

ARTICLE

TLR8 and complement C5 induce cytokine release and thrombin activation in human whole blood challenged with Gram-positive bacteria

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Abstract

We recently showed that TLR8 is critical for the detection of Gram-positive bacteria by human monocytes. Here, we hypothesized that TLR8 and complement together regulate antibacterial responses in human blood. Anticoagulated blood was treated with selective inhibitors of TLR8 and/or complement C5, and then challenged with live *Streptococcus agalactiae* (Group B streptococcus, GBS), *Staphylococcus aureus*, or *Escherichia coli*. Cytokine production, plasma membrane permeability, bacterial survival, phagocytosis, and activation of coagulation was examined. GBS and *S. aureus*, but not *E. coli*, triggered TLR8-dependent production of IL-12p70, IL-1 β , TNF, and IL-6 in fresh human whole blood. In purified polymorphonuclear neutrophils (PMN), GBS and *S. aureus* induced IL-8 release in part via TLR8, whereas PMN plasma membrane leakage and extracellular DNA levels increased independently of TLR8. TLR8 was more important than C5 for bacteria-induced production of IL-12p70, IL-1 β , and TNF in blood, whereas IL-8 release was more C5 dependent. Both TLR8 and C5 induced IL-6 release and activation of prothrombin cleavage, and here their combined effects were additive. Blocking of C5 or C5aR1 attenuated phagocytosis and increased the extracellular growth of GBS in blood, whereas TLR8 inhibition neither reduced phagocytosis nor intracellular killing of GBS and *S. aureus*. In conclusion, TLR8 is more important than C5 for production of IL-12p70, IL-1 β , and TNF upon GBS and *S. aureus* infection in blood, whereas C5 is central for IL-8 release and phagocytosis. Both TLR8 and C5 mediate IL-6 release and activation of coagulation during challenge with Gram-positive bacteria in blood.

KEYWORDS

innate immunity, bacterial infection, blood, phagocytes, cytokines, phagocytosis, prothrombin

1 | INTRODUCTION

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection.¹ Despite much effort, no specific therapy has been developed.^{2,3} When the host loses local

containment of an infection, the body is systematically exposed to microbes and products of damaged tissue which is sensed by the innate immune system.^{4,5} The subsequent rapid inflammatory response is fundamental to clear infections. On the other hand, the systemic activation of innate defense is potentially harmful and plays a major

Abbreviations: aC3, C3 inhibitor; aC5, anti-C5 monoclonal antibody; C5, complement factor 5; C5aR1, C5a receptor 1; C5aRA, C5a receptor 1 antagonist; GBS, Group B streptococcus; NETs, neutrophil extracellular traps; PMN, polymorphonuclear neutrophils; PTF₁₊₂, prothrombin fragment 1+2; pU, polyuridylic acid; TF, tissue factor.

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role in the early stages of sepsis pathophysiology, and leads both to hyperinflammation and immunosuppressive counter-reactions.⁶ It is therefore vital to understand the mechanisms of how pathogens are recognized and how the innate defense system is regulated in order to identify new targets for treatment of sepsis.

The TLR family and the complement system are key sensor and effector systems of innate immunity. The TLRs recognize microbial components and elicit potent proinflammatory responses when activated. TLR8 function as an endosomal sensor of the ssRNA degradation products uridine and short ssRNA oligomers, which bind cooperatively to the preformed TLR8 dimer and activate signaling.⁷ We and others have earlier shown that TLR8 contributes to sensing of bacteria such as GBS, *S. aureus*, *S. pyogenes*, and *E. coli*.^{8–11} Using a selective small-molecule inhibitor of TLR8,¹² we recently showed that TLR8 is a dominant sensor of the Gram-positive bacteria GBS, *S. aureus*, and *S. pneumoniae* in monocytes, whereas it appears considerably less important for the detection of Gram-negative species.¹³

The complement system is a part of the innate defense system where it facilitates phagocytosis via C3b opsonization, induces inflammation via anaphylatoxins such as C3a and C5a, and lyses sensitive bacteria via terminal complement complex formation.¹⁴ Systemic activation of complement can be harmful, triggering excessive inflammation and organ damage in sepsis.^{14,15} The C5a–C5aR1 signaling axis can be central in such processes.¹⁶ Blocking complement together with CD14, a coreceptor for TLR2 and TLR4, efficiently attenuates the cytokine induction by *E. coli* in human blood.^{17,18} Combined C5- and CD14-blockade can be protective during experimental sepsis in large mammals,^{19–21} and attenuates inflammation in human whole blood challenged with *S. aureus*.²² Further, the complement system interacts with the clotting mechanism in several ways, such as the C5a-mediated induction of tissue factor (TF) expression and activity on several cell types, triggering the intrinsic clotting pathway.¹⁴ Sepsis-induced disseminated intravascular coagulation (DIC) is primarily TF driven,²³ and in the human blood model where the thrombin inhibitor lepirudin is used, *S. aureus* activates prothrombin cleavage mainly via C5a-induced TF activity on monocytes.²⁴ In this infection model all leukocytes, complement factors, and parts of the coagulation system are present and functional under normal physiologic conditions. Therefore, it mimics normal blood stream infections, and is more relevant than simpler cell monoculture infection models. We here examined the effects of selective TLR8 inhibition in whole blood, alone and in combination with blockade of C5, during bacterial infection.

2 | MATERIALS AND METHODS

2.1 | Materials

The TLR8 antagonists CU-CPT9a and CU-CPT9b are previously described,¹² and they were generously provided together with a control reagent by The Regents of the University of Colorado, a body corporate for and on behalf of the University of Colorado Boulder. FSL-1, CL075, LPS (O111:B4), and polyuridylic acid (pU) were purchased from InvivoGen (Toulouse, France). Poly-L-arginine,

Dulbecco's PBS supplemented with Ca²⁺ and Mg²⁺, and EDTA, and Saponin were from Sigma–Aldrich (Steinheim, Germany). The recombinant hirudin analogue lepirudin (Refludan) was from Pharmion (Windsor, UK). Complement C3 inhibitor Compstatin (CP40, 20 μM final concentration) was kindly provided by professor J.D. Lambris (University of Pennsylvania, USA). The humanized IgG2/4 anti-C5 inhibitory antibody (eculizumab/Soliris®) was from Alexion Pharmaceuticals (Cheshire, CT, USA), and was used at 100 μg/ml. Recombinant anti-CD14 IgG2/4 hybrid (r18D11, 10 μg/ml final concentration) is previously described,²⁵ as is the IgG2/4 hybrid (NHDL) control Ab.²⁶ The C5a receptor 1 antagonist (C5aRA) PMX53 (10 μg/ml) was kindly provided by professor Trent Woodruff (University of Queensland, Australia). Bio-Plex multiplex cytokine assays were from Bio-Rad (Hercules, CA, USA). Prothrombin fragment (PTF) 1+2 plasma levels were determined with Enzygnost PTF₁₊₂ (monoclonal) kit from Siemens Healthcare (Marburg, Germany) as per manufacturer's instructions. Erythrocyte (RBC) lysis was done with the EayLyse™ buffer (Dako, Glostrup, Denmark). Sucrose (D+ Saccharose) was from VWR International (Radnor, PA, USA).

2.2 | Bacteria

The GBS NEM316 strain and the *S. aureus* 113 strain were generously provided by professors Philipp Henneke (University of Freiburg, Germany) and Friedrich Göetz (University of Tübingen, Tübingen, Germany), respectively. The *E. coli* Seattle 1946-strain was purchased from the American Type Culture Collection (ATCC 25922). The bacteria were grown on tryptic soy agar or blood agar plates. To prepare bacteria for experiments, bacterial colonies were inoculated in 5 ml tryptic soy broth (*E. coli* and *S. aureus*) or Todd-Hewitt broth (GBS) during vigorous shaking at 37°C overnight (12–18 h). Bacteria were quantified by optical density, as previously described.⁹

2.3 | Whole blood model and cytokine analysis

The whole blood model was performed as described previously.²⁷ In short, peripheral blood was drawn by venipuncture from healthy volunteers into 4.5 ml vacuum tubes (Nalge Nunc, Rochester, NY, USA) containing the thrombin inhibitor lepirudin (50 μg/ml). It was not investigated whether the donors previously had been sensitized to the bacterial antigens used.

