Phagocytosis of live and dead *Escherichia coli* and *Staphylococcus aureus* in human whole blood is markedly reduced by combined inhibition of C5aR1 and CD14

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ABSTRACT

Background: Sepsis is a dysregulated host response to infection. The aim of this study was to investigate the effects of complement- and CD14 inhibition on phagocytosis of live and dead Gram-negative and Gram-positive bacteria in human whole blood.

Methods: Lepirudin-anticoagulated blood was incubated with live or dead *E. coli* or *S. aureus* at 37 °C for 120 min with or without the C5aR1 antagonist PMX53 and/or anti-CD14. Granulocyte and monocyte phagocytosis were measured by flow cytometry, and five plasma cytokines by multiplex, yielding a total of 28 mediators of inflammation tested for.

Results: 16/28 conditions were reduced by PMX53, 7/28 by anti-CD14, and 24/28 by combined PMX53 and CD14 inhibition. The effect of complement inhibition was quantitatively more pronounced, in particular for the responses to *S. aureus*. The effect of anti-CD14 was modest, except for a marked reduction in INF-β. The responses to live and dead *S. aureus* were equally inhibited, whereas the responses to live *E. coli* were inhibited less than those to dead *E. coli*.

Conclusion: C5aR1 inhibited phagocytosis-induced inflammation by live and dead *E. coli* and *S. aureus*. CD14 blockade potentiated the effect of C5aR1 blockade, thus attenuating inflammation.

1. Introduction

It is crucial to mount an adequate and proportionate innate immune response towards invading pathogens that pose a threat to the human host (Medzhitov, 2008). The complement system and the toll-like receptors (TLRs) expressed on a plethora of cells, leukocytes in particular, constitute a first line of defence against microbes and other immediate dangers (Beutler, 2004; Matzinger, 2002). Usually, their sensing of danger and prompt activation results in a swift and harmless resolution of the initial threat. However, innate immunity is sometimes overwhelmed by pathogens and their numerous pathogen-associated

molecular patterns (PAMPs) leading to dysregulated inflammation, as seen in sepsis (Brown et al., 2006; Ward and Gao, 2009). In this regard, early prevention of an overwhelming innate immune response may be a key to reduce associated morbidity and mortality (Dellinger et al., 2013).

By inhibiting key bottleneck molecules of the complement system, such as C5, and CD14 of the TLR system, we aim to reduce an otherwise detrimental, downstream inflammatory response (Mollnes et al., 2008). This inhibition has successfully reduced the otherwise great inflammatory responses to both unimicrobial (*Escherichia coli, Neisseria meningitidis* and *Staphylococcus aureus*) and polymicrobial threats in

human whole blood and porcine models of sepsis (Barratt-Due et al., 2013; Skjeflo et al., 2014, 2015; Hellerud et al., 2017; Mollnes et al., 2002). Hitherto, the combined regimen has not been tested for phagocytosis over time, nor on a possibly different inflammatory response induced by dead compared to live bacteria.

Likewise, systematic comparison of live and dead bacteria has rarely been performed. We argue that this is an important issue for two main reasons. First, the CFU counts in patients only represent a fraction of the total amount of bacteria identified in patients by qPCR, as qPCR counts both live and dead bacteria. Here we intended to compare the potency of live and killed bacteria to induce a broad innate immunity based inflammatory response. Secondly, *in vitro* experiments are frequently made with killed bacteria for several reasons, e.g. to keep control with the number of bacteria during the experiment, or for safety reasons. Thus, it is of importance to investigate whether dead bacteria reflect the same responses as live bacteria under experimental conditions.

We hypothesize that inhibiting overwhelming phagocytosis of both live and dead Gram-positive and Gram-negative bacteria, with subsequent reductions in the levels of inflammatory mediators including cytokines and reactive oxygen species (ROS), can contribute to reduced inflammation in sepsis, and ultimately could be beneficial to patients concurrently treated with antibiotics.

2. Materials and methods

2.1. Whole blood model of bacteremia

Whole blood experiments were performed using single whole blood samples for all assays from 6 healthy donors and carried out as previously described (Mollnes et al., 2002). In brief, blood drawn by venipuncture was immediately distributed into polypropylene tubes containing Dulbecco's phosphate-buffered saline, PBS (Sigma-Aldrich, St Louis, MO), inhibitors or controls. The samples were preincubated for 10 min at 37 °C. After preincubation, PBS with $CaCl_2$ and $MgCl_2$ (Sigma-Aldrich, St Louis, MO), dead, *i.e.* heat-inactivated at 60 °C for 60 min, or live, Alexa-labelled *Staphylococcus aureus* (*S. aureus)* strains Cowan 1 (ATCC; American Type Culture Collection, Manassas, VA; 12598) or Alexa-labelled *Escherichia coli* (*E. coli,* strain LE392, ATCC 33572) were added to a final whole blood concentration of 1×10^8/mL and 1×10^7/mL, respectively. Each donor signed an informed consent form before participation and the regional ethics committee of the Northern Norway Regional Health Authority approved the study.

The time zero (T0) sample was processed immediately after blood sampling. After 60, 90, 120 and 240 min of incubation at 37 °C, blood was transferred to tubes containing ethylenediamine tetraacetic acid (EDTA, 10 mM), spun down at $3220 \times g$ at 4 °C for 15 min for plasma separation, and the plasma frozen for later cytokine analysis. Furthermore, the phagocytosis of Alexa-labelled bacteria in whole blood was assessed after 15, 45, 90 and 120 min. Separate experiments with the same donors were carried out where live *S. aureus* was titrated, 10^8/mL-10^7/mL-10^6/mL. Blood incubation was as described above. At time points zero, 60 and 120 min, blood was transferred to polypropylene tubes for analysis of phagocytosis. At time point zero and 120 min, blood was transferred to a multitier plate for PI staining and to DMEM/F-12 (Life Technologies, Paisley, UK) for colony forming units (CFU) quantification. Flow cytometric analysis on these experiments were performed on a NovoCyte (Acea, San Diego, Ca).

