hellerud,^a Tom Eirik Mollnes^a

Institute of Immunology, Oslo University Hospital Rikshospitalet and University of Oslo, Oslo, Norway^a; Department of Surgery, Ringerike Hospital and Bærum Hospital, Vestre Viken HF, Drammen, Norway^b; Division of Emergencies and Critical Care Medicine and The Interventional Centre, Oslo University Hospital Rikshospitalet and University of Oslo, Oslo, Norway^c; Research Laboratory, Somatic Research Centre, Nordland Hospital, Bodø, Norway^d

Sepsis is an infection-induced systemic inflammatory response syndrome. Upstream recognition molecules, like CD14, play key roles in the pathogenesis. The aim of the present study was to investigate the effect of systemic CD14 inhibition on local inflammatory responses in organs from septic pigs. Pigs ($n = 34$) receiving *Escherichia coli*-bacteria or *E. coli*-lipopolysaccharide (LPS) were treated with an anti-CD14 monoclonal antibody or an isotype-matched control. Lungs, liver, spleen, and kidneys were examined for bacteria and inflammatory biomarkers. *E. coli* and LPS were found in large amounts in the lungs compared to the liver, spleen, and kidneys. Notably, the bacterial load did not predict the respective organ inflammatory response. There was a marked variation in biomarker induction in the organs and in the effect of anti-CD14. Generally, the spleen produced the most cytokines per weight unit, whereas the liver contributed the most to the total load. All cytokines were significantly inhibited in the spleen. Interleukin-6 (IL-6) was significantly inhibited in all organs, IL-1 β and IP-10 were significantly inhibited in liver, spleen, and kidneys, and tumor necrosis factor, IL-8, and PAI-1 were inhibited only in the spleen. ICAM-1 and VCAM-1 was significantly inhibited in the kidneys. Systemic CD14-inhibition efficiently, though organ dependent, attenuated local inflammatory responses. Detailed knowledge on how the different organs respond to systemic inflammation *in vivo*, beyond the information gained by blood examination, is important for our understanding of the nature of systemic inflammation and is required for future mediator-directed therapy in sepsis. Inhibition of CD14 seems to be a good candidate for such treatment.

Sepsis is a serious and life-threatening systemic inflammatory response syndrome caused by infection (1). The inflammatory response is vast and complex. The innate immune system plays a key role in the onset and maintenance of the inflammation (2, 3). The main trigger of inflammation is the recognition of microbes and damaged tissue by receptors of the innate immune system (4, 5). Recognition molecules include both soluble and cell-bound molecules, e.g., the Toll-like receptors (TLRs) (6). Soluble recognition molecules extra- or intracellularly play in concert with the cell-bound receptors in initiating the inflammatory response (7). These receptors are targeting conserved features of microbes and thereby detect a wide range of microbial diversity (8, 9). The inflammation in sepsis becomes a threat to the host when it spreads out of control (10).

The TLRs are a broad group of recognition molecules in the innate immune system (6). They recognize a range of molecules (11). TLR4, MD-2, and CD14 form the TLR4 complex, which recognizes the lipopolysaccharides (LPS) of Gram-negative bacteria (12, 13). Interestingly, CD14 is also a comolecule for several other TLRs (14), showing its broad functionality and promiscuity as an innate immune receptor. It has been shown that CD14 plays a part in inflammation in organs, as in LPS-induced lung inflammation (15) and in the recruitment of neutrophils in liver microcirculation (16). A recent publication showed that CD14 controls the endocytosis of TLR4 upon the recognition of LPS and subsequent intracellular signaling (17). These findings point to CD14 as a key upstream receptor of innate immunity.

Studies of human and animal sepsis models have examined the inflammatory response in blood (2, 18, 19). The effects of sepsis on single organs, such as the heart or liver (20, 21), or on the endothelium have also been studied clinically and molecularly

(22). However, the inflammatory response to sepsis in organs *in vivo* is less well known. Thus, the aim of the present study was to investigate the inflammatory responses in various organs to systemic *E. coli* whole bacteria or LPS-induced inflammation and the effects of inhibiting CD14 on these responses. For this purpose, we used a porcine model which, in light of a recent publication, perhaps represents a more relevant model for mimicking human disease and in particular inflammation compared to murine models (23).

MATERIALS AND METHODS

Animals and experimental groups. The animals and experimental design used here have been described in detail previously (24). Briefly, ~7-week-old pigs (*Sus scrofa domesticus*, outbred stock), weighing 15 kg (range, 14.5 to 17 kg), were used. Clinically healthy pigs were allocated into one of two groups. The *E. coli* group consisted of subgroups receiving *E. coli* and anti-CD14 ($n = 7$), *E. coli* and isotype-matched control antibody ($n = 6$), *E. coli* only ($n = 6$), or bacterial culture medium ($n = 6$). The LPS group consisted of subgroups receiving LPS and anti-CD14 ($n = 6$) or LPS and isotype-matched control antibody (positive control group) ($n = 3$). The

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Address correspondence to Ebbe Billmann Thorgersen, ebtho@rr-research.no.