The baseline sample (T0) was collected immediately after blood was drawn with addition of EDTA to 10 mM to block further complement activation. Lepirudin blood was quickly aliquoted into 1.8 ml round bottom cryotubes (Nalge Nunc). For the experiments performed with TLR8 inhibitors in combination with anti-C5 and C5aRA (PMX53), blood was preincubated with CU-CPT9b (5 μM) or control compounds/vehicle on a tube roller at 37°C for 90–120 min. Subsequently, a T0 sample after preincubation was collected and ligands or live bacteria were added and incubated further. For the experiments performed with CP40, anti-C5, and anti-CD14, blood was mixed with the inhibitors a few min prior to the addition of ligands or bacteria. The TLR8 ligand pU was premixed with poly-L-arginine to allow complex

formation before addition to blood. After incubation for 120–240 min, aliquots were sampled and EDTA was added. Plasma was isolated by centrifugation of blood at $1700 \times g$ for 15 min, and stored at -20°C until analyses. Cytokine analysis was done using Bio-Plex assays and analysis on a Bio-Plex™ 200 System using a Bio-Plex Pro™ Wash Station, as described by the manufacturer (Bio-Rad).

2.4 | Leukocyte viability

To examine the viability of monocytes and granulocytes (PMN) in blood after 240 min of infection with GBS or *S. aureus*, 50 μl of blood was labelled with anti-CD14 (FITC; Dako) for 30 min on ice. RBC were lysed with 1.5 ml lysis reagent for 15 min at RT, and leukocytes (WBC) were centrifuged at $270 \times g$ for 5 min and resuspended in 400 μl PBS. The plasma membrane integrity was then examined by addition of propidium iodide (PI) at 3 $\mu\text{g}/\text{ml}$ approximately 5 min prior to the analysis on by flow cytometry using a BD FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA). Monocytes and granulocytes were gated according to FITC staining and side scatter, and the fraction of PI positive cells (PE gate) was determined.

2.5 | Neutrophil isolation and infection

Human polymorphonuclear neutrophils (granulocytes; PMN) were obtained from fresh peripheral blood drawn by venipuncture as described.²⁸ Briefly, venous blood from healthy volunteers was collected in isocitrate anticoagulant solution and centrifuged at $250 \times g$ for 10 min. WBC were obtained from the pellet following RBC sedimentation in 2.0% Dextran T-500 (Sigma–Aldrich). PMN were then separated by centrifugation on a 10 ml cushion of Lymphoprep™ (Axis-Shield). Contaminating RBC were removed by 20 seconds of hypotonic lysis in water, before adding concentrated HBSS. Viability was higher than 98%, based on trypan blue dye exclusion and Cell Hostess Counter quantification (Thermo Fisher Scientific, Waltham, MA, USA). The entire procedure was carried out at RT under sterile conditions. PMN were resuspended in RPMI with 10% human serum, and 5×10^6 cells were seeded per well in 24-well culture plates (Costar). The cells were pretreated with CU-CPT9a (5 μM) for 60 min to block TLR8 function, or with DMSO as a vehicle control. Subsequently, 1×10^7 or 1×10^8 live GBS or *S. aureus* were added to a final volume of 1 ml per well. After 60 min of incubation at 37°C and 5% CO_2 , Gentamicin (100 $\mu\text{g}/\text{ml}$) was added and the cultures were incubated for additional 5 h. Cell free supernatants were collected after brief centrifugation and stored at -20°C until analyses. Extracellular DNA was determined using the fluorescent DNA-binding dye CyQUANT® GR (Thermo Fisher Scientific), according to the manufacturer's procedure. The fluorescence intensity was quantified by excitation/emission at 485/535 nm using the Victor3™ 1420 multilabel counter (PerkinElmer, Waltham, MA, USA).

2.6 | Bacterial survival

Anticoagulated blood was incubated with CU-CPT9b or DMSO control for 90–120 min. Subsequently, live bacteria were added and mixed by pipetting. Immediately (T0) and after 240 min of incubation on a

tube roller at 37°C , 20 μl blood was lysed by dilution into 180 μl ice cold sterile water with 0.3% saponin. The lysed blood samples were further diluted in a 10-fold series, and 30 μl of each dilution was plated in triplicates on blood agar. After incubation at 37°C over night, the number of CFU were counted and the corresponding CFU concentration blood was calculated. Similar experiments were performed using lepirudin plasma.

The number of intracellular viable bacteria in WBC was determined by a previously described method,²⁹ using some modifications. After bacterial challenge for 240 min, 1 ml blood was diluted in 9 ml of RBC lysis buffer using 15 ml tubes (Nunc) at RT for 10 min. The lysed blood was centrifuged at $300 \times g$ for 10 min at 4°C , and the plasma/lysis phase containing extracellular bacteria was carefully removed. The WBC pellet was dissolved in 1.7 ml ice cold PBS and centrifuged in round-well cryotubes (Nunc) at $130 \times g$ for 5 min at 4°C . The supernatant was removed by careful pipetting and the wash step was repeated 1 time. For stringent elimination of the remaining extracellular bacteria, the WBC pellet was dissolved in 0.5 ml ice cold PBS and underlayered with 1 ml ice cold 30% sucrose in PBS. Cells were pelleted at $200 \times g$ for 10 min at 4°C , and the supernatant was carefully removed. Next, the WBC pellet was dissolved in ice cold PBS to the original blood volume of 1 ml. Finally, 20 μl of dissolved WBC was lysed, serially diluted, and plated for CFU quantification, as described above.

2.7 | Phagocytosis quantification

GBS and *S. aureus* were grown in broth over night and dissolved in 0.2 M NaHCO_3 at 1×10^{10} bacteria/ml. The bacteria were labelled with Alexa Fluor™ 488 TFP ester (Thermo Fisher Scientific, 100 $\mu\text{g}/\text{ml}$) at RT for 1 h, washed, and dissolved in DPBS supplemented with Ca^{2+} and Mg^{2+} at 5×10^7 bacteria/ml. Lepirudin anticoagulated whole blood was pretreated with inhibitors for 90 min before labeled bacteria were added (5×10^6 bacteria/ml). Blood aliquots were sampled after 60 and 120 min and placed on ice. Phagocytosis was quantified using the PHAGOTES™ reagent kit, according to the protocol of the manufacturer (Glycotope Biotechnology, Heidelberg, Germany). Leukocytes were labeled using anti-CD45-PerCp and anti-CD14-PE (BD Biosciences), and analysis was done on a BD FACSCanto™ II flow cytometer. Populations were gated according to side scatter and PerCp and PE staining, and the mean fluorescence intensity (MFI, FITC channel) of the monocyte and granulocyte populations were determined.

2.8 | Statistics

Statistical analyses were done on data sets from independent, consecutive experiments, and the number of consecutive independent experiments performed are indicated in the figure legends (N). Since such data typically can have a log-normal distribution, log-transformation was done prior to the analysis. Data sets were analyzed with GraphPad Prism software, using 1way repeated-measures analysis of variance (RM-ANOVA) and Dunette's multiple comparison test, or 2-way RM-ANOVA and Bonferroni's multiple comparison test. Significant differences between the control/vehicle—and the

treatment—conditions are indicated with the symbol “*”, whereas horizontal bars indicate comparisons of specific treatments. Significance levels correspond to * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

2.9 | Safety and ethics

The research was carried out according to the standard institutional safety procedures (biosafety level II) at the Norwegian University of Science and Technology. The use of human whole blood for our experiments was approved by the Regional Committee for Medical and Health Research Ethics in Central Norway (REC Central), The Norwegian Ministry of Education and Research, no. 2009/2245.

