Neisseria meningitidis and Escherichia coli are protected from leukocyte phagocytosis by binding to erythrocyte complement receptor 1 in human blood☆

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ABSTRACT

The initial interaction of Gram-negative bacteria with erythrocytes and its implications on leukocyte phagocytosis and oxidative burst in human whole blood were examined. Alexa-labeled *Escherichia coli*, wild-type H44/76 *N. meningitidis* and the H44/76*lpxA* lipopolysaccharide (LPS)-deficient mutant were incubated with whole blood using lepirudin as anticoagulant which has no adverse effects on complement. Bacteria free in plasma, bound to erythrocytes or phagocytized by granulocytes and monocytes were quantified using flow cytometry. The effects of the C3 inhibitor compstatin, a C5a receptor antagonist (C5aRa) and a complement receptor 1 (CR1)-blocking antibody (3D9) were examined. Most bacteria (80%) immediately bound to erythrocytes. The binding gradually declined over time, with a parallel increase in phagocytosis. Complement inhibition with compstatin reduced erythrocyte binding and bacterial C3 opsonization. In contrast, the C5aRa efficiently reduced phagocytosis, but did not affect the binding of bacteria to erythrocytes. The anti-CR1 blocking mAb dose-dependently reduced bacterial binding to erythrocytes to nil, with subsequent increased phagocytosis and oxidative burst. LPS had no effect on these processes since similar results were obtained using an LPS-deficient *N. meningitidis* mutant. *In vivo* experiments in a pig model of sepsis showed limited binding of bacteria to erythrocytes, consistent with the facts that erythrocyte CR1 receptors are absent in non-primates and that the bacteria were mainly found in the lungs. In conclusion, complement-dependent binding of Gram-negative bacteria to erythrocyte CR1 decreases phagocytosis and oxidative burst by leukocytes in human whole blood.

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1. Introduction

E. coli and *N. meningitidis* are important Gram-negative pathogens causing sepsis (Martin et al., 2003; Stephens et al., 2007). These bacteria activate complex inflammatory pathways, involving both the innate and the adaptive immune systems (Castellheim et al., 2009). Complement activation is a key feature important for activating the defense mechanisms opsonophagocytosis (Castellheim et al., 2009; Mollnes et al., 2002) and serum bactericidal activity. It also exerts potent inflammatory effects in sepsis through the release of anaphylatoxins, including C5a (Ward, 2004), and excessive complement activation in meningococcal diseases is related to disease severity (Brandtzaeg et al., 1989). *N. meningitidis* activates complement mainly through the alternative and lectin pathways, whereas the classical pathway is only slightly activated (Sprong et al., 2003). In contrast, *E. coli* mainly activates the alternative pathway (Mollnes et al., 2002). The opsonization of the bacterial surface with complement components, such as C1q, C3 and C4, are important for bacterial recognition by the

immune system (Castellheim et al., 2009). In addition, ficolins (Matsushita and Fujita, 2002), mannose-binding lectin (MBL) (Jack et al., 2005), properdin (Hourcade, 2006) and Igs may function as opsonins. The complement-opsonized bacteria are recognized by the immune system and binding to specific receptors such as complement receptor 1 (CR1) occurs (Birmingham and Hebert, 2001). CR3 or CD11b/CD18 is important in the phagocytosis (Mollnes et al., 2002) of bacteria by blood leukocytes. In the fluid phase, the anaphylatoxin C5a is released and binds to specific receptors on various cells, such as granulocytes, monocytes and endothelial cells (Lee et al., 2008). Interestingly, the inhibition of the anaphylatoxin C5a or its receptors has been reported to greatly enhance the survival of sepsis in animal models (Parrish et al., 2008; Ward, 2004). The C3 convertase inhibitor compstatin was also recently shown to decrease E. coli-induced coagulation activation in a sepsis model in baboons (Silasi-Mansat et al., 2010). These observations indicate that complement inhibitors are potential therapeutic agents for sepsis.

Erythrocytes are generally viewed as simple hemoglobin carriers involved in oxygen transport in humans. However, in 1930, using serum-opsonized trypanosomes, it was first observed that erythrocytes may bind microorganisms and play a role in the pathogenesis of bloodstream infections (Duke and Wallace, 1930). Later, Nelson demonstrated that opsonized particles and Gram-positive pneumococci bind to erythrocytes in a complement-dependent manner (Nelson, Jr., 1953). Subsequently, erythrocyte CR1 was identified as the high-affinity binding site of C3b, although it can also bind iC3b, C4b, iC4b (Cooper, 1969), C1q and MBL with lower affinity (Ghiran et al., 2000), linking complement-coated bacteria to erythrocytes (Birmingham and Hebert, 2001). CR1 is a large transmembrane glycoprotein, consisting of several homologous motifs (Birmingham and Hebert, 2001). It is expressed in varying numbers from approximately 100 to 1000 per human erythrocyte (Birmingham and Hebert, 2001) and distributed in clusters on the erythrocyte membrane after ligation (Ghiran et al., 2008; Paccaud et al., 1988). CR1 is also found on human monocytes, granulocytes (Ross et al., 1978) and B lymphocytes (Birmingham and Hebert, 2001). Immune complexes (ICs) opsonized with C3b and C4b bind erythrocyte CR1; they can then be cleared from the circulation and destroyed in the liver and spleen (Cornacoff et al., 1983; Schifferli et al., 1988). Another interesting feature of CR1 is its ability to inhibit complement activation by functioning as a cofactor of factor I that cleaves C3b and C4b into inactive forms (Iida and Nussenzweig, 1981). Through binding C3b and C4b, CR1 also accelerates the decay of the alternative (Fearon, 1979) and classical pathway C3 convertases (Iida and Nussenzweig, 1981). Many studies have examined the binding of ICs to erythrocyte CR1 and its involvement in IC clearance (Birmingham and Hebert, 2001). However, the roles of complement in the binding of different Gram-negative bacteria to erythrocytes and in leukocyte phagocytosis in human whole blood using lepirudin as an anticoagulant, have not been studied.