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results from the LPS group are presented in the supplemental material. Exclusion criteria included unhealthy animals prior to the experiments, hemoglobin at <5 g/dl during the stabilization phase, or $\text{SaO}_2 < 90\%$ with $\text{FiO}_2 = 0.30$ in the stabilization phase. No animals were excluded. Animals were anesthetized and surgery was conducted according to procedures described in detail previously (25). Mouse anti-porcine CD14 monoclonal antibody clone MIL-2, isotype IgG2b, was purchased from AbD Serotec (Oxford, United Kingdom). A mouse anti-human IgG2b (clone BH1, product no. 2070) was purchased from Diatec monoclonal antibodies AS (Oslo, Norway) and used as an isotype-matched control. Live *E. coli* (strain LE392; ATCC no. 33572) was obtained from American Type Culture Collection (Manassas, VA). *E. coli* was infused intravenously at an increasing rate: 3.75×10^7 bacteria/h from 0 to 60 min, 1.5×10^8 bacteria/h from 60 to 90 min, and 6.0×10^8 bacteria/h from 90 to 240 min. Infusions of bacteria started immediately after anti-CD14 or control treatment was given. A total amount of 1.075×10^8 bacteria/kg, corresponding to 1.1×10^6 bacteria/ml of blood, was infused into each animal in the *E. coli* group. Ultra Pure *E. coli* LPS (strain O111:B4) was purchased from InvivoGen (San Diego, CA). The LPS was dissolved in sterile water and thereafter in sterile saline water. A total amount of 0.03 mg of LPS/kg was infused intravenously over 30 min, immediately after anti-CD14 or isotype-matched control antibody was given.

Preparation of tissues and homogenization. Tissue samples from the right lung, liver, spleen, and kidney were harvested immediately after euthanasia, put on ice, cut, and transferred to Nunc tubes (Roskilde, Denmark) on dry ice, before storage at -70°C . All tissue samples were cut, weighed, and transferred to gentle MACS M tubes (Thermo Fischer Scientific, Gothenburg, Sweden) for homogenization, during which the samples, and all equipment used were kept on dry ice.

For the cytokine analysis, 50 mg of tissue sample was added to a mix of 495 μl of CytoBuster protein extraction reagent (Novagen, San Diego, CA) and 5 μl of protease inhibitor cocktail set 1 (Calbiochem, Darmstadt, Germany), followed by homogenization with Xiril Dispomix (Xiril AG, Hombrechtikon, Switzerland). After completion, the samples were incubated for 5 min on ice and thereafter centrifuged at $2,500 \times g$ for 20 min at 4°C . The supernatants were transferred to Nunc tubes and stored at -70°C until analysis.

For the *E. coli* analysis, 50 mg of tissue sample was added to 400 μl of MagNa Pure DNA tissue lysis buffer (Roche Diagnostics GmbH, Indianapolis, IN), followed by homogenization with Xiril Dispomix. After completion, the samples were incubated for 30 min at room temperature for better lysis. The samples were transferred to Safe-Lock tubes, PCR Clean (Eppendorf AG, Hamburg, Germany) was added, and the samples were stored at -70°C until analysis.

For the LPS analysis, 50 mg of tissue sample was added to 500 μl of LAL reagent water (Associates of Cape Cod), followed by homogenization with Xiril Dispomix. After completion, the samples were transferred to Nunc tubes and stored at -70°C until analysis.

For the RNA analysis, 20 mg of tissue sample was added to 800 μl of TRIzol reagent (Invitrogen, Carlsbad, CA), followed by homogenization with Xiril Dispomix. After completion, the samples were incubated for 5 min at room temperature, transferred to Safe-Lock tubes, and stored at -70°C until analysis. Precautions were taken to avoid RNase and DNA contamination. All conditions of homogenization were optimized and validated before analysis.

Total protein measurement. The total protein amount in all tissue cytokine homogenates was measured by using a DC protein assay (Bio-Rad, Hercules, CA), using 30% albumin solution from bovine serum (Sigma-Aldrich, St. Louis, MO) diluted with CytoBuster protein extraction reagent for the standard curve. Conditions were optimized and validated before analysis.

***E. coli* DNA analysis.** *E. coli* DNA from 80 μl of homogenized sample was isolated by robotic DNA purification using a MagNa Pure LC DNA isolation kit II (tissue variant) on a MagNa Pure LC instrument (Roche Applied Science, Mannheim, Germany). Quantification of *E. coli* DNA

was performed by detection of the *seqA* gene as described earlier (24) using real-time PCR (AB 7500 Fast; Applied Biosystems, Warrington, United Kingdom). The PCR amplification was performed in a total volume of 10 μl , using 2 μl of DNA, 5 μl of $2\times$ TaqMan Universal FAST Master Mix (Applied Biosystems), 0.5 μl of gene-specific $20\times$ gene expression assay mix (Applied Biosystems), and 2.5 μl of RNase/DNase-free water. The cycling conditions were as follows: 20 s of polymerase activation at 95°C was followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. All samples were amplified in triplicates.

LPS quantification. Endotoxin levels were quantified in a Pyrochrome *Limulus* amoebocyte lysate (LAL) assay by an endpoint chromogenic method using a diazo-coupling assay kit (Associates of Cape Cod). Homogenates were diluted in depyrogenated Pyrotube-D tubes with LAL reagent water (both from Associates of Cape Cod). The diluted samples were heat treated at 75°C for 10 min, mixed with pyrochrome solved in Glucashield a β -glucan inhibiting buffer, and incubated in a 96-well Pyroplate (both from Associates of Cape Cod) at 37°C on a dry block incubator. Normal human serum was used as negative control. After incubation, the procedure was followed according to the instructions from the manufacturer.