3 | RESULTS

3.1 | Efficacy of the TLR8 inhibitor CU-CPT9b in blood

To determine the minimum concentration of CU-CPT9b required to block TLR8 function, anticoagulated blood was pretreated with the inhibitor or the control compound at different concentrations for 120 min, and subsequently the TLR8 agonists pU or CL075, the TLR4 agonist LPS, or vehicle control (PBS) were added. Cytokine analyses in plasma after 240 min of treatment revealed that the TLR8 inhibitor efficiently blocked the CL075 induced production of TNF, IL-1 β , and IL-6 at the lowest dose tested (2.5 μ M), whereas the pU induced response was fully blocked at 5–10 μ M (Supplemental Fig. 1A). IL-8 showed a similar tendency but was weakly induced. The LPS-induced cytokine release was not affected by CU-CPT9b, whereas the control compound (Supplemental Fig. 1B) did not influence the TLR8-induced responses (Supplemental Fig. 1A). Hence, we considered 5 μ M CU-CPT9b to be optimal for inhibition of TLR8 activation in blood, which is similar to our recent findings with the analogue CU-CPT9a in cultures of purified monocytes.¹³

3.2 | TLR8-inhibition attenuates cytokine induction by GBS and *S. aureus* in whole blood

To determine the impact of TLR8 for the sensing of viable bacteria in blood, we pretreated blood with 5 μ M of CU-CPT9b or the control reagent for 120 min, and subsequently added pU, LPS, FSL-1 (TLR2/6 agonist), or live GBS, *S. aureus* or *E. coli* at different doses. Blood plasma was separated 240 min after the initiation of the challenge and cytokine levels were examined. As expected, TLR8 blockade almost eliminated the cytokine induction by pU, whereas it had no effect on the cytokine induction by LPS or FSL-1 (Fig. 1). More importantly, CU-CPT9b clearly reduced the production of cytokines after GBS and *S. aureus* challenge. The effect was most pronounced for IL-12p70, IL-1 β , and TNF, but also significant for IL-6 for the lower bacterial concentrations. There were no major effects of CU-CPT9b treatment on the release of IL-8 or MCP-1 (data not shown). Moreover, cytokine induction by *E. coli* was mainly TLR8 independent, which is in agreement with our recent findings with this strain in monocyte

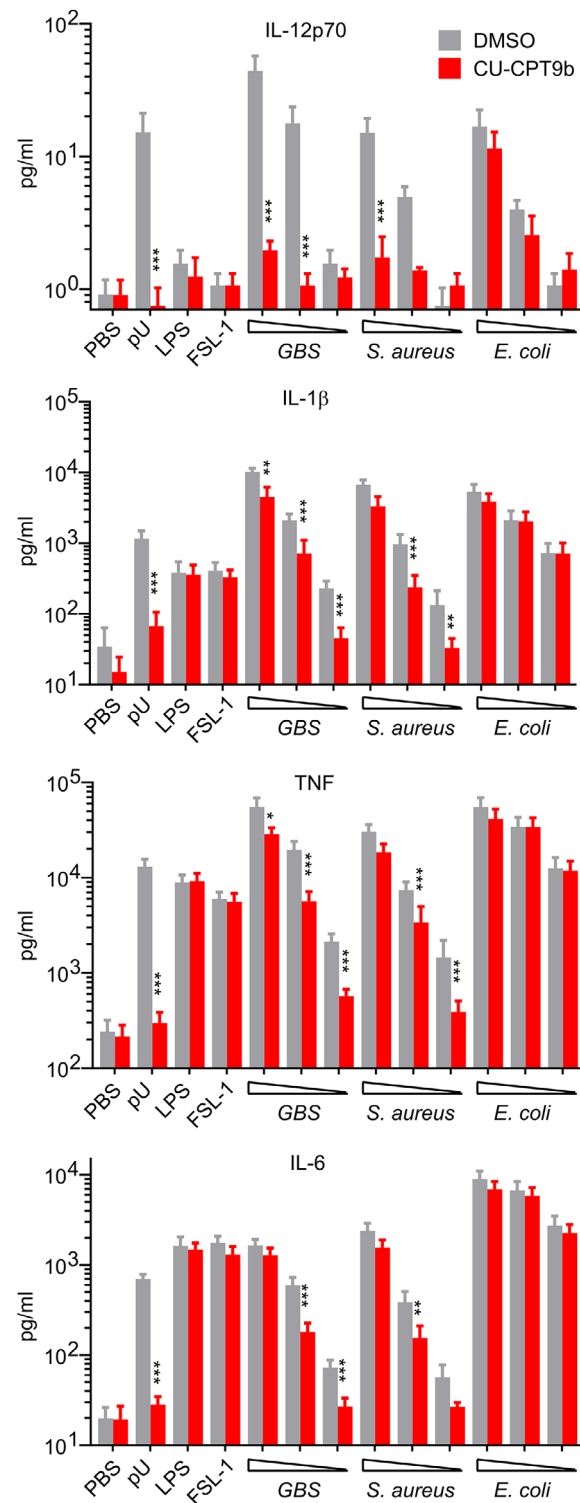


FIGURE 1 GBS and *S. aureus* challenge of whole blood induces cytokine release in part dependent on TLR8, whereas this is not seen with *E. coli*. Anticoagulated blood was incubated with the TLR8 inhibitor CU-CPT9b or a control reagent. Subsequently, challenge with live GBS or *S. aureus* (1×10^6 – 1×10^5 – 1×10^4 bacteria/ml) or *E. coli* (1×10^5 – 1×10^4 – 1×10^3 bacteria/ml) was done for 240 min. Agonists for TLR8 (pU, 1 μ g/ml), TLR4 (LPS, 100 ng/ml), and TLR2 (FSL-1, 100 ng/ml) were included as controls. The graphs show the mean plasma cytokine levels (log-scale) + SEM. $N = 6$ consecutive experiments

cultures.¹³ We also included 2 clinical isolates of *E. coli* that to some extent activate TLR8 in monocytes, but no clear effect of CU-CPT9b on cytokine induction in blood was found (data not shown). Over all, cytokine induction by live GBS and *S. aureus* challenge of human blood is partially TLR8 dependent, whereas *E. coli* is sensed mainly in a TLR8-independent manner.

3.3 | Monocyte and PMN viability and release of DNA and IL-8 by PMN

The integrity of the plasma membrane of monocytes and PMN during GBS and *S. aureus* challenge of blood for 240 min was examined by PI staining and flow cytometry analysis. The membrane integrity of PMN, but not monocytes, was compromised during challenge with the highest bacterial load, but TLR8 inhibition did not influence this (Fig. 2A). To further examine whether TLR8 has a functional role in PMN during bacterial infection, purified PMN were seeded in cell culture plates and pretreated with CU-CPT9a (5 μ M), and challenged with live bacteria for 6 h. The highest concentrations of GBS and *S. aureus* significantly increased the levels of extracellular DNA in the culture supernatant (Fig. 2B). The extracellular levels of DNA correlate with the loss in PMN plasma membrane integrity in blood, and may represent neutrophil extracellular traps (NETs) or DNA released via other forms of necrotic cell death.³⁰ Anyway, both effects occurred independently of TLR8 signaling. In contrast, IL-8 production by PMN in response to both bacteria was partially reduced by TLR8 inhibition, most strongly during GBS challenge (Fig. 2B). This suggests that TLR8 directly contribute to the sensing of bacteria in PMN.