In the present study, we therefore examined the interaction of E. coli and N. meningitidis with erythrocytes and how the interaction affects phagocytosis in a human whole-blood model. The roles of membrane lipopolysaccharide (LPS) and bacterial opsonization in the initial binding of E. coli, wild-type N. meningitidis H44/76 with LPS and the LPS-deficient H44/76lpxA mutant to erythrocyte CR1 were examined. The specific thrombin inhibitor lepirudin was used as anticoagulant because it does not affect complement activation, in contrast to calcium-binding anticoagulants and heparin (Mollnes et al., 2002). Our data shed new light on the interaction of Gram-negative bacteria with various blood cells and indicate that initial binding of the bacteria to erythrocytes reduces phagocytosis and oxidative burst by leukocytes in human whole blood.

2. Materials and methods

2.1. Equipment and reagents

All equipment, including polypropylene tubes (Nalgene NUNC, Roskilde, Denmark) and tips used in the whole-blood experiments, was endotoxin-free. Phosphate buffered saline (PBS) with or without Ca^{2+} and Mg^{2+} was obtained from Life Technologies (Paisley, UK). Lepirudin (Refludan®) was obtained from Hoechst (Frankfurt am Main, Germany). Protein G Spin Kit columns (0.2 mL) for antibody purification were obtained from Thermo Fisher Scientific (Pierce, Rockford, IL). Burst test and Phago test kits were obtained from ORPEGEN Pharma (Heidelberg, Germany). LDS-751, Alexa 488, a BacLight green kit for the direct fluorescent staining of unlabeled bacteria, and dimethylsulfoxide (DMSO) were obtained from Invitrogen Molecular Probes (Eugene, OR). Zymosan A, EDTA and bovine serum albumin were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Monoclonal antibodies and inhibitors

Mouse anti-human CR1 blocking mAb (clone 3D9) which inhibits the binding of CR1 to C3b/C4b has been extensively characterized previously (O'Shea et al., 1985). Using protein G columns, the mAb 3D9 was purified from 50 μL of sterile ascites fluid containing approximately 1 g/L mAb. The concentration of the purified 3D9 IgG1 antibody in the eluate (0.46 g/L) was measured at 280 nm using a SmartSpec™Plus Spectrophotometer from Bio-Rad (Hercules, CA). An isotype-matched mouse anti-human IgG1 control mAb (clone BH1) was purchased from Diatec. Antibodies were tested for LPS contamination using a chromogenic Limulus Amebocyte Lysate (LAL) assay (QCL-1000) from BioWhittaker (Walkersville, MD). When necessary, LPS was removed from the mAbs using END-X B15 from Associates of Cape Cod Inc. (East Falmouth, MA), and final LPS concentrations in the low pg/mL range were obtained. Compstatin is a 13-amino acid cyclic peptide that binds C3 and inhibits the cleavage of C3. We used the compstatin analogue Ac-IT-NH2, which is 264 times more active than the parent peptide I[CVVQDWGH HRC]T-NH2. Both compstatin and the control peptide IAVVQ DWGHHRAT-NH2 were synthesized as previously described (Katragadda et al., 2006). The cyclic hexapeptide AcF[OPdChaWR], a C5a receptor antagonist (C5aRa), was synthesized as previously described (Mastellos et al., 2001). The murine anti-human mAbs anti-C2 and anti-factor D, as well as the isotype-matched murine control mAb G3-519, were previously described in detail (Lappegard et al., 2005; Brekke et al., 2007).

2.3. Bacterial strains and counting

The E. coli strain LE-392 (ATCC 33572) was obtained from the American Type Culture Collection (Manassas, VA). E. coli was grown overnight on a Lactose dish, and 5-10 colonies were transferred to LB medium (1% tryptone, 0.5% (w/v) yeast extract and 1% NaCl) from Becton Dickinson (Sparks, MD) and grown overnight. The bacteria were harvested and washed once, using Dulbecco's PBS without Ca^{2+} and Mg^{2+} by centrifugation for 10 min at $3220 \times g$ (4 °C). Subsequently, the bacteria were aliquoted, heat-inactivated for 1 h at 60 °C and stored at −80 °C. A frozen ampoule was thawed at ambient temperature and was washed six times with PBS and centrifuged for 10 min at $3220 \times g$ (4 °C) to remove extracellular LPS. Bacteria for Alexa staining were separated, whereas the rest were washed three more times. For counting, the bacteria were stained for 5 min using SytoBC (Invitrogen Molecular Probes) and were counted in Truecount tubes (Becton-Dickinson) using a FACScalibur or a LSRII flow cytometer with FACSDiva software (Becton-Dickinson). The heat-inactivated E. coli bacteria were

stored in PBS at 4 °C for up to 2 months. The LPS concentration in the supernatant of the bacterial preparation remained unchanged during the storage period.

For the phagocytosis assay, heat-inactivated *E. coli* (6×10^9) or *N. meningitidis* were washed six times as described above, and the supernatant was discarded (Mollnes et al., 2002). Afterward, $NaHCO_3$ (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 three times with PBS and centrifuged for 5 min at $8000 \times g$, resuspended in PBS and counted as described above.