Protein quantification of inflammatory mediators. Cytokines were analyzed using commercially available kits. Quantikine porcine immunoassay kits from R&D Systems (Minneapolis, MN) were used for analyses of tumor necrosis factor (TNF), interleukin- 1β (IL- 1β), IL-6, and IL-8. IL-10 was analyzed by swine IL-10 enzyme-linked immunosorbent assay kit from BioSource Invitrogen (Camarillo, CA).

qPCR analyses. (i) RNA preparation. Total RNA was isolated from the homogenates using chloroform extraction with an RNeasy MinElute cleanup kit (Qiagen, Hilden, Germany). Sufficient RNA quantity and quality was assessed by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Contaminating DNA was removed from the samples using DNA-free DNase treatment and removal reagent (Ambion, Inc., Austin, TX) as recommended by the manufacturer. DNase-treated RNA samples were then stored at -70°C .

(ii) cDNA synthesis. cDNA was produced from 500 ng of each RNA sample by a one-vial reverse transcriptase PCR using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). PCR conditions were set to 120 min of reverse transcription at 37°C and 5 s at 85°C to stop the reaction.

(iii) Candidate genes. FAM-MGB dye-labeled TaqMan qPCR primers for E-selectin (Ss03394520_m1), ICAM-1 (Ss03392384_m1), VCAM-1 (Ss03390909_m1), PAI-1 (Ss03392656_u1), IL- 1β (Ss03393804_m1), IP-10 (Ss03391846_m1), IL-10 (Ss03382372_u1), CD14 (Ss03818718_s1), TLR-4 (Ss03389780_m1), Caspase-1 (Ss03394227_m1), and C5aR1 (Ss03375530_u1) were obtained from Applied Biosystems. GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Ss03374854_g1) was used as an endogenous control since it was confirmed stably expressed in all groups and organs in control experiments.

(iv) qPCRs. Three qPCRs per sample were performed for each candidate gene. A 15- μl reaction volume consisting of 5 ng of cDNA, 0.75 μl of $20\times$ TaqMan gene expression assay mix (Applied Biosystems), 7.5 μl of TaqMan Universal PCR Master Mix (Roche, Branchburg, NJ), and 6.25 μl of nuclease-free water (Sigma-Aldrich) was added to 96-well MicroAmp optical reaction plates (Applied Biosystems) for analysis. PCR amplification was performed in a StepOnePlus machine (Applied Biosystems) with the following cycling conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 two-segment cycles (95°C for 15 s and 60°C for 1 min). Fluorescence was automatically measured during each two-segment cycle.

Data presentation and statistical analysis. All data are presented per mg of tissue. The two positive control groups in the *E. coli* group (i.e., the *E. coli* and isotype-matched control antibody group and the *E. coli*-only group) showed identical results and were therefore merged into one group for the LPS analyses, *E. coli* DNA analyses, and the cytokine analyses. (For an outline of the groups, please see reference 24.) Differences in

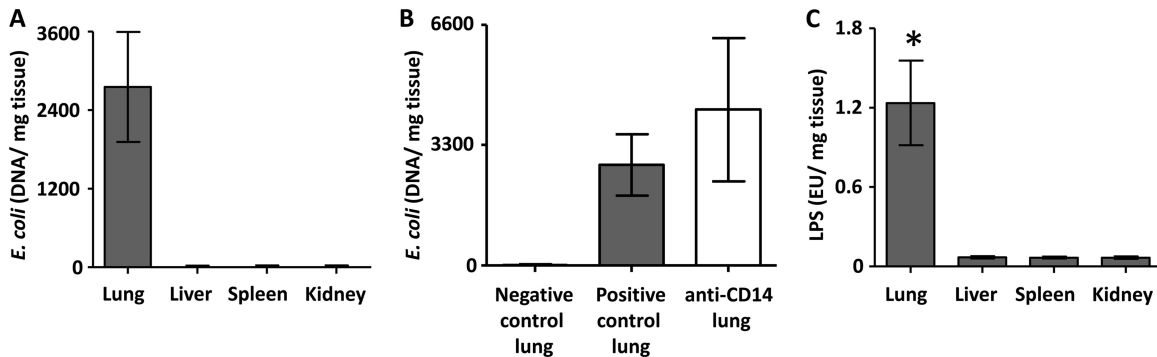


FIG 1 *E. coli* DNA and LPS load in the organs and effect of anti-CD14. *E. coli* DNA levels in the organs in the positive control group in the *E. coli* group (A) and *E. coli* DNA levels in the lung in the anti-CD14 group compared to the positive control group are shown (B). (C) LPS loads in organs in the positive control group in the *E. coli* group. The data are presented as means and 95% confidence intervals. *, $P < 0.05$.

organ LPS load in the control group of the *E. coli* group was analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons *post hoc* test. Differences in organ LPS load between the treatment group and the positive control group were analyzed using a two-sample *t* test. Differences in organ cytokine load in the *E. coli* control group were analyzed using one-way ANOVA with Tukey's multiple-comparison *post hoc* test. Differences in organ cytokine load between the treatment group and the positive control group were analyzed using a Mann-Whitney U test, as normal distribution of the data could not be assumed. The mRNA analyses were only performed on the *E. coli* group. The group that only received *E. coli* was used as a positive control group in the mRNA analyses. qPCR results were quantified by calculating the fold changes using the $2^{-\Delta\Delta CT}$ method and normalized to the negative control group (culture medium group). Differences between the groups were calculated using one-way ANOVA with the Bonferroni *post hoc* test corrected for multiple pairwise comparisons.