All equipment, tips and working solutions were endotoxin-free. Polypropylene tubes (4.5 mL; Nunc, Roskilde, Denmark) with lepirudin (Refludan®; Celgene, Uxbridge, UK; 50 mg/L) were used as anticoagulant.

2.2. Inhibitors and antagonists

The recombinant anti-human CD14 IgG2/4 hybrid monoclonal antibody (r18D11) was produced as previously described (Lau et al.,

2013) and added at a final concentration of 10 μg/mL. The cyclic hexapeptide PMX53 (sequence Ace-Phe-[Orn-Pro-dCha-Trp-Arg]) blocking the C5a receptor 1 (C5aR1) was synthesized and purified as previously described (Croker et al., 2016), and used at a final concentration of 10 μM. Each inhibitor has been carefully titrated in our laboratory to give maximum blockade and established, fixed doses were therefore used.

2.3. Alexa-labelling of bacteria

For Alexa-labelling, the bacteria were washed, and the supernatant was discarded. Afterward, $NaHCO3$ (0.2 M, 600 μL, pH 8.35, sterile-filtered and heat-inactivated 1 h at 60 °C) was added together with 6 μL of Alexa FLUOR® 488 carboxylic acid succinimidyl ester (10 g/L) in DMSO. The tube was packed in tinfoil and was rotated for 1 h at ambient temperature. Bacteria were washed additional three times with PBS and centrifuged for 5 min at $8000 \times g$ before resuspension in PBS. The bacteria were counted in Truecount tubes (Becton-Dickinson, San José, CA) using a LSRII flow cytometer with FACSDiva software (Becton-Dickinson).

2.4. Cytokine-assay

EDTA-plasma was prepared by centrifugation, as described above. The cytokine levels in the plasma were analyzed using the microsphere-based Bio-Plex Human Cytokine 4-plex Assay (Bio-Rad, Hercules, CA). The assay was performed according to instructions from the manufacturer and comprised the following: Interleukin (IL-) 1β, IL-6, IL-8 and tumor necrosis factor (TNF). Concentrations of IFN-β were determined with the VeriKine-HS human IFN-β serum ELISA kit (PBL Assay Science, Piscataway, NJ).

2.5. Phagocytosis assay

Whole blood samples were transferred to polypropylene tubes stored on ice, containing an equal amount of quenching solution (from the Phagotest kit, Glycotope Biotechnology GmbH, Heidelberg, Germany) to stop further phagocytosis and to quench the fluorescence of surface-bound but not phagocytosed bacteria. Next, washing solution (Phagotest, Glycotope Biotechnology) was added and the tubes were spun down at $300 \times g$ for 5 min. The supernatant was discarded before a second wash. The cells were then stained with anti-CD14 PE and anti-CD45 PerCp (both Becton-Dickinson) for 15 min before adding the lysing buffer (Phagotest, Glycotope Biotechnology). The lysing buffer was left to act for 20 min at ambient temperature before the cells were spun down at $300 \times g$ for 5 min. The supernatant was discarded and the cells were washed once more, PBS was added and the samples were transferred to a 96-well polypropylene storage plate (Falcon, Corning Inc., NY) for flow cytometry. Threshold was set on forward scatter and PMNs and monocytes were gated for by using a CD14/side scatter dot plot on CD45 positive events. The mean fluorescence intensity (MFI) of the Alexa fluorochrome was used as a measure for phagocytosis. An example of the gating strategy and its corresponding histogram is shown in the Supplementary Fig. 1A.

2.6. PI-staining

For the monocyte viability study, 70 μL of whole blood was mixed with 2 μL of EDTA at time point zero or after 120 min of incubation. 10 μL of this was incubated with 2 μL anti-CD45 PE and 2 μL anti-CD14 APC for 15 min on ice and then stained with 236 μL PBS PI (1,3 μg/mL). Threshold was set at CD45 PE and monocytes were gated for in APC/SSC plot. Dead cells were positive events in the 675/30 nm filter.
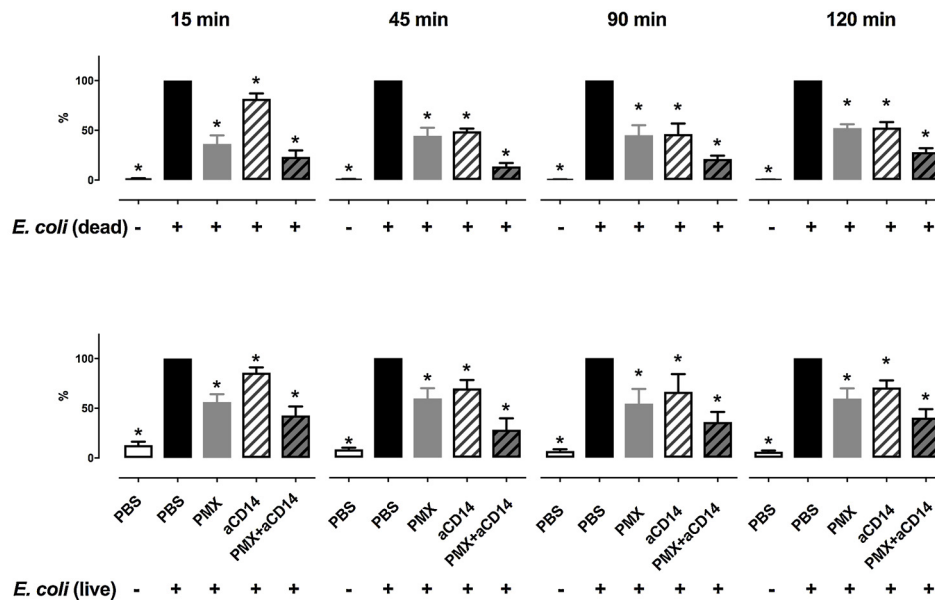
Phagocytosis in granulocytes