Heat-inactivated, wild-type *N. meningitidis* 44/76 (H44/76) was obtained from the National Institute of Public Health. This international reference strain is characterized as B:14:P1.7,16:L3,7,9 and was originally isolated from a patient with an invasive meningococcal disease (Holten, 1979). The *N. meningitidis* 44/76*lpxA* mutant strain that completely lacks LPS in the outer membrane was created by L. Steeghs and P. van der Ley (National Institute of Public Health and Environment, the Netherlands) (Steeghs et al., 2001) and donated to the National Institute of Public Health, Oslo, for research purposes. Both strains are encapsulated. Staining of these bacteria was performed according to the same procedures of *E. coli* staining.

2.4. In vitro whole-blood model of sepsis

The whole-blood model has been previously described in detail (Mollnes et al., 2002). Briefly, venous blood samples were collected into 4.5-mL NUNC tubes containing lepirudin (50 mg/L), from healthy donors who had provided informed written consent. Experiments using each blood sample were individually performed on different days. Tubes with complement inhibitors or PBS controls were prepared individually. Blood samples were added immediately after sampling, and tubes were preincubated for 4 min at 37 °C. Thereafter, PBS (control), Alexa-stained bacteria or unstained bacteria were added, and the samples were further incubated at 37 °C.

2.5. Flow cytometry of erythrocyte-bound bacteria and free bacteria in plasma

Whole blood incubated with inhibitors and Alexa-stained *E. coli* or *N. meningitidis* was fixed with 0.25% paraformaldehyde for 4 min at 37 °C. Samples were diluted at 1:320 using PBS to avoid coincidences from erythrocytes not carrying bacteria; they were then counted using Truecount tubes. Whole blood with EDTA (10 mM) served as a control. Samples were analyzed using a FACSCalibur or LSRII flow cytometer (Becton Dickinson), with forward scatter (FSC) and side scatter (SCC) in a log mode and with the threshold on the green channel. Gates were set around the beads, the erythrocytes and the free bacteria for analysis. The erythrocyte population was verified in control experiments using mouse anti-human CD235a (Glycophorin A) (clone JC159, Dako, Glostrup, Denmark). To confirm that the bacteria/erythrocyte conjugates were not formed due to the presence of paraformaldehyde, we performed control experiments without paraformaldehyde and obtained similar results.

2.6. Enzyme immunoassay

After the incubation, complement activation was stopped by adding EDTA (10 mM final concentration). Complement activation was analyzed in plasma by measuring the soluble C5b-9 terminal complement complex (TCC) using ELISA as previously described (Mollnes et al., 1985). In brief, the mouse anti-human mAb aE11

was used as capture antibody reacting with neoepitope exposed in C9 after incorporation in the C5b-9 complex, and a biotinylated anti-C6 mAb (Quidel Corporation, San Diego, CA) was used as the detection mAb. Streptavidin-horseradish peroxidase was then added. Results are expressed as arbitrary units/mL (AU/mL). Normal human serum activated with zymosan (10 mg/mL) was used as standard and defined to contain 1000 AU/mL.

2.7. Flow cytometry of bacterial opsonization

Lepirudin plasma was obtained from whole blood added lepirudin (50 mg/L) after centrifugation for 15 min at $3220 \times g$ (4 °C). The plasma was preincubated with PBS or the indicated inhibitors for 4 min at 37 °C in NUNC tubes. Unopsonized *E. coli*, *N. meningitidis* or PBS were added, followed by an additional incubation for 10 min at 37 °C. Bacteria were washed twice with PBS and centrifuged for 15 min at $3220 \times g$ (4 °C) and resuspended in PBS containing 0.1% (w/v) BSA. C1q, C3 and C4 opsonization was analyzed using rabbit anti-human C1q, FITC-conjugated rabbit anti-human C3c (F0201) and rabbit anti-human C4c mAbs, respectively. FITC-conjugated rabbit anti-mouse Ig (F0261) was used as control. All antibodies were from Dako. Results were expressed as median fluorescence intensity (MFI).

2.8. Phagocytosis assay

Whole blood incubated with inhibitors and Alexa-stained *E. coli* or *N. meningitidis* was processed according to kit instructions (ORPEGEN Pharma); phagocytosis of bacteria was analyzed in the presence of a quenching solution. The assay performed in the absence of the quenching solution, reflects both the phagocytosis and the extracellular binding of the stained bacteria to leukocytes. Whole blood was stained with the nuclear dye LDS-751 (fluorescence 3, FL3) to separate leukocytes from erythrocytes. Granulocytes and monocytes were gated separately in a plot displaying SCC versus FL1 (anti-CD14 FITC) from Becton Dickinson (San Jose, CA, USA). Samples were analyzed using a LSRII flow cytometer (Becton Dickinson). Data were expressed as MFI of the whole granulocyte or monocyte population.