The results for the LPS group are presented without statistical analysis, since the positive control group was limited to three animals. For all of the statistical analyses, a P value of < 0.05 was considered statistical significant. The results were analyzed using GraphPad Prism (La Jolla, CA) and PASW Statistics 18 (IBM, Chicago, IL).

Ethics. The study was approved by the Norwegian Animal Research Authority, and animals were treated according to the Norwegian Laboratory Animal Regulations.

RESULTS

Total protein amount. The total protein amount was measured in all of the samples and correlated to the weight of the tissue. No differences were found between the homogenates (data not shown).

***E. coli* group.** (i) ***E. coli* DNA.** *E. coli* DNA was only detected in the homogenates from the lungs (Fig. 1A). Since the threshold for detection was 7,000 copies per ml of homogenate, the presence of lower amounts of *E. coli* in the other organs could not be detected. There was a nonsignificant trend ($P = 0.086$) toward a higher count of *E. coli* DNA in the anti-CD14 group compared to the positive control group in the lungs (Fig. 1B). No *E. coli* DNA was found in the negative control group.

(ii) **LPS load.** There was a substantially and significantly higher amount of LPS in the lung samples compared to the other organs ($P < 0.0001$), and no difference between the other organs (Fig. 1C). In the liver and spleen, there was a significantly higher LPS amount in the anti-CD14 group compared to the positive control group ($P = 0.0003$ and 0.0141 , respectively), and no difference in lungs and kidneys (Fig. 2).

Inflammatory biomarkers. (i) **Cytokines in the positive control group measured by enzyme immunoassays.** TNF, IL-1 β , IL-6, and IL-8 were increased in all of the organs in the positive control group (Fig. 3). There was an overall highly significant difference between the loads of TNF ($P = 0.0004$), IL-1 β , IL-6, and IL-8 ($P < 0.0001$) in the organs. TNF was significantly higher in the spleen compared to the liver and kidney and in the lung compared to the kidney ($P < 0.05$). IL-1 β was significantly higher in the spleen compared to the lung, liver, and kidney, in the lung compared to the kidney, and in the liver compared to the kidney ($P < 0.05$). IL-6 was significantly higher in the spleen compared to the lung and kidney and in the liver compared to the lung and kidney ($P < 0.05$). IL-8 was significantly higher in the spleen compared to the lung, liver, and kidney ($P < 0.05$).

IL-10 was detected only in the liver and kidney, but no increase was found in the positive control group compared to the negative control group (data not shown). None of the cytokines, except for IL-10 in the liver and kidney, was detected in the negative control group (data not shown).

(ii) **Effect of anti-CD14 on the cytokine load.** In the lung, IL-6 was significantly reduced ($P = 0.024$) in the anti-CD14 group compared to the positive control group (Fig. 4). TNF, IL-1 β , and IL-8 were not significantly affected by anti-CD14. In the liver, IL-1 β ($P = 0.0023$) and IL-6 ($P = 0.0022$) were significantly reduced in the anti-CD14 group compared to the positive control group (Fig. 4). TNF and IL-8 were not significantly affected by anti-CD14. In the spleen, all of the measured cytokines—TNF ($P = 0.017$), IL-1 β ($P = 0.0076$), IL-6 ($P = 0.0009$), and IL-8 ($P = 0.01$)—were significantly reduced in the anti-CD14 group compared to the positive control group (Fig. 4). In the kidney, IL-1 β ($P = 0.013$) and IL-6 ($P = 0.033$) were significantly reduced in the anti-CD14 group compared to the positive control group (Fig. 4). TNF and IL-8 were not significantly affected by anti-CD14.

(iii) **Contribution of the different organs to the total cytokine load and the effect of anti-CD14.** The data reported thus far were all calculated per mg of tissue. The total contribution from each of the four organs was then examined according to their weight (Table 1). The total load, the percent contribution of each of the organs to total cytokine load, and the percent change in cytokine load by anti-CD14 in each organ are presented. The liver contributed by $> 50\%$ to the load of TNF, IL-1 β , and in particular IL-6 with 79% of the total load. The lungs contributed substantially by

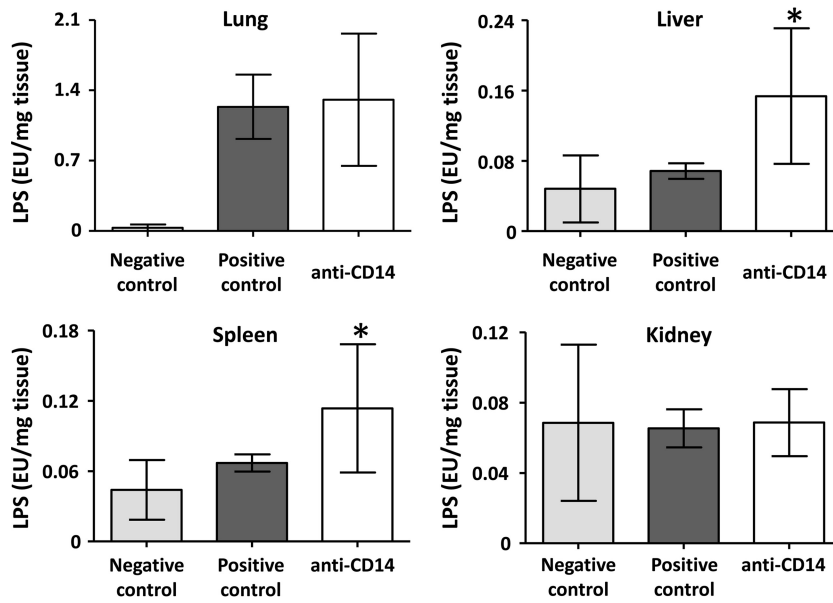


FIG 2 Effect of anti-CD14 on LPS load in the organs. The LPS loads in the organs in the negative control group, in the positive control group, and in the anti-CD14 group are shown for various organs. The anti-CD14 group is statistically compared to the positive control group. The data are presented as means and 95% confidence intervals. *, $P < 0.05$.