2.7. Statistics and data presentation

The data were analyzed with GraphPad Prism 6.0 h and 7.0c (GraphPad Software, San Diego, CA). In accordance with the paper of Matthews and colleagues concerning analysis of serial measurements in medical research (Matthews et al., 1990), we summarized total responses as areas under the curve. Normality was then tested for using the Kolmogorov–Smirnov test and any non-normal or skewed (skewness ≥ ± 1) data was log-transformed before testing by one-way repeated measures ANOVA and Dunnet's post hoc test comparing all columns with that of stimulated, uninhibited whole blood. *P* values < 0.05 were considered significant and all graphs and tables show means with 95% confidence intervals (CI).

3. Results

3.1. Granulocyte phagocytosis of E. coli: effect of, time, single-inhibition and viability (Fig. 1)

Firstly, the *E. coli* induced a significant (*P* < 0.05) increase in the phagocytic activity in granulocytes compared to PBS alone (Fig. 1). Secondly, this activity was significantly (*P* < 0.05) reduced at all time-points for both inhibitors and their combination. Notably, the effect of anti-CD14 increased markedly from 15 to 45 min whereas the effect of PMX53 was consistently strong at all time-points. The effects were similar for dead (Fig. 1, upper panels) and live (Fig. 1, lower panels) bacteria. The relative level of phagocytosis was reduced by single inhibition of C5aR1 and CD14 at all time-points, both in response to live and dead bacteria. The corresponding data for Fig. 1 with absolute values for MFI are shown in the Supplementary Fig. 2.

3.2. Monocyte phagocytosis of E. coli: effect of time, single-inhibition and viability (Fig. 2)

The *E. coli* induced a significant (*P* < 0.05) increase in the phagocytic activity in monocytes compared to PBS alone (Fig. 2). C5aR1 inhibition significantly reduced the phagocytosis of the dead bacteria throughout the whole experiment (Fig. 2, upper panels), and the phagocytosis of the live bacteria at 15 and 45 min (Fig. 2, lower panels). In contrast to the phagocytosis in granulocytes, there were no effect of inhibiting CD14 alone on the phagocytosis in monocytes of neither live

nor dead bacteria (Fig. 2).

3.3. Effect of combined inhibition of C5aR1 and CD14 on the monocyte and granulocyte phagocytosis of E. coli

The data above describes the effects of single inhibition of C5aR1 and CD14. Notably, he combination of PMX53 and anti-CD14 reduced the phagocytosis of both live and dead bacteria by both granulocytes and monocytes at all time-points (15, 45, 90 and 120 min), i.e. in all 16 cases (Figs. 1 and 2).

3.4. Granulocyte phagocytosis of S. aureus: effect of single-inhibition, time and viability (Fig. 3)

Firstly, the *S. aureus* induced a significant (*P* < 0.05) increase in the phagocytic activity in granulocytes compared to PBS alone (Fig. 3). Secondly, this activity was significantly (*P* < 0.05) reduced at all time-points in response to both live and dead bacteria when using PMX53 alone. In contrast, single inhibition of CD14 had no effect.

3.5. Monocyte phagocytosis of S. aureus: effect of single-inhibition, time and viability (Fig. 4)

Firstly, the *S. aureus* induced a significant (*P* < 0.05) increase in the phagocytic activity in monocytes compared to PBS alone (Fig. 4). Secondly, this activity was significantly (*P* < 0.05) reduced at all time-points but 120 min in response to dead bacteria (Fig. 4, upper panels) when using PMX53 alone, and at 15 and 45 min for the live bacteria (Fig. 4, lower panels). At 120 min, there were insufficient monocytes for adequate analysis. As for the phagocytosis in granulocytes, CD14 inhibition had no effect (Fig. 4).

3.6. Effect of combined inhibition of C5aR1 and CD14 on the granulocyte and monocyte phagocytosis of S. aureus

As described above, anti-CD14 exerted no inhibitory effect on the phagocytosis of *S. aureus* when used alone, yet the level of phagocytosis in monocytes at 45 min for PMX53-treated samples was reduced further when combining PMX53 with anti-CD14 (Fig. 4). This was the case both for dead and live bacteria. In contrast to *E. coli,* the single inhibition of C5aR1 was the main reason for the reduced level of phagocytosis in 16
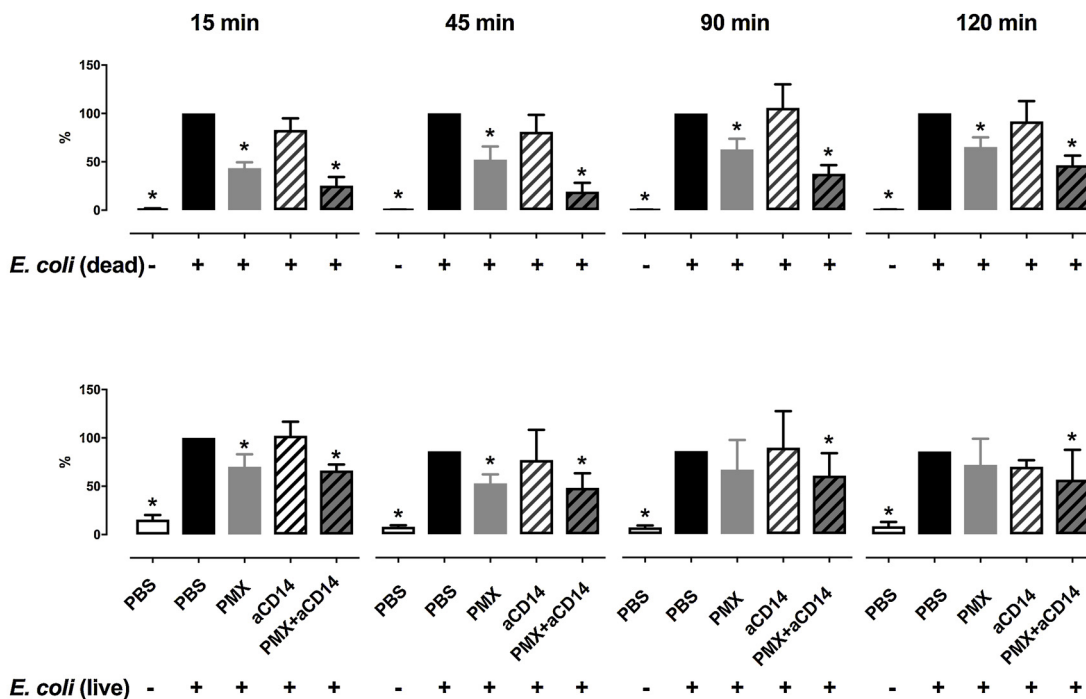
Phagocytosis in monocytes