2.9. Microscopy

Human whole blood was supplemented with PBS containing Alexa-stained *E. coli* or *N. meningitidis* at a final concentration of 72×10^6 mL^{-1} and was incubated for 10 min at 37 °C. Blood films were prepared and immediately air-dried. The films were stored in the dark and examined using either transmitted light observation microscopy or reflected fluorescence microscopy with corresponding filters for FITC. Combined reflected fluorescence and transmitted light microscopy was performed according to the instructions using a very weak transmitted light intensity. As a control, a portion of the whole blood was diluted with PBS and was examined as an unstained wet preparation by fluorescence microscopy. An Olympus BX51TRF microscope was equipped with a ColorView IIIu digital camera with 5-megapixel resolution and was controlled by the CellP program (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Immunofluorescence histology was performed on cryosections obtained from the lung, liver and spleen of the pigs infused with Alexa-stained *E. coli*. Cryosections (5 μm thick) were cut from tissue embedded and snap-frozen in O.C.T Compound (Tissue-Tek; BDH, Lutterworth, UK). Sections were air-dried and fixed for 10 min in ice-cold acetone. Fc receptors were blocked by incubating the sections for 30 min with PBS containing 5% pig serum and 5% goat serum. To identify tissue macrophages, a pretitrated anti-porcine CD45 monoclonal antibody (a kind gift from Karin Haverson, Uni-

versity of Bristol, Bristol, UK) was applied and incubated for 2 h. Slides were thoroughly washed three times with PBS for 5 min each time. An isotype-specific goat anti-mouse antiserum (Southern Biotechnology, Birmingham, AL) conjugated to Texas Red was then applied and incubated for 1 h. The slides were washed three more times, and the nuclear dye DAPI was applied and incubated for 10 min. After the final wash, the sections were mounted in Fluoromount (Vector Laboratories, Burlingame, CA) and sealed with nail varnish. Stained slides were examined using a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with a combined excitation and emission filter block specific for the applied fluorescence staining.

2.10. Oxidative burst

Whole blood preincubated with inhibitors and *E. coli* was processed according to kit instructions (ORPEGEN Pharma) as previously described (Mollnes et al., 2002). In brief, after 10 min incubation with or without bacteria, 100 μL blood were transferred to 5 mL polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ). Substrate solution (20 μL) containing dihydrorhodamine 1,2,3 was then added and samples were further incubated 10 min (37 °C). Samples were diluted with PBS and analyzed using a LSRII flow cytometer (Becton Dickinson). Monocytes and granulocytes were gated separately in a FSC/SCC dotplot. Results were expressed as MFI.

2.11. In vivo porcine model of sepsis

Norwegian Landrace swine (Sus scrofa domesticus) were anesthetized and surgically treated as previously described (Castellheim et al., 2008; Nielsen et al., 2009). Two pigs received heat-inactivated, Alexa-labeled *E. coli*, and two pigs received Alexa-labeled *N. meningitidis* intravenously. A low dose of bacteria, 5.7×10^{10} in total, was intravenously infused during the first 120 min through a central venous catheter. The initial dose was 4.5×10^8 bacteria/h; the infusion rate was doubled every 30 min until 120 min. After 120 min, a bolus, containing 9.2×10^{10} bacteria, was injected in approximately 1 min. Blood samples for cytospin preparations, real-time quantitative PCR (RT-qPCR) of bacterial DNA in whole blood, plasma, buffy coat and erythrocyte fractions, blood gas analysis, routine hematology and flow cytometry were collected at the indicated time points. Samples for RT-qPCR were aliquoted and stored at −70 °C. Hematological parameters, including leukocyte differential count, were analyzed in EDTA tubes on a CELL-DYN 4000 (Abbot Diagnostics, Abbot Park, IL) as previously described (Castellheim et al., 2008). The pigs were treated with noradrenalin, and intravenous fluid when needed. A small dose of sildenafilcitrate was given orally before the start of the bacterial infusion in order to prevent pulmonary hypertension.

2.12. RT-quantitative PCR for bacterial DNA in whole blood

Whole blood anticoagulated with lepirudin (50 μg/mL) was centrifuged for 15 min at $145 \times g$ (4 °C) without braking. Thereafter, the plasma, buffy coat and red cell fractions were collected and stored at −80 °C. DNA from whole blood, plasma, buffy coat and red cell fractions were isolated on a MagNA Pure LC instrument from Roche Applied Science (Mannheim, Germany) using a MagNA Pure LC DNA Isolation Kit I (Roche), according to the manufacturer's instructions. Genomic DNA from *E. coli* O157, strain EDL 933, from the Institute for Reference Materials and Measurements (Geel, Belgium), was diluted with water, quantified by optical density measurement and used as a standard. The standard was diluted 10-fold with whole blood (10^8–10^3 *E. coli* DNA copies/mL) anti-coagulated with lepirudin. Total DNA was isolated from 200 μL of each dilu-

tion; 5 μL of the DNA extract was used for RT-qPCR. The negative control was whole blood with sterile PBS; the positive control was DNA extracted from lepirudin-anticoagulated whole blood mixed with DNA from *E. coli* strain B from Sigma-Aldrich. *E. coli* DNA was quantified by RT-qPCR using a ABI 7500 instrument from Applied Biosystems (Warrington, UK) as previously described (Thorgersen et al., 2010). *N. meningitidis* DNA was quantified by RT-qPCR using a LightCycler instrument (Roche Diagnostics, Basel, Switzerland) as previously described (Ovstebo et al., 2004). The lower detection limits of the analyses for *E. coli* and *N. meningitidis* DNA were 1×10^4 and 1×10^3 DNA copies/mL, respectively.

2.13. Statistical analysis

Results were analyzed using SigmaStat version 3.5 (SPSS Science Software Gmbh, Erkrath, Germany). Data were analyzed using one-way repeated measurements ANOVA followed by Holm-Sidak post-test, using the bacteria plus PBS as a control. Significance was assigned where $P < 0.05$.

2.14. Ethics

The study on humans was approved by the Regional ethics committee. The animal experiments were performed according to the Norwegian laboratory animal regulations. The study was approved by the University Animal Care Committee.