ca. 30% to the load of TNF, IL-1 β , and IL-8. The spleen and kidney contributed with 2 to 15% of the total cytokine load. Anti-CD14 substantially reduced the total cytokine load in the organs, with the exception of TNF, IL-1 β , and IL-8 in the lungs and IL-8 in the kidneys. The load of IL-6 was reduced by 65 to 87% by anti-CD14 in all of the organs. The cytokines in the spleen, which was the most efficient organ in producing cytokines per weight unit,

were overall most extensively reduced by anti-CD14, with a reduction from 63 to 82% for all four cytokines.

Adhesion molecules measured by qPCR. VCAM-1 mRNA was upregulated in all organs except for the spleen and ICAM-1 in all organs (Fig. 5). The fold change for both was highest in the liver and kidney. Anti-CD14 significantly ($P < 0.05$) reduced the up-regulation for both VCAM-1 and ICAM-1 in the kidney, in con-

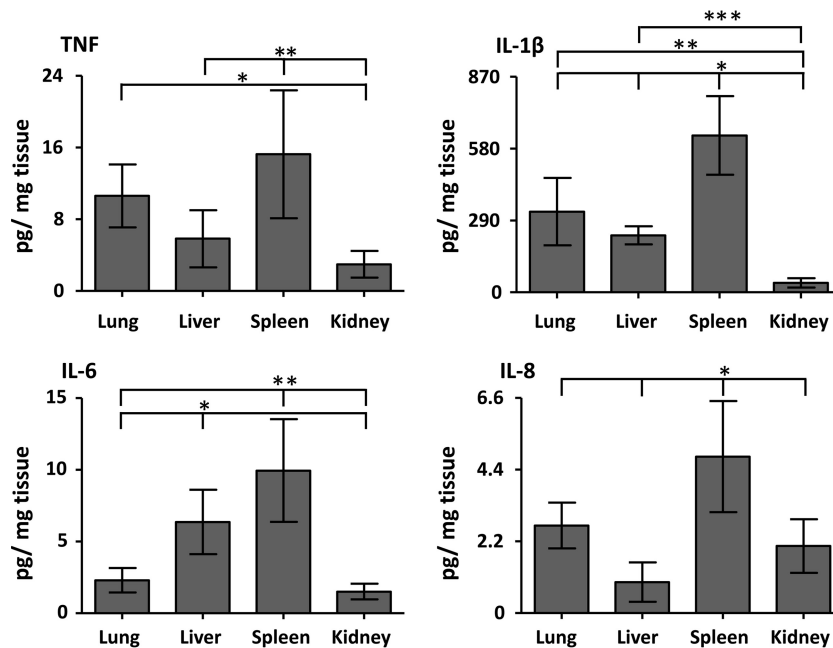


FIG 3 Cytokine load in the organs. The organ load of the proinflammatory cytokines TNF, IL-1 β , IL-6, and IL-8 in the organs in the positive control group is shown in the *E. coli* group. The data are presented as means and 95% confidence intervals. TNF: *, lung versus kidney; **, spleen versus liver and kidney. IL-1 β : *, spleen versus lung, liver, and kidney; **, lung versus kidney; ***, liver versus kidney. IL-6: *, liver versus lung and kidney; **, spleen versus lung and kidney. IL-8: *, spleen versus lung, liver, and kidney. All, $P < 0.05$.