Fig. 2. Effects of C5a receptor 1 (C5aR1) blockade and CD14 inhibition on the monocyte phagocytosis of live and dead *E. coli*.
The relative inhibition of the phagocytosis of dead (top panels) and live (bottom panels) *E. coli* when using the C5aR1 antagonist PMX53 (labelled PMX), a neutralizing anti-CD14 antibody (labelled aCD14) or the combination thereof (PMX + aCD14) was analyzed at 15, 45, 90 and 120 min. * $P < 0.05$, analyzed using repeated measures, two-way ANOVA with Dunnet's multiple comparisons post-hoc test, comparing all columns with the second reference column in black.

Phagocytosis in granulocytes

Fig. 3. Effects of C5a receptor 1 (C5aR1) blockade and CD14 inhibition on the granulocyte phagocytosis of live and dead *S. aureus*.
The relative inhibition of the phagocytosis of dead (top panels) and live (bottom panels) *S. aureus* when using the C5aR1 antagonist PMX53 (labelled PMX), a neutralizing anti-CD14 antibody (labelled aCD14) or the combination thereof (PMX + aCD14) was analyzed at 15, 45, 90 and 120 min. * $P < 0.05$, analyzed using repeated measures, two-way ANOVA with Dunnet's multiple comparisons post-hoc test, comparing all columns with the second reference column in black.
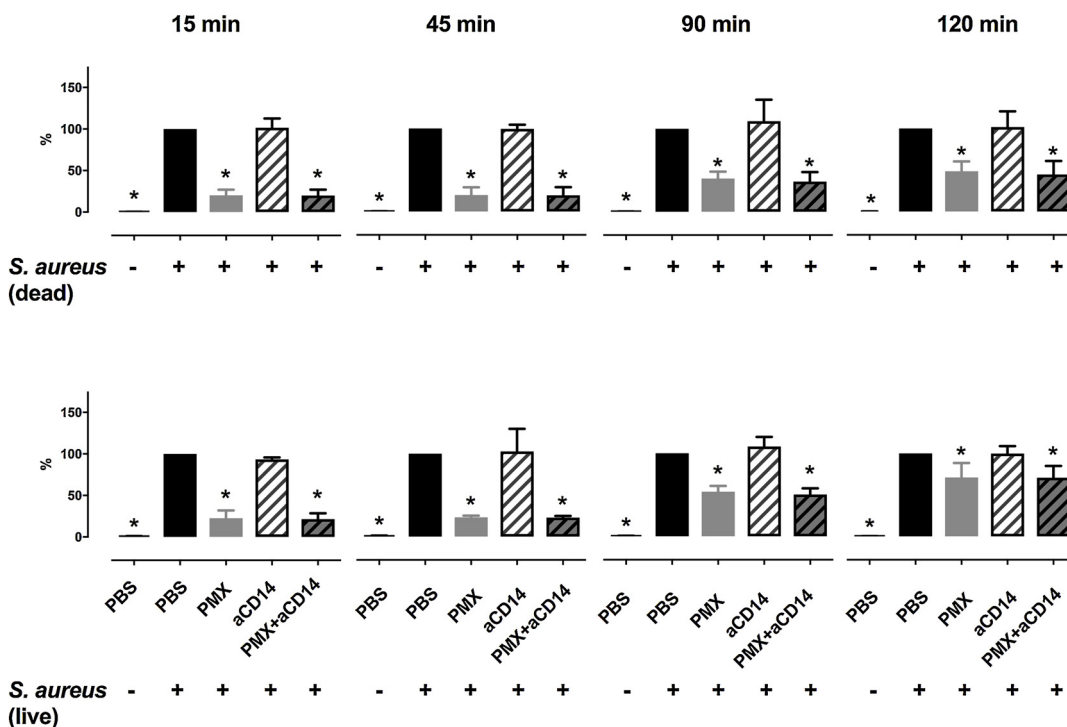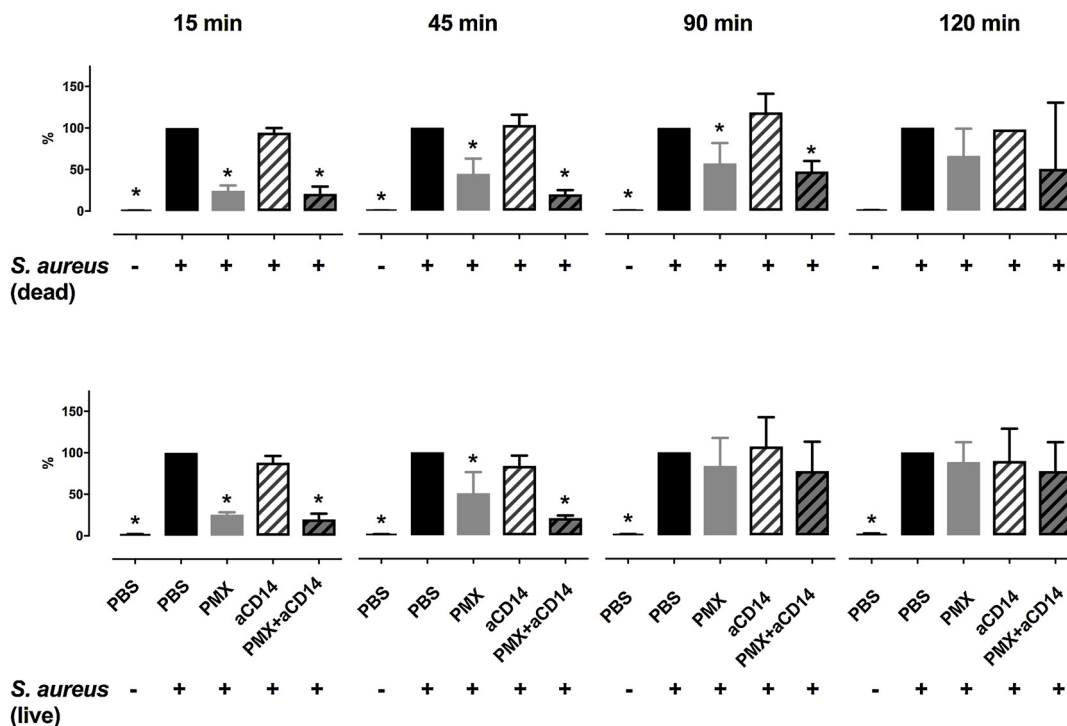
Phagocytosis in monocytes