3.4 | Impact of complement and CD14 for cytokine responses to live bacteria in blood

Inhibition of complement at the level of C3 or C5 combined with CD14-blockade attenuates cytokine release in blood challenged with *E. coli* and *S. aureus*.^{18,22} We compared the efficacy of the C3-inhibitor Compstatin (CP40; α C3), the C5-blocking antibody eculizumab (α C5), and a CD14-blocking antibody during challenge with GBS, *S. aureus* and *E. coli*. Plasma cytokine levels were determined after challenge for 120 (Supplemental Fig. 2A) and 240 min (Supplemental Fig. 2B). C3- or C5-inhibition partially attenuated IL-1 β , TNF, IL-6, and IL-8 induction by the Gram-positive bacteria during 120 min of challenge (Supplemental Fig. 2A). GBS-induced cytokine production was minimally affected by CD14 blockade, whereas the cytokine induction by *S. aureus* was more attenuated, in particular the IL-6 release. Anti-CD14 strongly reduced the cytokine induction by *E. coli*, and when combined with inhibitors of C3 or C5 even further reduction was obtained (Supplemental Fig. 2A). The effects of C3 and C5 inhibitors were less clear after 240 min, although the IL-6 induction by *S. aureus* was still significantly reduced when combined with anti-CD14, and the *E. coli* induced cytokine release remained strongly attenuated. Inhibition at the level of C3 and C5 were almost equally efficient (Supplemental Fig. 2B). Since C5 blocking does not interfere with C3b-dependent opsonization, this seems to be a better and more selective target than C3.

3.5 | Effects of combined TLR8 and C5 inhibition in blood upon GBS and *S. aureus* challenge

In agreement with our hypothesis, the cytokine induction by the Gram-positive bacteria in blood is both TLR8 and complement dependent. Next, we examined if combined inhibition of TLR8 and C5 could further attenuate the cytokine responses. *E. coli* was excluded from this experiment, since it mainly triggered CD14-dependent and TLR8-independent cytokine production. CU-CPT9b markedly decreased the induction of IL-1 β and TNF during 120 min of challenge with GBS and *S. aureus*, with the strongest effect for GBS (Fig. 3A). C5 inhibition had similar but weaker effects, whereas the combined inhibition of C5 and TLR8 did not significantly reduce the IL-1 β and TNF levels more than TLR8 inhibition alone. On the other hand, IL-8 induction by both bacteria during 120 min of challenge was strongly dependent on C5, but not TLR8. Also, combined inhibition did not reduce IL-8 more than C5 inhibition alone (Fig. 3A). Induction of IL-6 during 120 min of challenge with GBS was dependent on both C5 and TLR8, whereas *S. aureus* triggered early IL-6 release mainly via C5. Combined inhibition did not reduce the IL-6 release significantly more than single inhibition. After 240 min of challenge IL-12p70 was induced, mainly in a TLR8-dependent manner (Fig. 3B). Moreover, the effect of C5 inhibition on the other cytokines was diminished, except for IL-6, whereas the effect of CU-CPT9b had increased and partially attenuated also the IL-8 production for the low bacterial dose. For the high bacterial dose, no significant reduction in IL-8 levels was obtained with the inhibitors (Fig. 3B). IL-6 was most strongly reduced when combining the C5- and TLR8-inhibitors. Taken together, cytokine induction in blood by the Gram-positive bacteria is dependent on both TLR8 and C5, as hypothesized. We observed an increased impact of TLR8 and a reduced effect of C5 at the later time point. Combined blockade of C5 and TLR8 had additive effects, most notably on IL-6 production.

3.6 | Impact of TLR8 and C5 on growth and survival of bacteria in blood

To examine whether inhibiting TLR8 or C5 influenced the bacterial survival in blood, an aliquot of blood samples was lysed, diluted, and plated on blood agar. The viability (CFU/ml) of GBS strongly increased during 240 min of challenge, whereas the *S. aureus* viability declined (Fig. 4A). Inhibition of C5 alone or in combination with TLR8 further increased the number of viable GBS in blood for the highest bacterial dose, whereas CU-CPT9b alone did not have any effect (Fig. 4A). For *S. aureus*, neither inhibitor influenced the bacterial numbers. Gram-positive bacteria are generally resistant against direct complement mediated lysis by the membrane attack complex, and the GBS and *S. aureus* strain used replicated efficiently in lepirudin plasma, with no direct effect of CU-CPT9b on the bacterial growth (Supplemental Fig. 3). To discriminate intracellular and extracellular viable bacteria, leucocytes (WBC) were isolated after 240 min of bacterial challenge, washed, lysed, and plated for CFU quantification. Compared with whole blood, few viable bacteria were present in the WBC fraction, suggesting that phagocytes kill both GBS and *S. aureus* efficiently

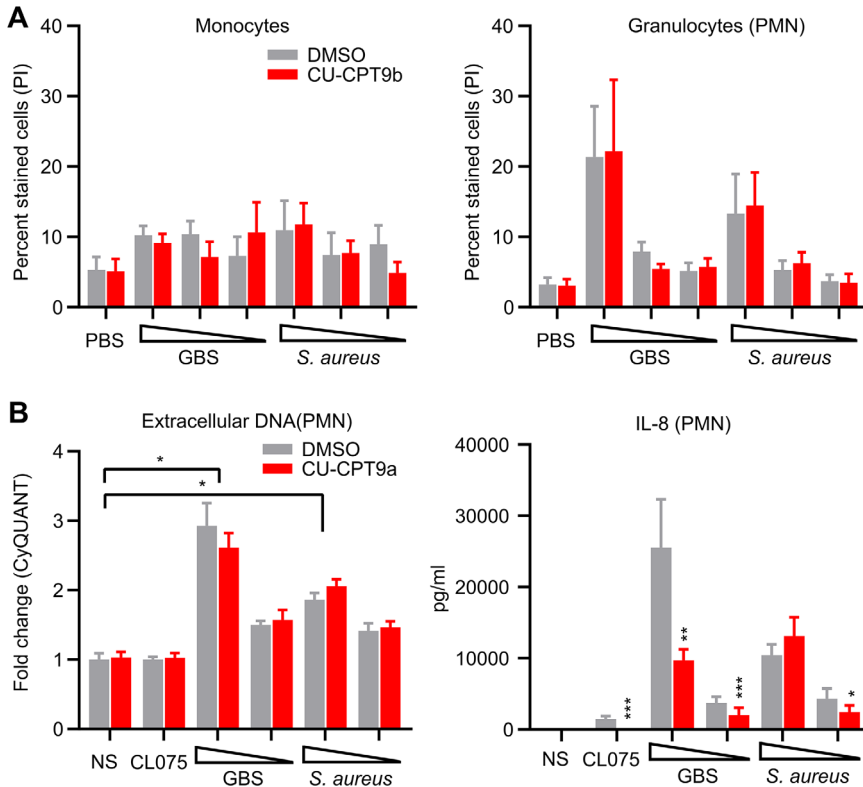


FIGURE 2 Effect of TLR8 inhibition on monocyte and granulocyte (PMN) plasma membrane integrity during GBS and *S. aureus* challenge in whole blood, and role of TLR8 in purified PMN during bacterial challenge. (A) Anticoagulated blood was treated with TLR8 inhibitor (CU-CPT9b) or vehicle (DMSO) and challenged with live GBS or *S. aureus* (1×10^6 – 1×10^5 – 1×10^4 bacteria/ml) for 240 min. The fraction of monocytes and granulocytes with compromised plasma membrane integrity was determined with propidium iodide staining and flow cytometry. The graphs show mean + SEM ($N = 3$). (B) PMN were isolated from whole blood and treated with TLR8 inhibitor (CU-CPT9a) or vehicle and challenged with GBS and *S. aureus* (1×10^6 – 1×10^5 bacteria/ml) for 6 h. The amounts of extracellular DNA and IL-8 in the supernatants were determined. The graphs show means + SEM ($N = 6$)

after phagocytosis, and this was independent of TLR8 (Fig. 4B). To clarify if TLR8 or C5 directly affected phagocytosis, uptake of fluorescently labelled bacteria by monocytes and PMN in blood was quantified by flow cytometry. Anti-C5 or a C5a receptor antagonist (C5aRA) reduced the uptake of GBS by monocytes and PMN during 60 and 120 min of challenge, whereas TLR8 inhibition had no clear effect (Supplemental Fig. 4A). Anti-C5 or C5aRA treatment also reduced the uptake of *S. aureus* during 60 min of challenge, whereas the effect declined during longer incubation, and TLR8 inhibition had no clear effects (Supplemental Fig. 4B).