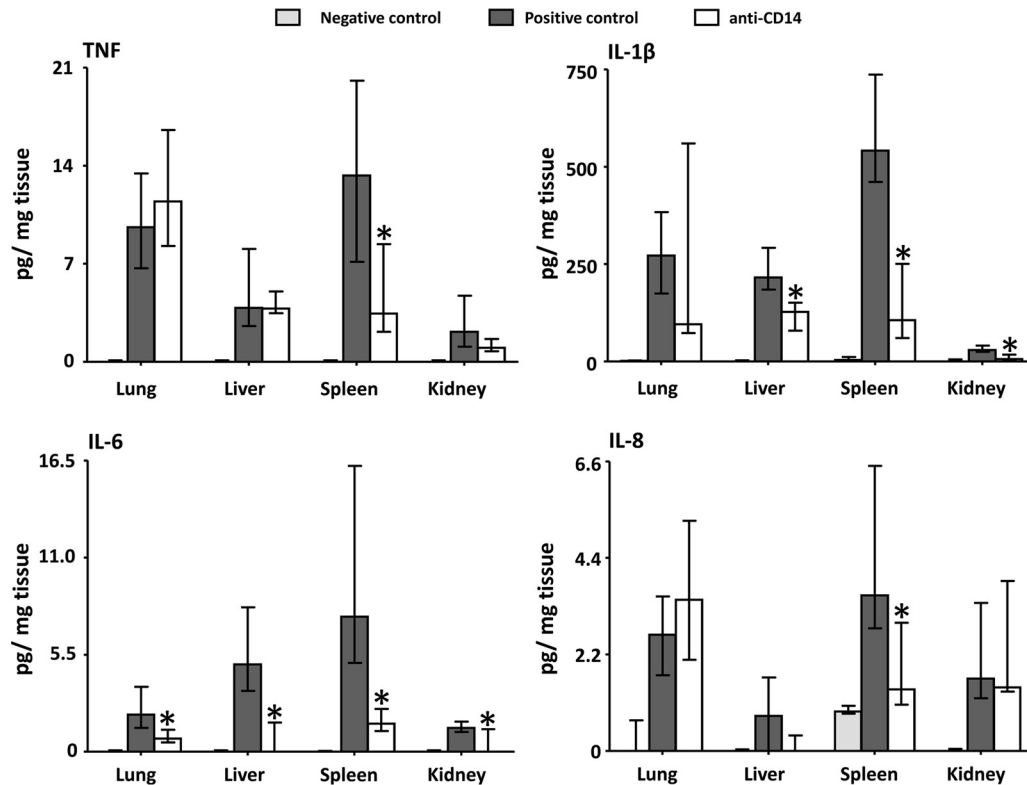


FIG 4 Effect of anti-CD14 on the proinflammatory cytokine load in the organs. The loads of the proinflammatory cytokines TNF, IL-1 β , IL-6, and IL-8 in the negative control group, the positive control group, and the anti-CD14 group were determined. The anti-CD14 group is statistically compared to the positive control group. The data are presented as medians and interquartile ranges. *, $P < 0.05$.

trast to the other organs, where no effect was seen. E-selectin was substantially upregulated in all organs (data not shown) with no significant effect of anti-CD14.

Selected cytokines measured by qPCR. IP-10 mRNA was upregulated in all organs (Fig. 5). The fold change in upregulation

was highest in the spleen and kidney. Anti-CD14 significantly ($P < 0.05$) reduced the upregulation in the liver, spleen, and kidney, in contrast to the lung, where the difference was not significant. IL-1 β mRNA was upregulated in all organs (data not shown). Anti-CD14 had no significant effect on this upregulation.

TABLE 1 Contribution of organs to cytokine load and effect of anti-CD14^a

Cytokine	Organ	Cytokine load (pg/mg of tissue)	Total cytokine load ^b (ng)	% contribution of each organ to the total load ^c	% change in load per organ by anti-CD14
TNF	Lungs	10	1,733	33	19
	Liver	5.8	2,761	53	-23
	Spleen	15	435	8.4	-69
	Kidneys	3.0	245	4.7	-58
IL-1 β	Lungs	325	53,187	29	8.8
	Liver	230	109,067	59	-48
	Spleen	633	18,046	9.8	-73
	Kidneys	37	3,121	1.7	-70
IL-6	Lungs	2.3	376	9.9	-65
	Liver	6.4	3,014	79	-87
	Spleen	10	284	7.5	-82
	Kidneys	1.5	124	3.3	-70
IL-8	Lungs	2.7	439	36	30
	Liver	1.0	450	37	-75
	Spleen	4.8	137	11	-62
	Kidneys	2.1	170	14	27

^a Four organs were included (lungs, liver, spleen, and kidneys).

^b Organ size was calculated from the mean weight of organs in 15-kg pigs (50).

^c That is, of the four organs.