Fig. 4. Effects of C5a receptor 1 (C5aR1) blockade and CD14 inhibition on the monocyte phagocytosis of live and dead *S. aureus*.
The relative inhibition of the phagocytosis of dead (top panels) and live (bottom panels) *S. aureus* when using the C5aR1 antagonist PMX53 (labelled PMX), a neutralizing anti-CD14 antibody (labelled aCD14) or the combination thereof (PMX + aCD14) was analyzed at 15, 45, 90 and 120 min. * $P < 0.05$, analyzed using repeated measures, two-way ANOVA with Dunnet's multiple comparisons post-hoc test, comparing all columns with the second reference column in black.

out of 16 cases studied.

3.7. Effect of staphylococcal concentration on phagocytosis

Live *S. aureus* was incubated with whole blood for 120 min at final concentrations of 1×10^8/mL, 1×10^7/mL and 1×10^6/mL. The level of phagocytosis was assessed at 60 and 120 min. All three concentrations induced phagocytosis at both time points in both granulocytes (Fig. 5) and monocytes (Fig. 6). The phagocytic response was dose-dependent with increasing amounts of bacteria (note that different scales were needed on the y-axis). PMX-53 alone reduced the level of phagocytosis at all time points for all concentrations except for 1×10^8/mL at 120 min in monocytes, whereas combined PMX-53 and anti-CD14 reduced phagocytosis at all time-points in response to all concentrations.

3.8. Total levels of phagocytosis and cytokine release as area under the response-time curve

Due to the time-dependent differences we observed at single time points, we summarized the total effect of single and combined inhibition on the total phagocytosis and cytokine release throughout the experiment. The % reduction is shown for both live and dead *E. coli* (Table I) as well as for *S. aureus* (Table 2). The corresponding absolute data are shown in Supplementary Tables I and II. Because the dead bacteria were more heavily stained with the Alexa dye, these supplementary tables indicate a higher ingestion of dead than live bacteria. In the Supplementary Fig. 1B we have therefore adjusted for the difference in staining, illustrating comparable levels of phagocytosis in granulocytes and monocytes for both live and dead *E. coli*.

E. coli. PMX alone reduced total phagocytosis for both cell types and both viabilities (Table 1). The IL-8-response was the only one of the five

cytokines measured that was reduced for both live and dead bacteria (Table 1). Single CD14 inhibition reduced granulocyte but not monocyte phagocytosis. IFN-β levels were markedly reduced for both live (68%) and dead (78%) bacteria, whilst the IL-6-response was only reduced for dead bacteria (Table 1). However, the combination of PMX53 and CD14 substantially reduced the levels of all seven cases for the dead bacteria and five for the live, i.e. 12 of 14 conditions (Table 1). The effect of combined inhibition was generally greater on the responses to dead *E. coli* than for live *E. coli*.

S. aureus. PMX53 alone reduced the total phagocytosis for both cell types in response to both live and dead *S. aureus* (Table 2). Of the five cytokines measured, three were reduced by PMX53 (IL-1β, TNF and IL-8) (Table 2). Single CD14 inhibition did not reduce phagocytosis. However, IL-8 levels were reduced by CD14 inhibition. Note in particular that IFN-β release in response to *S. aureus* was not reduced by CD14 inhibition. In contrast to the marked inhibition seen for *E. coli*, IFN-β levels tended to increase in response to *S. aureus* when CD14 was inhibited. Notably, when combining PMX53 and anti-CD14, INF-β levels were the only ones unaffected. Thus 12 of 14 cases were substantially reduced (Table 2). The effect of the combined inhibition was similar for the responses to dead and live *S. aureus*.

3.9. Temporal cytokine release in response to E. coli and S. aureus

E. coli induced increased levels of IL-1β, IL-6, IL-8, TNF and IFN-β for both live and dead bacteria at 120 min. In contrast to single inhibition, combined inhibition with PMX53 and anti-CD14 attenuated the release of all cytokines except TNF at 120 min (Suppl. Figs. 2–5).