Taken together, TLR8 appears dispensable for phagocytosis and intracellular bacterial killing in the whole blood model, whereas C5 limits the extracellular growth of GBS by facilitating phagocytosis via C5aR1 activation. Uptake of *S. aureus* in the early phase also depends on C5a–C5aR1 signaling, but this becomes redundant during prolonged challenge, and is not critical to control the infection.

3.7 | Effect of TLR8 and C5 on coagulation activation by bacteria

Coagulation disturbances are believed to be of major importance in sepsis. Therefore, we examined the effects of inhibiting TLR8 and C5 on activation of coagulation during infection. Because clotting in this model is prevented by the thrombin inhibitor lepirudin, initiation of coagulation was examined at the stage of prothrombin cleavage. Prothrombin fragments (PTF₁₊₂) in plasma were detected with ELISA after 240 min of bacterial challenge. GBS and *S. aureus* activated coagulation in a C5-dependent manner at both doses of inoculum (Fig. 5). With CU-CPT9b alone, there was also a tendency towards reduction

of PTF₁₊₂ levels. Combined C5- and TLR8-inhibition gave an additive effect, and strongly reduced the initiation of coagulation triggered by the bacteria.

Taken together, our data indicate that TLR8 is important for the production of central proinflammatory cytokines in human blood exposed to live GBS and *S. aureus*, whereas *E. coli* is sensed mainly in a TLR8-independent manner. TLR8 in PMN contribute to IL-8 release during Gram-positive challenge, although in whole blood IL-8 release is mainly C5 driven. IL-6 production and initiation of coagulation is dependent on both TLR8 and C5, and combined inhibition is an efficient means of attenuating these responses. Phagocytosis and intracellular bacterial killing do not require TLR8 signaling, whereas signaling via C5aR1 enhance phagocytosis and appears especially important for limiting the extracellular growth of GBS.

4 | DISCUSSION

We here examined the importance of TLR8 and C5 for the activation of antibacterial responses in a human whole blood model of bacteremia. The recently developed small molecule inhibitor of TLR8¹² is highly efficient and selective also in human blood. Inhibiting TLR8 reveals its importance for the production of IL-12p70, IL-1 β , and TNF during *S. aureus* and GBS challenge in blood, and is in accordance with our recent findings of a dominating role of TLR8 in the sensing of Gram-positive bacteria in monocytes, mediating strong induction of IFN β ,^{8,9} IL-12p70, IL-1 β , TNF, IL-6, and IL-10.¹³ The Gram-positive bacteria induced IL-12p70 production in whole blood almost exclusively via TLR8. This is also seen in

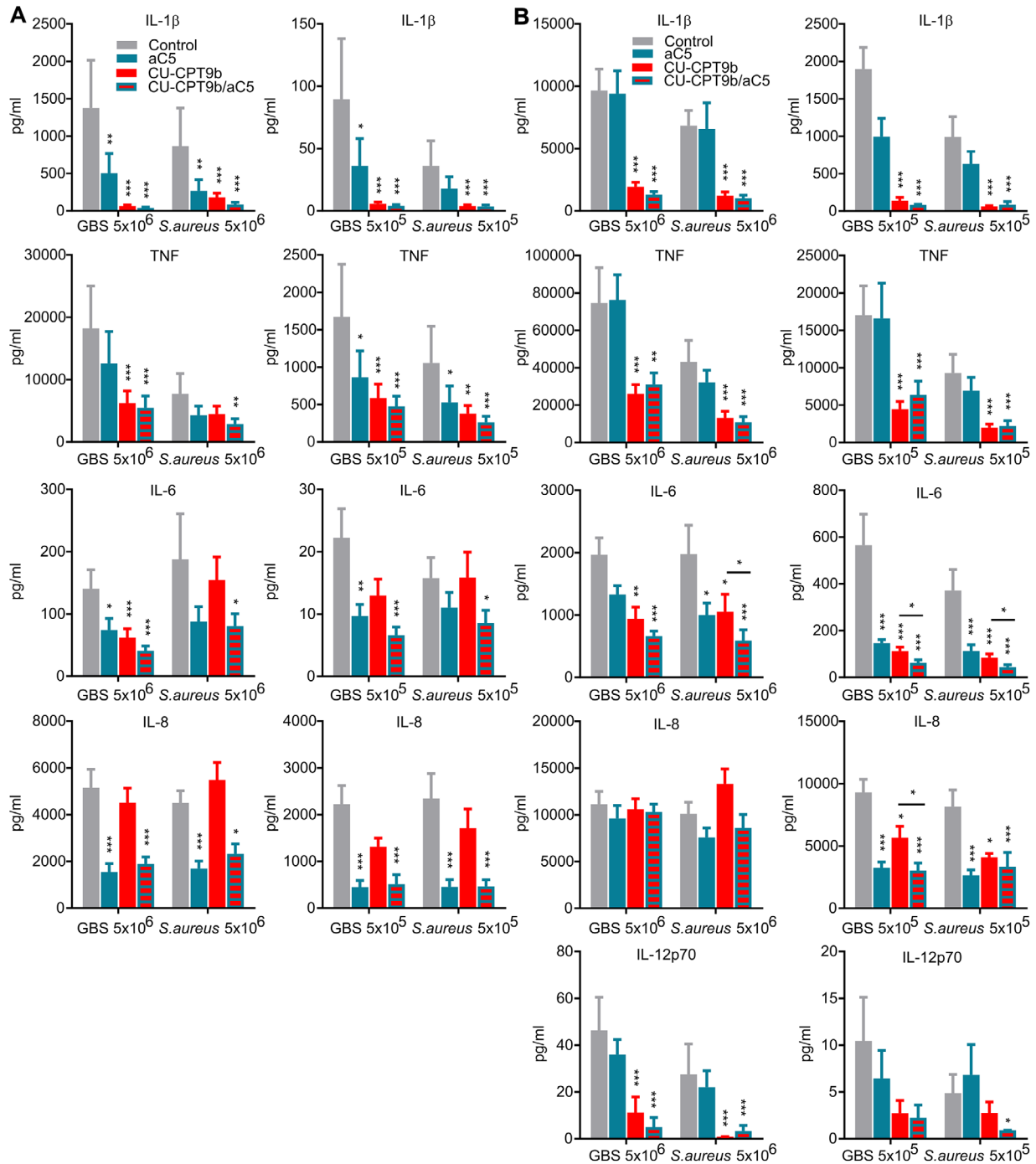


FIGURE 3 Cytokine induction by GBS and *S. aureus* in whole blood is variably dependent on TLR8 and C5. Anticoagulated blood was treated with TLR8 inhibitor CU-CPT9b or anti-C5 (eculizumab; aC5), individually or in combination, and a nonspecific antibody in combination with a control reagent served as control. Subsequently, the blood was challenged with live GBS or *S. aureus* (5×10^6 bacteria/ml or 5×10^5 bacteria/ml). Cytokine levels in plasma were examined after (A) 120 min and (B) 240 min of infection. In noninfected blood the mean cytokine level (pg/ml) after 120/240 min of incubation were: IL-1 β (2/11), TNF (138/231) IL-6 (7/14), IL-8 (866/2489), IL-12p70 (–/1). The graphs show means + SEM ($N = 6$)

purified monocytes, where TLR8 agonists induce much more IL-12p70 compared with TLR2- or TLR4-agonists.¹³ Induction of IL-12p70 and IFN β via TLR8 requires IRF5 nuclear translocation, which is not triggered by surface TLR signaling.^{8,9,13} TLR2 and TLR4 trigger robust production of IL-1 β , TNF and IL-6 by monocytes,¹³ probably via canonical NF- κ B- and MAPK-pathways. IRF5 activation is not

essential, but still potentiates the induction of IL-1 β , TNF, and IL-6 in TLR8 signaling.³¹