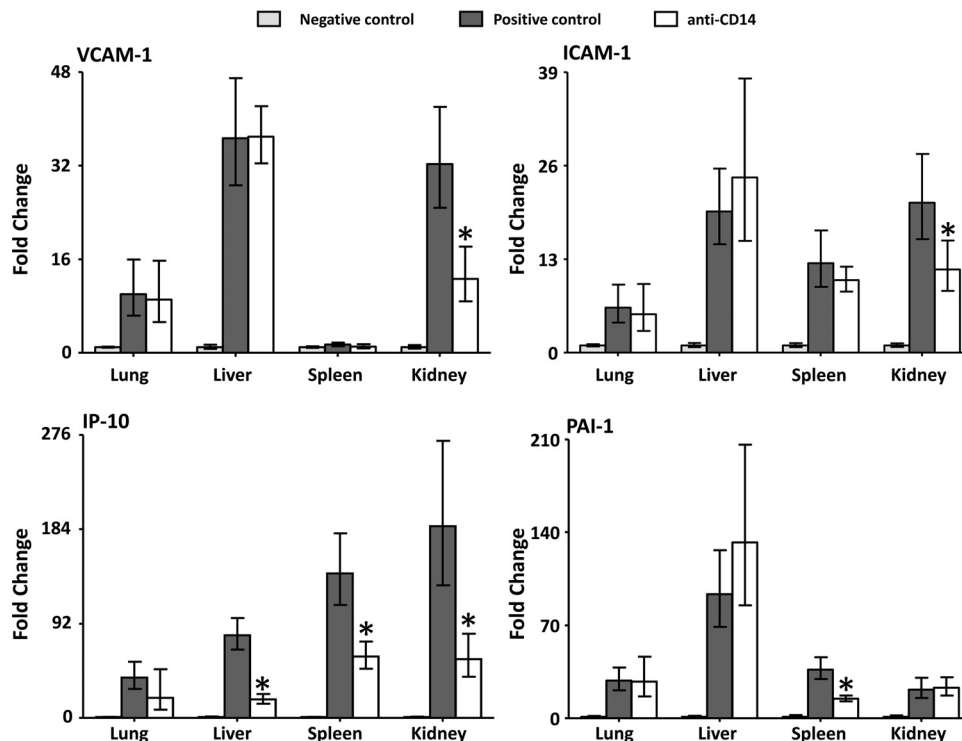


FIG 5 Effect of anti-CD14 on the mRNA induction of the adhesion molecules IP-10 and PAI-1. Fold change in the mRNA expression of the adhesion molecules VCAM-1 and ICAM-1, the chemokine IP-10, and the inhibitor of fibrinolysis, PAI-1, in the negative control group, the positive control group, and the anti-CD14 group. The anti-CD14 group is statistically compared to the positive control group. The data are presented as means and 95% confidence intervals. *, $P < 0.05$.

IL-10 mRNA was upregulated in the spleen and largely unchanged in the other organs (data not shown). Anti-CD14 reduced the upregulation in the spleen nonsignificantly.

Hemostatic marker (PAI-1) measured by qPCR. PAI-1 mRNA was upregulated in all organs (Fig. 5). The fold change was highest in the liver. Anti-CD14 significantly reduced ($P < 0.05$) the upregulation only in the spleen.

DISCUSSION

This study documents the organ distribution of bacteria and bacterial LPS and the subsequent differential local inflammatory responses in a pig model of *E. coli* sepsis. This inflammatory response was inhibited in an organ-dependent manner by anti-CD14. The data indicate an organ-specific accumulation of the bacteria and LPS to the lungs, with very low levels in other organs. However, the inflammatory response in the organs, measured as the cytokine load, did not reflect the skewed *E. coli* distribution. Detailed knowledge on how the different organs respond to systemic inflammation *in vivo*, beyond the information gained by blood examination, is important for our understanding of the nature of systemic inflammation and is required for future mediator-directed therapy in sepsis. Importantly, large-animal models, such as the one described here, are needed to better mimic the situation in humans.

In order to compare the *E. coli* whole-bacterium group, which we consider a clinically more relevant sepsis model than the frequently used endotoxin (LPS) model, we included an LPS group for comparison. The LPS group showed by far the same trends as the *E. coli* group. We used a laboratory bacterial strain. Although an isolate of a human *E. coli* strain from a sepsis patient would

have been clinically more relevant, the currently used strain works well to induce septic shock in the pigs. Since Gram-negative sepsis with whole bacteria was the main focus here, our discussion below focuses on the *E. coli* group.

LPS from the *E. coli* bacteria accumulated in the lungs of the pigs and was detected only in very low quantities in other organs. *E. coli* DNA was only detected in the lungs. Compared to the organ biopsy specimens, including the lungs, high detectable levels of LPS and moderate levels of *E. coli* DNA were found in blood in these animals (24). The lungs have a special position in the immune system in pigs, in particular since they harbor intravascular macrophages similar to liver Kupffer cells in humans (26). It seems that the bacteria were trapped in the lungs throughout the experiment since the tissue samples were obtained after a 4-h observation time. Interestingly, treatment with anti-CD14 did not have any significant effect on the *E. coli* or LPS levels in the lungs. This finding is in accordance with a previous study showing no effect of the TLR4 pathway in the elimination of bacteria in *E. coli*-induced pneumonia in mice (27). No effect of anti-CD14 inhibition was seen on *E. coli* or the LPS levels in the same animals in blood (24). Of interest, anti-CD14 significantly increased the LPS levels in the liver and spleen. One might speculate that the saturation of CD14 molecules by anti-CD14 leads to increased levels of free, and thus measurable, LPS in these organs. However, the LPS levels in the spleen and liver were very low, so the importance of this finding is uncertain.

The cytokine load showed striking differences between the organs. Some of the differences observed between control and treatment groups, although statistically significant, were small, and these should thus be interpreted with caution regarding biological

significance. The huge LPS load in the lungs did not lead to an overwhelming increase in proinflammatory cytokines per mg of tissue compared to the other organs. Two studies have shown that already stimulated alveolar macrophages are hyporesponsive to further LPS stimuli in terms of cytokine production (28, 29). Thus, an overwhelming LPS load from the bacteria, as seen in our study, could thus be an explanation for the moderate cytokine production. Of the other organs, the spleen, in particular, had significantly higher levels of IL-1 β , IL-6, and IL-8 per mg of tissue compared to the lungs. Even in the kidneys where LPS could not be detected, IL-6 and IL-8 reached approximately the same levels as in the lungs per mg of tissue. The spleen showed the highest levels of all measured cytokines compared to the other organs. Interestingly, the load of all cytokines was reduced by 60 to 80% with anti-CD14 in the spleen, which overall was by far the most efficient inhibition. In a pig model study wherein *E. coli* LPS was infused in an experimental period from 60 to 240 min, the authors also found a differential cytokine pattern in various organs (30). The spleen was not found to have the same prominent cytokine content as in our study. There could be several reasons for this in terms of the experimental design, the sizes of the pigs, the types of LPS, and the fact that we used a more clinically relevant whole-bacterium model and not only LPS as an inductor of inflammation.