S. aureus induced increased levels of IL-1β, IL-6, IL-8, TNF and INF-β at 120 min. In contrast to single inhibition, combined inhibition with PMX53 and anti-CD14 attenuated the release of all cytokines at 120 min except IFN-β for live bacteria (Suppl. Figs. 6–8).
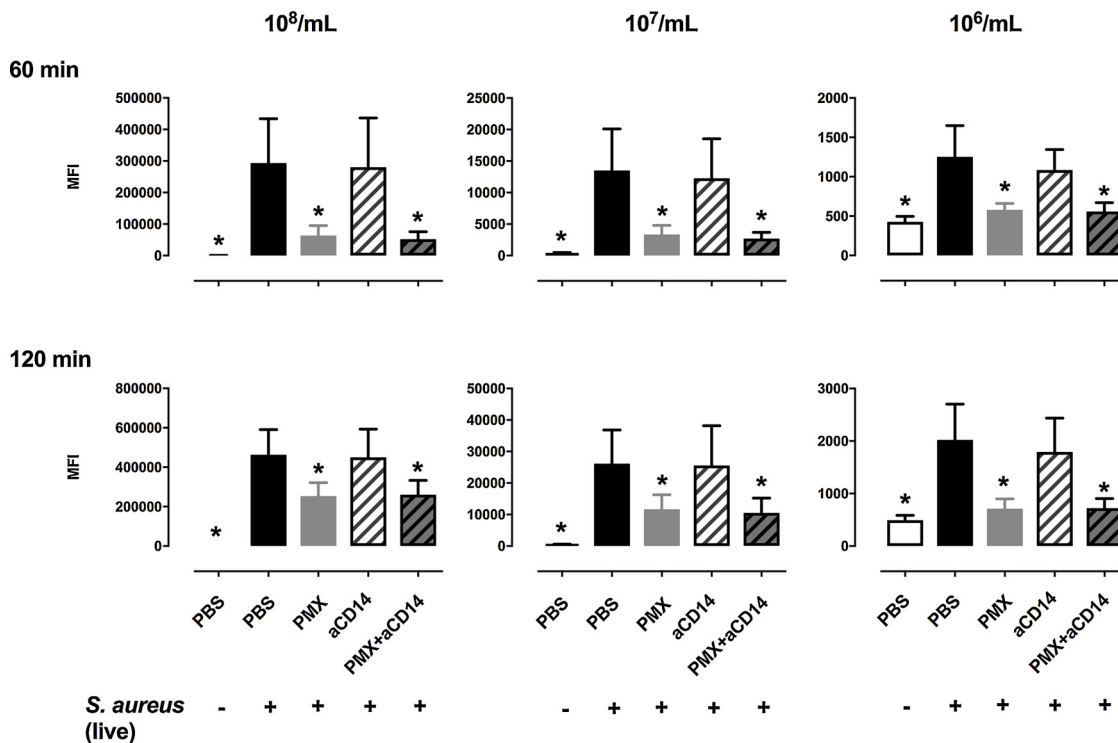
Phagocytosis in granulocytes

Fig. 5. Effects of C5a receptor 1 (C5aR1) blockade and CD14 inhibition on the granulocyte phagocytosis by different concentrations of live *S. aureus*.
The inhibition of the phagocytosis in granulocytes of 1×10^8, 1×10^7 and 1×10^6 staphylococci per mL at 90 min (top panels) and 120 min (bottom panels) when using the C5aR1 antagonist PMX53 (PMX), a neutralizing anti-CD14 antibody (aCD14) or the combination thereof (PMX + aCD14). * $P < 0.05$, analyzed using repeated measures, two-way ANOVA with Dunnet's multiple comparisons post-hoc test, comparing all columns with the second reference column in black. All columns are shown with 95% CI.

4. Discussion

In this study, we reveal a crucial dependence on the C5a-C5aR1-axis, with an additional dependence on the Toll-like co-receptor CD14, for the level of phagocytosis and cytokine release in response to both live and dead Gram-positive and Gram-negative bacteria in human whole blood. This corroborates and extends the scope of our previous work in human whole blood identifying complement and CD14 as key players in the immediate (10–15 min) CD11b expression, phagocytosis and oxidative burst in response to *S. aureus* (Skjeflo et al., 2014) and *E. coli* in whole blood incubations (Mollnes et al., 2002; Brekke et al., 2007; Barratt-Due et al., 2010).

As in the aforementioned papers, despite exerting very little effect on its own, combining CD14 inhibition with central C5a-C5aR1-complement inhibition often potentiated the effect of complement inhibition alone. This is particularly striking when comparing the effect of PMX53 to that of PMX53 combined with anti-CD14 in the two tables summarizing total response in the present paper.

Overall, the responses to both bacteria were largely complement dependent. However, complement inhibition gradually lost its effect after 90 min and even more so after 120 min, particularly on the monocyte phagocytosis of live bacteria. At this time point, however, few monocytes were detectable by flow cytometry, probably indicating that they had disintegrated or somehow succumbed to the bacterial load. We use 200 monocytes/sample as a minimum to represent the global monocyte population and we counted 1600 monocytes per sample on average, of which only two were below 200. Analyses at this time point therefore had large confidence intervals and low statistical power. To further examine the reason for these low counts, we conducted experiments with three concentrations of staphylococci (1×10^8/mL, 1×10^7/mL and 1×10^6/mL) and assayed both the

effect of complement inhibition and the monocyte counts and viability. Importantly, complement inhibition had an even greater effect on the lower concentrations of bacteria, both alone and in combination with anti-CD14. Interestingly, monocyte numbers were indistinguishable between the three groups, yet the monocyte death was dose-dependent on the bacteria. Still, less than 10% of the monocytes counted in flow cytometry were dead at the highest concentration, suggesting that monocytes could be disintegrated or bound to the surface of the tubes. Whether or not phagocyte loss occurs during clinical bacteremia and sepsis is a fundamental and very interesting question which merits further testing in the clinical setting.