Human PMN express all TLRs except TLR3 and TLR7.³² Synthetic TLR8 ligands induce high levels of IL-8 release in highly pure PMN cultures, whereas the induction of other cytokines is much lower.³³ We here show that TLR8 contributes to the release of IL-8 by PMN

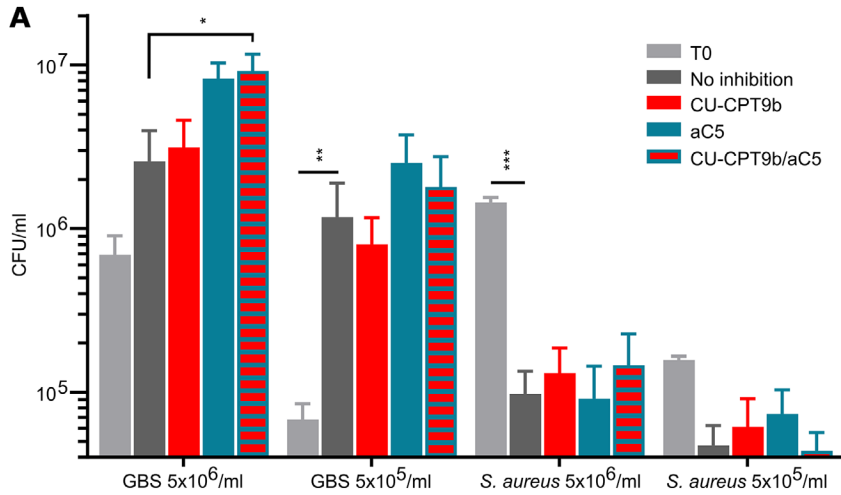


FIGURE 4 C5 cleavage but not TLR8 signaling attenuates the growth GBS in human blood, whereas both processes are dispensable for controlling the growth of *S. aureus*. (A) Anticoagulated blood was treated with inhibitors of TLR8 or C5, individually or in combination, or with PBS as a vehicle control (no inhibition). Live GBS or *S. aureus* (5×10^6 bacteria/ml or 5×10^5 bacteria/ml) were added, and blood aliquots were sampled immediately (T0) or after 240 min of incubation, lysed and plated on blood agar to calculate the numbers of CFU. The graph shows means + SEM ($N = 6$). (B) Effect of TLR8 inhibition on intracellular bacterial survival. Anticoagulated blood was treated with TLR8-inhibitor CU-CPT9b or DMSO as vehicle control. Subsequently, live bacteria were added (5×10^6 or 5×10^5 bacteria/ml), and blood aliquots were sampled immediately (T0) or after 240 min of incubation. WBC were isolated, washed, and lysed to determine the number of intracellular viable bacteria. The graphs show means + SEM ($N = 4$)

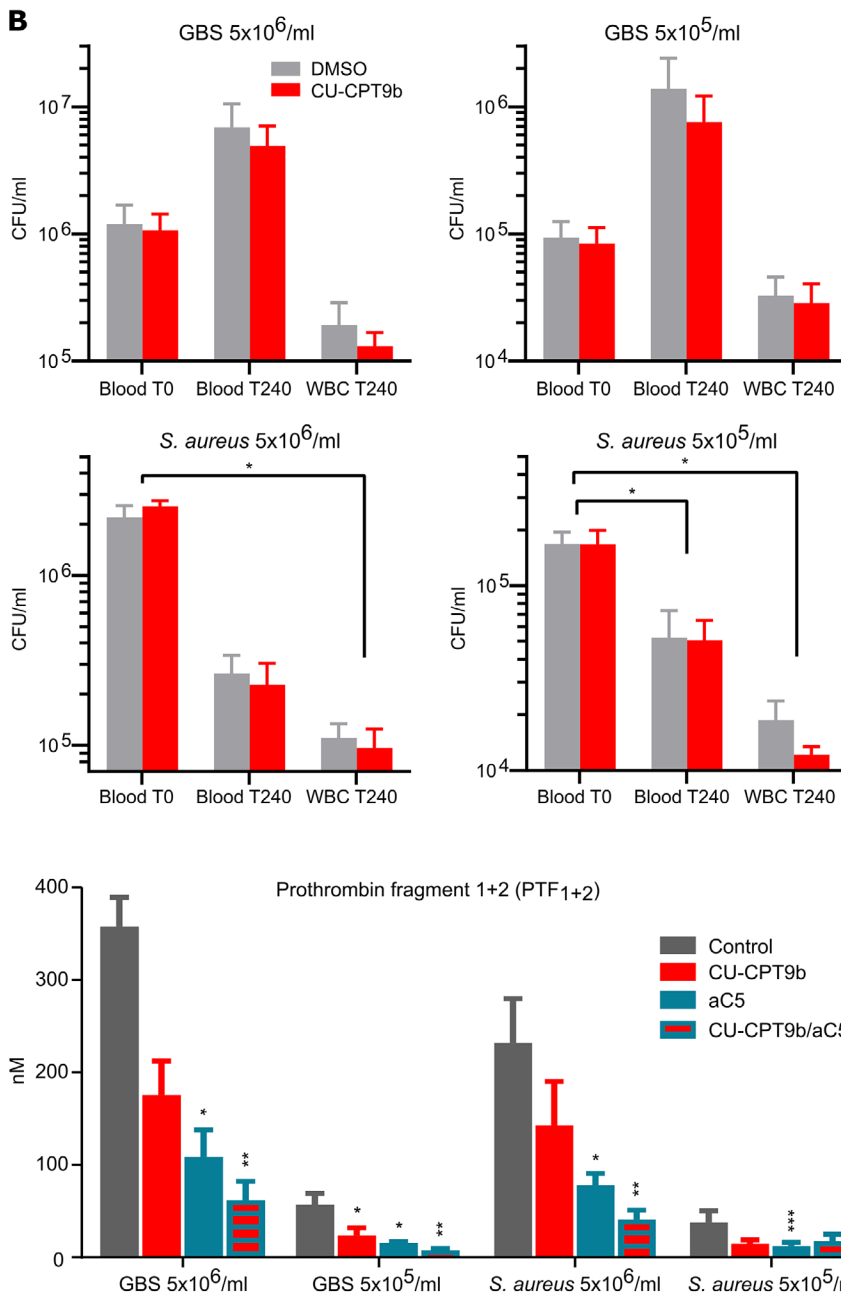


FIGURE 5 Prothrombin cleavage induced by GBS and *S. aureus* is partially dependent on TLR8 and C5. Anticoagulated blood was treated with TLR8 inhibitor CU-CPT9b or aC5, individually or in combination, or with a control mAb combined with control reagent (Control). Live GBS or *S. aureus* (5×10^6 bacteria/ml or 5×10^5 bacteria/ml) were incubated for 240 min, and the plasma levels of prothrombin fragment 1+2 (PTF₁₊₂) were determined with ELISA. In non-infected blood the mean level of PTF₁₊₂ after 240 min of incubation was 17.5 nM. The graph shows means + SEM ($N = 5$)

during Gram-positive infections, but this did not occur in monocyte cultures.¹³ In whole blood, bacteria triggered IL-8 release mainly in a C5-dependent manner, although TLR8 contributed nonredundantly in some conditions. Bacterial infection also resulted in increased permeability of the PMN plasma membrane and increased the levels of extracellular DNA. This could be a result of accidental or programmed necrosis, or it might be due to NET formation, which can be of vital or suicidal type.³⁰ TLR signaling may influence the PMN life span by triggering of NET formation, but TLR signaling in PMN also attenuates spontaneous apoptosis.³² However, our findings that may be related to these processes occurred independently of TLR8.