The concept of “compartmentalization,” referring to different pathophysiological events in different organs (compartments) of the inflammatory response in sepsis, is well established (31). Gene profiles in sepsis, for instance, may vary substantially between organs, as shown in a CLP model in rats (32). In the CLP-rat model, some gene clusters were upregulated in the liver and downregulated in the spleen and vice versa. There are also indications that downstream intracellular signaling of common inflammatory pathways may differ from organ to organ, e.g., NF- κ B activation by *E. coli* LPS was dependent on TNF and IL-1 β receptors in the liver but not in the lungs in a mouse model (33). These findings support our data showing that the cytokine load varies substantially between the organs in sepsis. Basic knowledge of these inflammatory differences between the organs leads to a better understanding of the inflammatory process and potentially to a more selective, organ-targeted therapy in sepsis.

IL-1 β , which seems to be more CD14 dependent in pigs than in humans (34, 35), was significantly inhibited in all organs except for the lungs. IL-1 β is one of the principle products of caspase-1 of the inflammasome (36). It is known that the CD14/TLR4-complex “primes” inflammasomes upon activation (36), but it has also been shown that caspase-1 is downregulated and inert to inhibition in Gram-negative sepsis (37). We found a profound inhibition of IL-1 β on the protein level by anti-CD14, but no inhibition of the mRNA upregulation. These data suggest that the inhibition of IL-1 β by anti-CD14 is due to inhibition of the processing of pro-IL-1 β to its active form by the inflammasome and not the pretranslational mRNA upregulation. It seemed that the lungs were in a special position versus the other organs in regard to the missing effect of anti-CD14 inhibition. The overwhelming inflammatory response caused by rapid accumulation of the bacteria might have blurred an effect of the treatment and thereby visible inhibition.

IL-6 was significantly inhibited in all four organs by anti-CD14. This is consistent with our previous finding in blood (24). Induction of IL-6 in Gram-negative sepsis seems to be closely tied

to CD14 in several species, including primates (38). Reduction of induction is seen both when the receptor is inhibited by, for instance, an antibody (39), or by short interfering RNA (siRNA) targeting the CD14 gene (40). Human whole blood challenged *in vitro* with Gram-negative bacteria revealed IL-6 as one of the most CD14-dependent proinflammatory cytokines (35), indicating a species similarity between human and pigs with respect to CD14-dependent IL-6 production.

IL-8 is in general more dependent on complement, the other main inflammation-inducing system of innate immunity, *in vitro* both in humans and in pigs (34, 35). However, *in vivo* in pigs anti-CD14 abolished the IL-8 response in blood (24). We found in the present study a significant reduction of IL-8 in the spleen but not in the other organs. It seems that the inhibition of CD14 *in vivo* has a good effect on blood cells (39), which are indeed abundantly present in the spleen. The same pattern of inhibition was found for TNF and PAI-1. Thus, it seems that these mediators first and foremost are inhibited where blood cells are abundant.

Interestingly, IP-10 was virtually abolished by anti-CD14 on the mRNA level in all of the organs except the lungs. IP-10 is a multifunctional chemokine and is produced by a range of cells, including endothelial cells and T cells. It has various biological functions, such as the chemoattraction of T cells, the promotion of T-cell adhesion to endothelial cells, angiogenesis, and antitumor effects (41). We have previously shown that IP-10 was inhibited significantly by anti-CD14 *in vitro* in whole blood in humans (39) and show here for the first time that the same striking effect is seen *in vivo* in organs in pigs. IP-10 is also important in sterile inflammation, i.e., the rejection of liver grafts (42, 43). Importantly, because of its profound effect on T cells, IP-10 seems to be a link in the inflammatory network between innate and adaptive immunity.

Adhesion molecules are found on the endothelium and on white blood cells. These molecules direct the immune cells to the site of inflammation by the process of rolling, activation, adhesion, and transmigration (44). The selectins are essential in the initial rolling (45). We found that E-selectin, which is known to be upregulated on inflamed endothelium, was upregulated in all of the organs examined. The upregulation was not inhibited by anti-CD14. In an *ex vivo* xenotransplantation model, upregulation of E-selectin was found to be highly and almost completely complement dependent (46). Furthermore, in an *E. coli* sepsis model in baboons, endothelial cell integrity was preserved by inhibition of complement (47). Consequently, a combined treatment with both CD14 and complement could be rational in sepsis to attenuate the inflammatory responses initiated by innate immune danger signaling, as we have proposed previously (48). VCAM-1 and ICAM-1 are also expressed on inflamed endothelium and contribute to rolling and firm adhesion of the white cells. We found that VCAM-1 and ICAM-1 were highly upregulated in the liver and kidneys, only moderately upregulated in the lungs and, with regard to VCAM-1, not at all upregulated in the spleen. It is known that there are some differences between the organs in the way the leukocyte adhesion cascade is organized. For instance, the leukocyte adhesion cascade differs in particular in respect to the role of the selectins in liver sinusoids compared to other tissues (49). Tissue differences such as these probably also explain the difference in effect of anti-CD14 inhibition, which efficiently inhibited both VCAM-1 and ICAM-1 in the kidneys, but not in the liver,

despite similarly increased expression of the molecules in these organs.

In conclusion, we have demonstrated here how the systemic inflammatory response makes its footprint in the organs in sepsis and how anti-CD14 has an inhibitory effect on these responses. Detailed knowledge on how the different organs respond to systemic inflammation *in vivo*, beyond the information gained by blood examination, is important for our understanding of the nature of systemic inflammation and is required for future mediator-directed therapy in sepsis. The inhibition of CD14 seems to be a good candidate for such a treatment regimen.

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