For the individual cytokines, some notable inter-bacterial differences were seen, e.g. for inhibition of IFN-β production. *E. coli* is shown to mainly induce IFN-β via endosomal or phagosomal TLR4-TRAM-TRIF-IRF3 activation (Husebye et al., 2010), while *S. aureus* mainly induces IFN-β via phagosomal TLR8-IRF5 signaling (Bergstrøm et al., 2015). *E. coli*-induced IFN-β production was CD14 dependent, which fits a model where LPS and TLR4 internalisation are strictly CD14-dependent, resulting in endosomal TLR4 signaling (Jiang et al., 2005; Zanoni et al., 2011). In comparison, TLR4-NF-kB signaling from the plasma membrane is only CD14 dependent at low concentrations of LPS or Gram-negative bacteria (Brekke et al., 2008; Christiansen et al., 2012). In the present study, a high dose of *E. coli* was used, which could explain the relatively weak effect of anti-CD14 on the NF-kB dependent cytokines. However, we have seen a stronger effect of anti-CD14 on the cytokine responses induced by *E. coli* in our previous work (Brekke et al., 2008). In the case of *S. aureus*, TLR2-activation blocks TLR8-IRF5 signaling (Bergstrøm et al., 2015) and because TLR2-activation is partly CD14 dependent (Christiansen et al., 2012) anti-CD14 could lead to increased IFN-β induction via TLR8.

Unexpectedly, however, with both bacteria we did not reveal a clear
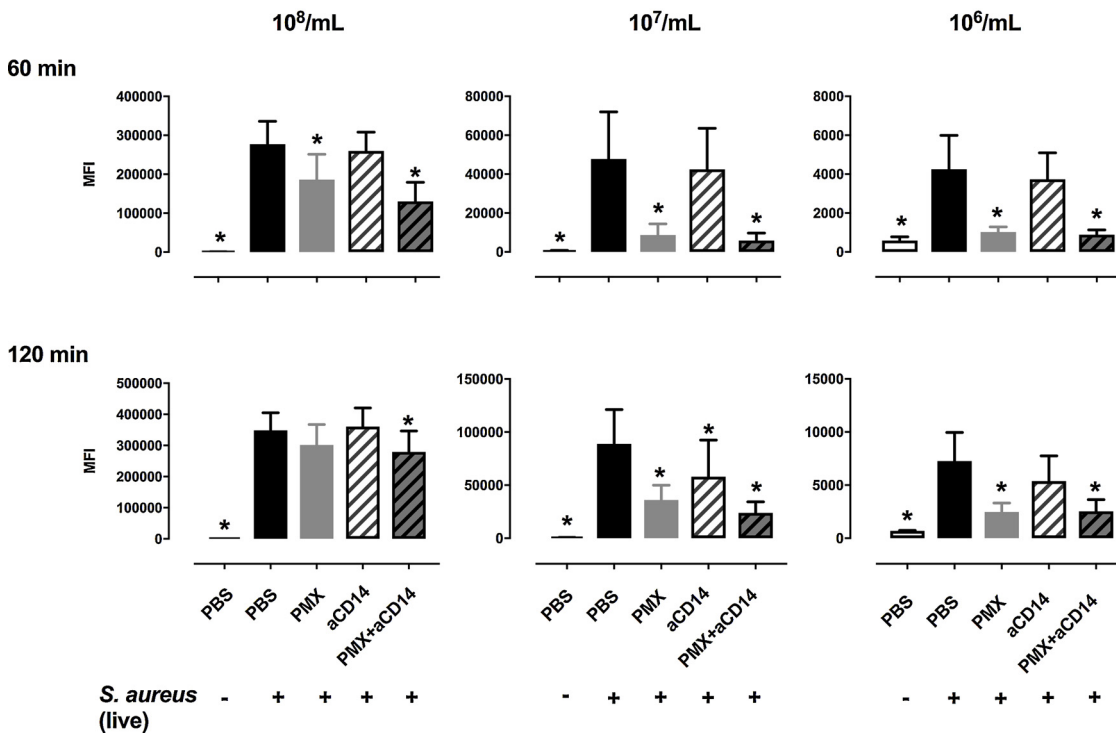
Phagocytosis in monocytes

Fig. 6. Effects of C5a receptor 1 (C5aR1) blockade and CD14 inhibition on the monocyte phagocytosis by different concentrations of live *S. aureus*.
The inhibition of the phagocytosis in monocytes of 1×10^8, 1×10^7 and 1×10^6 staphylococci per mL at 90 min (top panels) and 120 min (bottom panels) when using the C5aR1 antagonist PMX53 (PMX), a neutralizing anti-CD14 antibody (aCD14) or the combination thereof (PMX + aCD14). * $P < 0.05$, analyzed using repeated measures, two-way ANOVA with Dunnet's multiple comparisons post-hoc test, comparing all columns with the second reference column in black. All columns are shown with 95% CI.

Table 1
Percentage reduction of *E. coli*-induced inflammatory markers by PMX53, anti-CD14, or the combination thereof.

E. coli viability	PMX53 (% of uninhibited)		Anti-CD14 (% of uninhibited)		PMX53 + anti-CD14 (% of uninhibited)	
	Live	Dead	Live	Dead	Live	Dead
Granulocyte phagocytosis	43[*],[1]	54[*]	31[*]	50[*]	66[*]	80[*]
Monocyte phagocytosis	31[*]	43[*]	7	12	39[*]	70[*]
IL-1β	22	−10	−32	−28	38[*]	54[*]
IL-6	−29	−15	20	48[*]	21	67[*]
IL-8	43[*]	47[*]	1	15	43[*]	66[*]
TNF	4	−1	9	23	15	56[*]
IFN-β	−51	−15	67[*]	78[*]	70[*]	85[*]

[1] Data are from six consecutive time-response experiments in whole blood from six different donors and data are presented as the mean percentage area under the response-time (120 min) curve (AUC) in inhibited blood compared to uninhibited, stimulated blood.