3. Results

3.1. Initial erythrocyte binding and phagocytosis of E. coli and N. meningitidis in human whole blood

To examine whether *E. coli* and *N. meningitidis* bind to erythrocytes or are free in plasma, we added Alexa-stained bacteria to fresh human whole blood. The majority of the *E. coli* bacteria, added to human whole blood, bound to erythrocytes after 10-min incubation (Fig. 1A). Dose response experiments showed a linear relationship between the concentration of *E. coli* and the number of free bacteria in plasma and the number of erythrocyte-bound bacteria (Fig. 1A). Similar results were obtained using *N. meningitidis* (data not shown). We also observed a non-linear relationship between the phagocytosis and the added concentration of *E. coli* (Fig. 1B). Finally, a linear relationship between *E. coli* concentration and complement activation in plasma, shown by TCC measurement, was observed (Fig. 1C). Control experiments confirmed that Alexa-labeled and unlabeled bacteria activated complement at the same level (data not shown).

Fluorescence microscopy of blood films confirmed that *E. coli* and wild-type *N. meningitidis* 44/76 initially bound to erythrocytes (Fig. 1D and G). Some phagocytized bacteria were observed (Fig. 1E and H), but they comprised less that 5% of the total bacteria added after 10 min incubation (data not shown). Occasionally, free bacteria were seen in plasma (Fig. 1F and I). Using unlabeled bacteria and fluorescence microscopy, similar findings were obtained from the staining of blood films with fluorochrome-labeled anti-*N. meningitidis* or anti-*E. coli* mAbs or BacLight (data not shown), indicating that Alexa labeling of the bacteria does not affect their binding to erythrocytes. Furthermore, when microscopy was performed on diluted whole blood from wet fluid preparations, the bacteria were mainly found on erythrocytes, moving with the bacteria bound to their surface. Collectively, these data show that the majority of *E. coli* and *N. meningitidis* initially bound to erythrocytes in human whole blood.
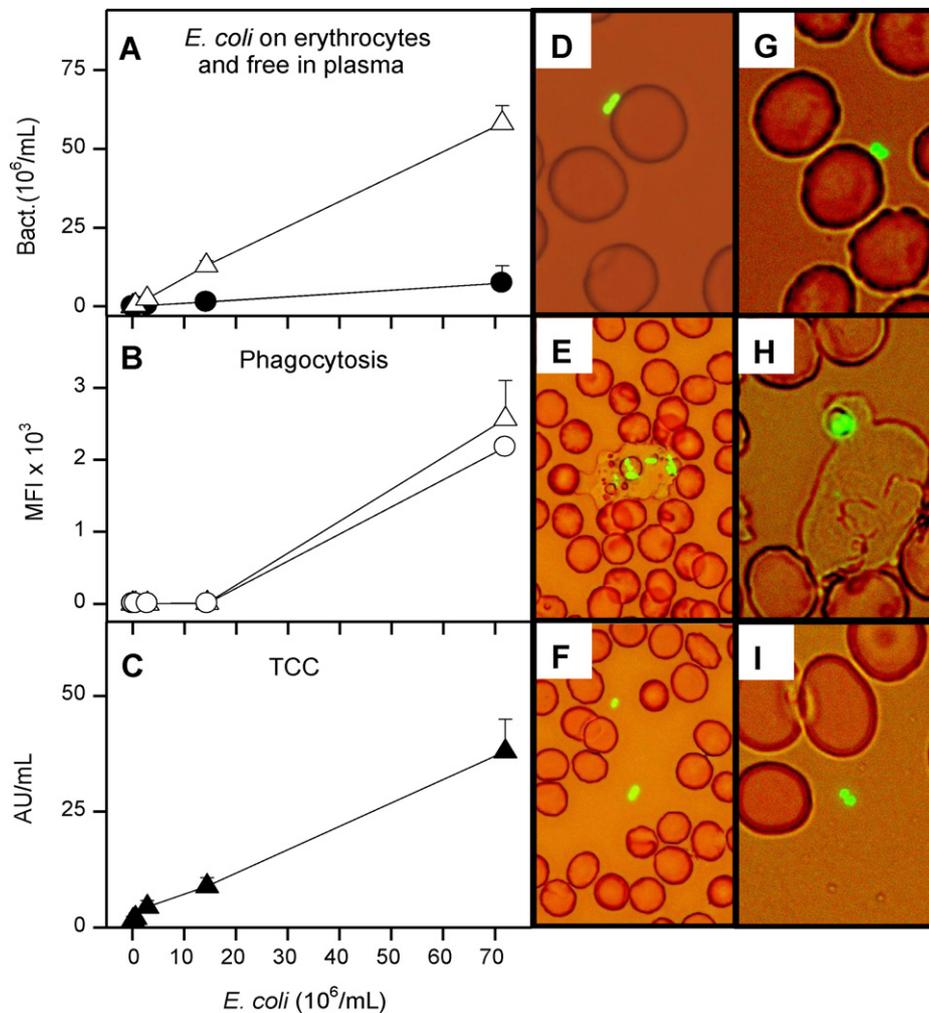
Fig. 1. Dose–response effect of *E. coli* on the initial binding to erythrocytes, phagocytosis and complement activation in human whole blood. Alexa-labeled *E. coli* was added to fresh whole blood and was incubated for 10 min at 37 °C. (A) Free bacteria in plasma (filled circles) and erythrocyte-bound bacteria (open triangles) were analyzed using flow cytometry; results are expressed as 10^6 bacteria/mL. (B) Phagocytosis of *E. coli* in granulocytes (open triangles) and monocytes (open circles) was analyzed using flow cytometry; results are expressed as median fluorescence intensity (MFI). (C) *E. coli*-induced complement activation was analyzed by measuring the terminal complement complex (TCC) in plasma using ELISA; results are expressed as arbitrary units/mL (AU/mL) (filled triangles). Data are presented as the means ± SD from three to six independent experiments, using samples from different blood donors each time. (D–F) Microscopy of Alexa-labeled *E. coli* and (G–I) *N. meningitidis* 44/76 bacteria in whole blood films prepared after 10-min incubation using 72×10^6 bacteria/mL blood. The samples were analyzed using combined fluorescence and transmitted light microscopy (1000× magnification for *E. coli* and 1600× magnification for *N. meningitidis*) on a Olympus BX51TRF microscope equipped with a ColorView IIIu digital camera. (D) *E. coli* and (G) *N. meningitidis* 44/76 bacteria bound to erythrocytes. (E) *E. coli* and (H) *N. meningitidis* 44/76 bacteria phagocytized by a leukocyte and (F) *E. coli* and (I) *N. meningitidis* 44/76 bacteria free in plasma. Results from one of five representative experiments are shown.