Cytokine induction by GBS challenge in blood appears more TLR8-dependent compared with *S. aureus*. TLR2 also contributes to *S. aureus* detection in blood,²² and TLR2 signaling reduces TLR8-mediated IRF5-activation.⁸ These findings are consistent with a more important role of CD14 on *S. aureus* induced cytokines, as CD14 is a cofactor in TLR2- and TLR4-signaling that enhances cytokine responses.³⁴ In contrast, the majority of GBS strains produce little TLR2 activating lipoproteins,³⁵ explaining why anti-CD14 treatment during challenge only gave minor effects. Combined inhibition of complement and CD14 attenuated the cytokine production more efficiently than either reagent alone during infection with *E. coli* and *S. aureus*.^{17,22} Similar effects by combining these inhibitors were seen in the current study, also with GBS challenge.

TLR8 can sense purified *E. coli* RNA and live *E. coli*.^{10,11,36} Here, the *E. coli* induced cytokine responses were highly dependent on CD14, but unaffected by TLR8 inhibition, which is in line with our recent findings with purified monocytes and this particular strain of *E. coli*.^{9,13} Still, TLR8 contributed significantly to IL-1 β and IL-12p70 induction by 2 clinical isolates of *E. coli* in monocytes, as well as with the Gram-negative bacterium *P. aeruginosa*. Also, *E. coli* activated TLR8 more strongly if CD14-dependent TLR4 signaling was blocked, as cell surface TLR signaling antagonizes TLR8-IRF5 activation and triggers redundant cytokine release.¹³ In the current study, also the *E. coli* isolates failed to mediate nonredundant cytokine production via TLR8 in blood, whereas the reasons for this discrepancy between these model systems are unclear.

Blocking C3 or C5 alone reduced IL-8 release by *E. coli*, but had otherwise minor effects on cytokine induction by this bacterium. C5a was previously identified as central for *E. coli* mediated IL-8 production.¹⁷ With GBS and *S. aureus* challenge, C3- or C5-inhibition reduced cytokine production more than with *E. coli*, and the C5a-C5aR1 interaction is known as important for *S. aureus* induced responses.²⁴ As expected, the Gram-positive bacteria induce cytokines in blood via both TLR8- and C5-dependent mechanisms, and combined inhibition gave an additive effect, most clearly seen for IL-6.

The number of viable GBS strongly increased during 240 min of incubation in blood, whereas the *S. aureus* numbers declined. This is in line with previous studies with these bacteria.^{24,37,38} Hence, phagocytes in blood more easily resolve infections with *S. aureus* than GBS. C5 inhibition further increased the growth of GBS, probably because C5aR1 signaling enhances phagocytosis. Similarly, distinct

complement inhibitors from scabies increased the growth of *S. pyogenes* in blood.³⁹ Phagocytosis of *S. aureus* was also reduced by C5- or C5aR-inhibition, in agreement with our previous findings.⁴⁰ Still, C5a-C5aR1 signaling gradually becomes dispensable for phagocytosis of this bacterium as time progresses, and C5 inhibition did not impair the resolution of infection in blood. Although costimulation with a TLR2 agonist increases the uptake of *S. aureus* by monocytes but not PMN in blood,⁸ anti-CD14 has only modest effects.⁴⁰ Sensing of bacteria via TLR8 occurs downstream of phagocytosis and bacterial degradation, and it is delayed relative to TLR2 activation. TLR8 signaling may therefore occur too late to influence the phagocytic process.

TLR8 inhibition neither affects the intracellular killing of the bacteria, even though TLR8 can trigger bactericidal effector mechanisms such as degranulation and respiratory burst in PMN in vitro.³³ This is in line with the finding that IRAK-4 deficiency does not impair intracellular killing of bacteria and fungi by human PMN.⁴¹

TLR8 also recognizes *S. pyogenes* in human monocytes.¹⁰ In murine macrophages TLR13 has a similar function as human TLR8, and deficiency in endosomal TLR signaling impaired early *S. pyogenes* recognition in a murine model of subcutaneous infection. Loss of endosomal TLR function led to local bacterial overgrowth and hyperinflammation, impaired bacterial containment, and systemic inflammation.⁴² Analogous, human TLR8 may be important for early detection of bacteria in the tissue, induction of inflammation, and leukocyte recruitment to the focus of infection. Furthermore, TLR8 can regulate adaptive immunity via IL-12 production.⁴³

Coagulation limits the spreading of invading bacteria, whereas DIC is a serious complication in sepsis. *E. coli* challenge triggers monocyte TF expression and coagulation in a C5a-C5aR1-dependent manner,¹⁷ and inhibition of C5 cleavage attenuated coagulopathy in a baboon model of *E. coli* sepsis.¹⁹ Activation of coagulation by *S. aureus* is C5 dependent,²⁴ and we here show that both C5 and TLR8 enhance prothrombin cleavage upon GBS and *S. aureus* infection, with an additive effect when these inhibitors are combined.

Numerous selective and nonselective anti-inflammatory reagents and strategies have been tested as adjuvant sepsis treatments, including TLR4 antagonist (eritoran), corticosteroids, anti-TNF strategies, as well as modulators of coagulation.⁴⁴ Although novel therapies have shown benefits in animal sepsis models, they have so far failed to translate to the clinical setting.⁴⁵ Anti-inflammatory intervention clearly has 2 sides: although it may reduce the harmful effects of excessive inflammation, it may also compromise the host's defense system against infections. Therefore, treating infections with immunomodulatory agents is a fine-tuned act of balance where patient stratification, timing and likely a combination of approaches is needed. We here show that TLR8 inhibition during Gram-positive challenge in blood results in strong and sustained reduction in proinflammatory cytokines, as well as reduction in the activation of coagulation, whereas at the same time phagocytosis and intracellular bacterial killing is sustained. Whether TLR8 inhibition can be useful as an adjuvant treatment in sepsis during phases of excessive inflammation remains an open question.

AUTHORSHIP

B.E., J.K.D., T.E., and J.S. contributed to the conception and design of the work. B.E. conducted blood infection experiments, viability studies, and cytokine analyses with assistance by J.F.K., L.R., J.S., and S.H.M. M.G. did the PMN experiments, L.R. analyzed phagocytosis, and J.S. examined bacterial growth and survival. Z.H. and H.Y. developed and provided the TLR8 inhibitors, and T.E.M provided inhibitors for complement and CD14, and instructions for their use. B.E. and J.S. wrote the original draft with critical comments by the coauthors, and J.S. did the final revision.

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DISCLOSURES

The authors declare no conflicts of interest.