* $p < 0.05$, repeated measures one-way ANOVA.

Table 2
Percentage reduction of *S. aureus*-induced inflammatory markers by PMX53, anti-CD14, or the combination thereof.

S. aureus viability	PMX53 (% of uninhibited)		Anti-CD14 (% of uninhibited)		PMX53 + anti-CD14 (% of uninhibited)	
	Live	Dead	Live	Dead	Live	Dead
Granulocyte phagocytosis	51[*],[1]	64[*]	−3	−3	53[*]	68[*]
Monocyte phagocytosis	36[*]	48[*]	13	−5	47[*]	63[*]
IL-1β	85[*]	74[*]	47	−3	84[*]	86[*]
IL-6	23	14	18	37	86[*]	80[*]
IL-8	65[*]	64[*]	40[*]	32[*]	79[*]	82[*]
TNF	58[*]	52[*]	24	9	68[*]	77[*]
IFN-β	29	29	−36	−20	26	43

[1] Data are from six consecutive time-response experiments in whole blood from six different donors and data are presented as the mean percentage area under the response-time (120 min) curve (AUC) in inhibited blood compared to uninhibited, stimulated blood.

* $p < 0.05$, repeated measures one-way ANOVA.

correlation with IFN-β production and phagocytosis. This might also be due to the relatively high dose of bacteria used in this study, as more than a 50% reduction of phagocytosis might be necessary to limit the phagosomal signaling. Another possible explanation for the opposing effects of complement inhibition on the IFN-β response to *E. coli* in particular could be that C5a receptor blockade exerted opposing effects. On the one hand, PMX53 could have increased the IFN-β production by suppressing the distinct innate cytosolic surveillance pathway described in a separate study of *Listeria monocytogenes* (Mueller-Ortiz et al., 2017). On the other, PMX53 could have reduced the IFN-β production by

reducing the phagocytosis. If so, the combined inhibition of complement and CD14, which efficiently reduced the phagocytosis of both bacteria, could explain the reduction of IFN-β as the effect on the phagocytosis was the most prominent.

Importantly, phagocytosis is both necessary and desirable in sepsis, especially in localized infection such as in the lung – where staphylococci readily localize. We argue, however, that reduced phagocytosis together with adequate antibiotic therapy might be beneficial in systemic inflammation and sepsis. A particular advantage of using PMX53 is that C3 opsonization remains unaltered. Arguably, bacteria are then

still targeted for a somewhat slower ingestion and will probably be transferred to the splenic and hepatic phagocytes, bound to erythrocyte complement receptor 1 (Brekke et al., 2011). Combined inhibition of C5aR1 and CD14, together with adequate antibiotic coverage, could then allow for killing and clearance of the bacteria with less collateral inflammatory damage.

Interestingly, the phagocytosis of *S. aureus* as a Gram-positive representative, was consistently more easily attenuated than that of *E. coli* as a Gram-negative counterpart, despite adding *S. aureus* to a final concentration of 1×10^8/mL and *E. coli* to a final concentration of 1×10^7/mL. We have previously identified these concentrations as equi-potent with respect to readouts like ROS and cytokines (unpublished data), so Gram-negative bacteria and lipopolysaccharide (LPS) are presumably more potent triggers of the innate immune response which is thus harder to inhibit. This also fits the findings of Günther and colleagues who observed a more sustained and reduced response to *S. aureus* when compared to the more acute response to *E. coli* in experimental bovine mastitis (Günther et al., 2011). Likewise, Duan et al. showed small inflammatory responses in mice challenged with *S. aureus* compared to *E. coli* over a 7-day observational period (Duan et al., 2016). Cohen and Opal also ask whether Gram-positive sepsis differs from Gram-negative sepsis to such an extent that the two should be investigated separately for possible therapeutic interventions (Opal and Cohen, 1999). Our current study, however, indicates that pre-emptive, combined inhibition of innate immunity works for both subsets. Indeed, combined inhibition of C5 and CD14 inhibition significantly reduced inflammatory mediators and improved survival in two recently published randomized trials of polymicrobial sepsis in mice and piglets (Skjeflo et al., 2015; Huber-Lang et al., 2014).

The 120-minute window of our whole blood model is an obvious limitation of the study, as is the lack of circulation and intact endothelium, and organ perfusion. The strength of our whole blood model addresses some of these limitations, since it enables all inflammatory systems in blood, both cellular and fluid-phase, to mutually interact. "Therapeutic" intervention in this model reveals important cross-talk between these systems, which is not possible when working with isolated cell systems. Still, future studies could very well address the question of both phagocyte and bacterial viability over time, perhaps within a longer time-frame using isolated cell systems. Equally, we need to assess the possible applicability of combined C5aR1 and CD14 inhibition in sepsis by conducting larger, *in vivo* studies to verify the findings of the data presented here.

4.1. Conclusion

C5aR1 was crucial for phagocytosis-induced inflammation of live and dead *E. coli* and *S. aureus,* which are both clinically relevant bacteria. Additionally, however, CD14 contributed to this effect as combined C5aR1 and CD14 inhibition was the most efficient in reducing the inflammatory response in general.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.03.014.

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Glossary

C5a: Complement protein 5 fragment a
C5aR1: C5a receptor 1 (CD88)
IFN: Interferon
IL: Interleukin
IRF: Interferon regulatory factor
LPS: Lipopolysaccharide
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMPs: Pathogen associated molecular pattern
PBS: Phosphate-buffered saline
TLR: Toll-like receptor
TNF: Tumor necrosis factor