3.2. Effect of complement inhibitors on the binding of bacteria to erythrocytes and on phagocytosis

The effect of complement inhibitors on the binding of *E. coli* and *N. meningitidis* to erythrocytes was then examined. Approximately 80% of *E. coli* (Fig. 2A and B), and at least 90% of *N. meningitidis* (Figs. 3A, B and 1G) initially bound to erythrocytes. A few *N. meningitidis* were found in plasma (Figs. 3A and 1I) and a few were phagocytized in leukocytes (Fig. 1H) after 10-min incubation. Complement inhibition by mAbs blocking factor D and C2 significantly reduced the binding of *E. coli* and *N. meningitidis* to erythrocytes and simultaneously increased the number of free bacteria in plasma (Figs. 2A, B and 3A, B). Complement inhibition completely blocked the phagocytosis of *E. coli* ($P < 0.05$) by granulocytes and monocytes after 10 min (Fig. 2C and D). In comparison, complement inhibition completely blocked the phagocytosis of *N. meningitidis* ($P < 0.05$) by granulocytes, while the phagocytosis in monocytes after 10 min was significantly, but not completely inhibited by compstatin (Fig. 3C and D). In contrast, an isotype control mAb had no such effect. As expected, a C5aRa and a control peptide had no

effect on the binding of the bacteria to erythrocytes. However, the C5aRa completely blocked granulocyte phagocytosis and partially blocked monocyte phagocytosis of *E. coli* after 10-min incubation ($P < 0.05$). EDTA, blocking both complement and leukocyte activation by binding calcium, almost completely inhibited the binding of both bacteria to erythrocytes. (Figs. 2B and 3B) It increased the free bacteria in plasma (Figs. 2A and 3A) and efficiently blocked the phagocytosis (Figs. 2C, D and 3C, D). As a control, bacteria were added in the blood-free PBS buffer to assess their number (Figs. 2A and 3A).

3.3. Time course study on the effect of complement inhibitors on the binding of E. coli to erythrocytes and on granulocyte phagocytosis

The numbers of free *E. coli* bacteria in plasma were generally low at all time points, except in the presence of EDTA, although a slight increase in the presence of compstatin was observed after 120 min (Fig. 4A). The binding of *E. coli* to erythrocytes was time-dependent and slowly decreased over time (Fig. 4B). Com-
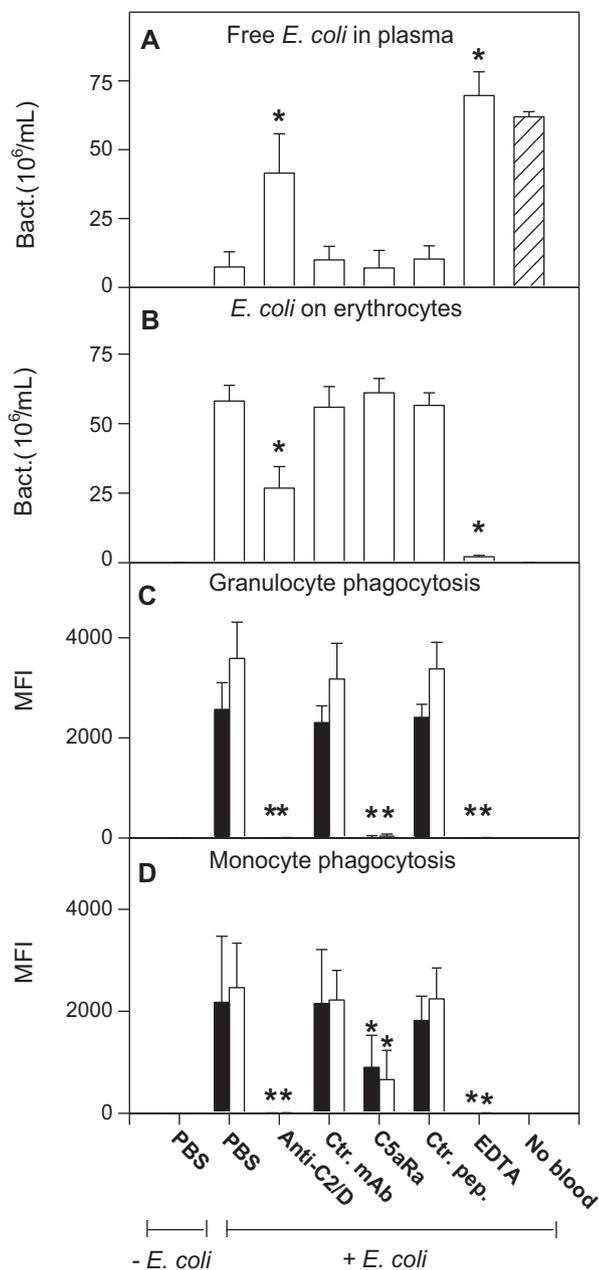
Fig. 2. Effect of complement inhibitors on the binding of *E. coli* to erythrocytes and phagocytosis in granulocytes and monocytes. Alexa-labeled *E. coli* (72×10^6 mL^{-1}) was added to human whole blood and was incubated at 37 °C for 10 min, in the presence of mAbs blocking C2 (71 mg/L) and factor D (36 mg/L; Anti-C2/D), an iso-type control mAb (Ctr. mAb; 107 mg/L), a C5aR antagonist (C5aRa; 10 mg/L) and its corresponding control peptide (Ctr. pep.; 10 mg/L) or EDTA (10 mM). (A) *E. coli* in plasma and (B) erythrocyte-bound *E. coli* were analyzed using flow cytometry; results are expressed as 10^6 bacteria/mL (Bact. 10^6 mL^{-1}). No blood: Bacteria added to blood-free PBS were included as a control (upper panel, hatched bar to the right). (C) Granulocyte and (D) monocyte phagocytosis of *E. coli* was analyzed using flow cytometry; results are expressed as median fluorescence intensity (MFI). Phagocytosis was analyzed with (black bars) or without a quenching solution (white bars), reflecting phagocytized and phagocytized plus surface-bound bacteria, respectively. Results are presented as the means ± SD of data from three to six different blood donors. *$P < 0.05$, compared to *E. coli* alone using one-way repeated measurements ANOVA followed by Holm–Sidak post-test.