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REFERENCES

- Singer M, Deutschman CS, Seymour C, et al. The third international consensus definitions for sepsis and septic shock (sepsis-3). *JAMA*. 2016;315:801-810.
- Gaieski DF, Edwards JM, Kallan MJ, et al. Benchmarking the incidence and mortality of severe sepsis in the united states. *Crit Care Med*. 2013;41:1167-1174.
- Weycker D, Akhras KS, Edelsberg J, et al. Long-term mortality and medical care charges in patients with severe sepsis. *Crit Care Med*. 2003;31:2316-2323.
- Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell*. 2010;140:805-820.
- Hotchkiss RS, Moldawer LL, Opal SM, et al. Sepsis and septic shock. *Nat Rev Dis Prim*. 2016;2:16045.
- Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. *N Engl J Med*. 2003;348(2):138-150.
- Tanji H, Ohto U, Motoi Y, et al. Autoinhibition and relief mechanism by the proteolytic processing of Toll-like receptor 8. *Proc Natl Acad Sci USA*. 2016;113:3012-3017.
- Bergström B, Aune MH, Awuh JA, et al. TLR8 senses Staphylococcus aureus RNA in human primary monocytes and macrophages and induces ifn- β production via a TAK1-IRK β -IRF5 signaling pathway. *J Immunol*. 2015;195:1100-1111.
- Ehrnström B, Beckwith KS, Yurchenko M, et al. Toll-like receptor 8 is a major sensor of group B Streptococcus but not Escherichia coli in human primary monocytes and macrophages. *Front Immunol*. 2017;8:1-14.
- Eigenbrod T, Pelka K, Latz E, et al. TLR8 senses bacterial RNA in human monocytes and plays a nonredundant role for recognition of Streptococcus pyogenes. *J Immunol*. 2015;195:1092-1099.
- Krüger A, Oldenburg M, Chebrolu C, et al. Human TLR8 senses UR/URR motifs in bacterial and mitochondrial RNA. *EMBO Rep*. 2015;16:1656-1663.
- Zhang S, Hu Z, Tanji H, et al. Small-molecule inhibition of TLR8 through stabilization of its resting state. *Nat Chem Biol*. 2018;14:58-64.
- Moen SH, Ehrnström B, Kojen JF, et al. Human Toll-like receptor 8 (TLR8) is an important sensor of pyogenic bacteria, and is attenuated by cell surface TLR signaling. *Front Immunol*. 2019;10:1-12.
- Markiewski MM, Nilsson B, Nilsson Ekdahl K, et al. Complement and coagulation: strangers or partners in crime. *Trends Immunol*. 2007;28:184-192.
- Ward PA. The dark side of C5a in sepsis. *Nat Rev Immunol*. 2004;4:133-142.
- Ward PA. The harmful role of C5a on innate immunity in sepsis. *J Innate Immun*. 2010;2:439-445.
- Lappegård TK, Christiansen D, Pharo A, et al. Human genetic deficiencies reveal the roles of complement in the inflammatory network: lessons from nature. *PNAS*. 2009;106:15861-15866.
- Gustavsen A, Nymo S, Landsem A, et al. Combined inhibition of complement and CD14 attenuates bacteria-induced inflammation in human whole blood more efficiently than antagonizing the Toll-like receptor 4-MD2 complex. *J Infect Dis*. 2016;214:140-150.
- Keshari RSS, Silasi R, Popescu NII, et al. Inhibition of complement C5 protects against organ failure and reduces mortality in a baboon model of Escherichia coli sepsis. *Proc Natl Acad Sci USA*. 2017;114:E6390-E6399.
- Skjeflo EW, Sagatun C, Dybwik K, et al. Combined inhibition of complement and CD14 improved outcome in porcine polymicrobial sepsis. *Crit Care*. 2015;19:1-8.
- Hellerud BC, Orrem HL, Dybwik K, et al. Combined inhibition of C5 and CD14 efficiently attenuated the inflammatory response in a porcine model of meningococcal sepsis. *J Intensive Care*. 2017;5:1-9.
- Skjeflo EW, Christiansen D, Espevik T, et al. Combined inhibition of complement and CD14 efficiently attenuated the inflammatory response induced by Staphylococcus aureus in a human whole blood model. *J Immunol*. 2014;192:2857-2864.
- Pawlinski R, Mackman N. Cellular sources of tissue factor in endotoxemia and sepsis. *Thromb Res*. 2010;125:570-3.
- Skjeflo EW, Christiansen D, Fure H, et al. Staphylococcus aureus-induced complement activation promotes tissue factor-mediated coagulation. *J Thromb Haemost*. 2018;16:905-918.
- Lau C, Gunnarsen KS, Hoydahl LS, et al. Chimeric anti-CD14 IGG2/4 hybrid antibodies for therapeutic intervention in pig and human models of inflammation. *J Immunol*. 2013;191:4769-4777.
- Landsem A, Fure H, Krey Ludviksen J, et al. Complement component 5 does not interfere with physiological hemostasis but is essential for Escherichia coli-induced coagulation accompanied by Toll-like receptor 4. *Clin Exp Immunol*. 2019;196:97-110.
- Mollnes TE, Brekke OL, Fung M, et al. Essential role of the C5a Page 14 receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of. *Blood*. 2002;100:1869-1877.
- Pouliot M, M-É F, Massé M, et al. Adenosine up-regulates cyclooxygenase-2 in human granulocytes: impact on the balance of eicosanoid generation. *J Immunol*. 2002;169:5279-5286.
- Drevets DA, Canono BP, Campbell PA. Measurement of bacterial ingestion and killing by macrophages. *Curr Protoc Immunol*. 2015;2015:14.6.1-14.6.17.
- Boeltz S, Amini P, Anders H-J, et al. To NET or not to NET: current opinions and state of the science regarding the formation of neutrophil extracellular traps. *Cell Death Differ*. 2019;26(3):395-408.
- Cushing L, Winkler A, Jelinsky SA, et al. IRAK4 kinase activity controls Toll-like receptor induced inflammation through the transcription factor IRF5 in primary human monocytes. *J Biol Chem*. 2017;292:18689-18698.

32. Thomas CJ, Schroder K. Pattern recognition receptor function in neutrophils. *Trends Immunol.* 2013;34:317-328.
33. Janke M, Poth J, Wimmenauer V, et al. Editors' choice articles Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8. *J Allergy Clin Immunol.* 2009;123:1026-1033.
34. Christiansen D, Brekke OL, Stenvik J, et al. Differential effect of inhibiting MD-2 and CD14 on LPS- Versus Whole E. coli bacteria-induced cytokine responses in human blood. *Adv Exp Med Biol.* 2012;946:237-251.
35. Henneke P, Takeuchi O, Malley R, et al. Cellular activation, phagocytosis, and bactericidal activity against group B Streptococcus involve parallel myeloid differentiation factor 88-dependent and independent signaling pathways. *J Immunol.* 2002;169:3970-3977.
36. Ugolini M, Gerhard J, Burkert S, et al. Recognition of microbial viability via TLR8 drives TFHcell differentiation and vaccine responses. *Nat Immunol.* 2018;19:386-396.
37. Ruppen C, Decosterd L, Sendi P. Is gentamicin necessary in the antimicrobial treatment for group B streptococcal infections in the elderly? An in vitro study with human blood products. *Infect Dis (Auckl).* 2017;49:185-192.
38. Askarian F, Lapek JD, Dongre M, et al. Staphylococcus aureus membrane-derived vesicles promote bacterial virulence and confer protective immunity in murine infection models. *Front Microbiol.* 2018;9:1-17.
39. Mika A, Reynolds SL, Pickering D, et al. Complement inhibitors from scabies mites promote streptococcal growth - A novel mechanism in infected epidermis. *PLoS Negl Trop Dis.* 2012;6:e1563.
40. Skjeflo EW, Christiansen D, Landsem A, et al. Phagocytosis of live and dead Escherichia coli and Staphylococcus aureus in human whole blood is markedly reduced by combined inhibition of C5aR1 and CD14. *Mol Immunol.* 2019;112:131-139.
41. Van Bruggen R, Drewniak A, Tool ATJ, et al. Toll-like receptor responses in IRAK-4-deficient neutrophils. *J Innate Immun.* 2010;2:280-287.
42. Hafner A, Kolbe U, Freund I, et al. Crucial role of nucleic acid sensing via endosomal Toll-Like receptors for the defense of Streptococcus pyogenes in vitro and in vivo. *Front Immunol.* 2019;10:1-13.
43. Ugolini M, Gerhard J, Burkert S, et al. Recognition of microbial viability via TLR8 promotes T follicular helper cell differentiation and vaccine responses. *Nat Immunol.* 2018;19:386-396.
44. Marshall JC. Why have clinical trials in sepsis failed. *Trends Mol Med.* 2014;20:195-203.
45. Fink MP. Animal models of sepsis. *Virulence.* 2014;5:143-153.

SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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