Fig. 3. Effect of complement inhibitors on the binding of *N. meningitidis* 44/76 to erythrocytes. Alexa-labeled *N. meningitidis* (72×10^6 mL^{-1}) was added to human whole blood and was incubated at 37 °C for 10 min in the presence or absence of PBS, anti-C2 and anti-factor D (Anti-C2/D), its corresponding control mAb, compstatin, C5aRa, a control peptide (Ctr. pep.) or EDTA. The inhibitor concentrations used are described in the legend to Fig. 2. (A) *N. meningitidis* in plasma and (B) erythrocyte-bound *N. meningitidis* were analyzed using flow cytometry; the results are expressed as 10^6 bacteria/mL (Bact. 10^6 mL^{-1}). (C) Granulocyte and (D) monocyte phagocytosis of bacteria was analyzed using flow cytometry; results are expressed as median fluorescence intensity (MFI). Phagocytosis was analyzed with (black bars) or without a quenching solution (white bars), reflecting phagocytized and phagocytized plus surface-bound bacteria, respectively. Results are presented as the means ± SD of three different blood donors. *$P < 0.05$, compared to *N. meningitidis* alone using one-way repeated measurements ANOVA followed by Holm–Sidak post-test.

plement inhibition, using the C3 convertase inhibitor compstatin, significantly decreased the binding to erythrocytes. Granulocyte phagocytosis of bacteria increased with time in the absence of the inhibitors (Fig. 4C). The data indicate that the complement-dependent binding of bacteria to erythrocytes *in vitro* last several
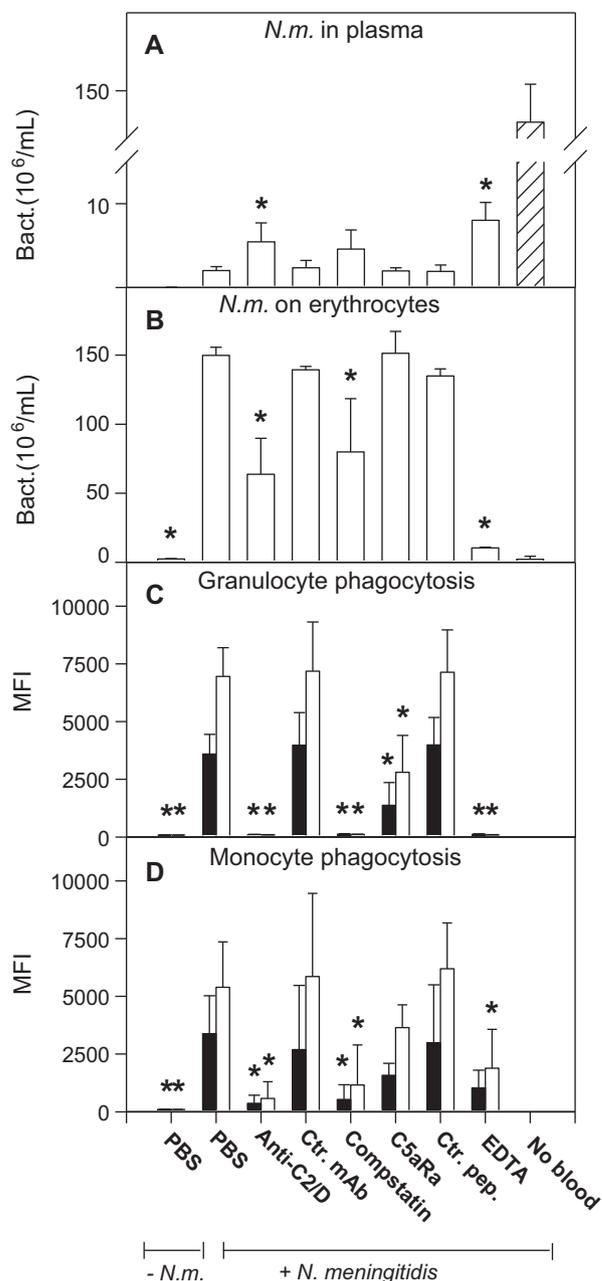
h and that the number of erythrocyte bound bacteria decreases when the bacteria are either released from erythrocytes or removed by phagocytosis. However, neither compstatin nor the C5aRa completely inhibited phagocytosis after 60 and 120 min, indicating that the complement-independent phagocytosis of *E. coli* increases over
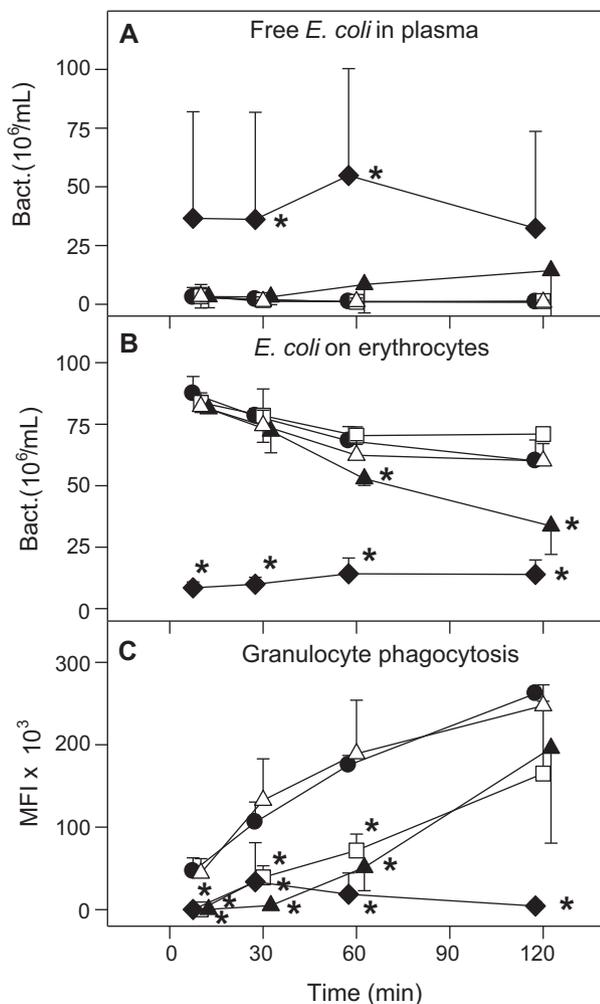
Fig. 4. Time course of *E. coli* binding to erythrocytes, phagocytosis and the effect of complement inhibitors. Alexa-labeled *E. coli* (90×10^6 mL^{-1}) was added to human whole blood and incubated for up to 120 min (37 °C). (A) *E. coli* in plasma, (B) erythrocyte-bound *E. coli* and (C) granulocyte phagocytosis with the quenching solution were analyzed using flow cytometry. The added complement inhibitors and controls are as follows: compstatin (black triangle), its control peptide (white triangle), C5aRa (white square) and PBS control (black circles). The general complement inhibitor EDTA (black diamond) was included as a control. The inhibitor concentrations used are described in the legends to Figs. 2 and 3. Results are presented as the means ± SD ($N=3$), using samples from different blood donors. *$P < 0.05$, compared to *E. coli* plus PBS as a control, analyzed at each time point using one-way repeated measurements ANOVA followed by Holm–Sidak post-test.

time. In contrast, EDTA efficiently blocked phagocytosis even after 120-min incubation.

3.4. Effect of complement inhibitors on bacterial C3 and C4 opsonization

The effects of complement inhibitors compstatin and C5aRa on the C3 and C4 opsonization on *E. coli* and *N. meningitidis* were then examined in lepirudin plasma using flow cytometry (Fig. 5). Compstatin efficiently reduced C3 opsonization on both *E. coli* and *N. meningitidis* (Fig. 5A and C) but increased C4 opsonization (Fig. 5B and D). Notably, membrane LPS had no effect on binding or the inhibition of the opsonins, shown by the identical behavior of wild-type *N. meningitidis* and the LPS-deficient mutant (Fig. 5C and D). As expected, the C5aRa and its corresponding control peptide had no effect on bacterial opsonization. EDTA efficiently blocked both C3 and C4 opsonization because it efficiently inhibits all complement activation pathways. C1q opsonization was very low and
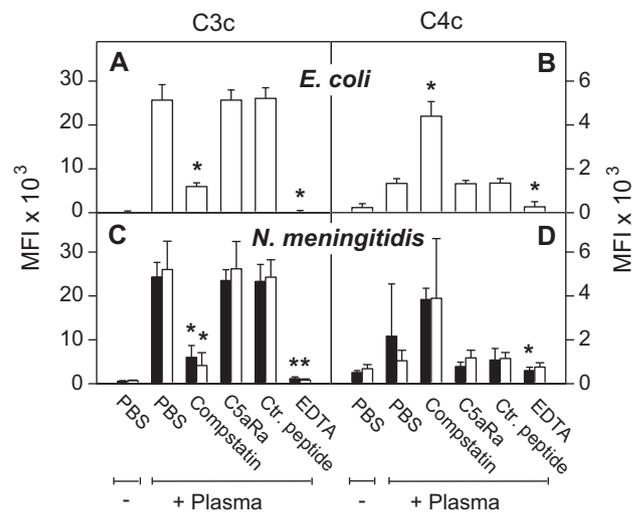

Fig. 5. Effect of complement inhibitors on *E. coli* and *N. meningitidis* C3 and C4 opsonization. The effect of complement inhibitors compstatin, its corresponding control peptide (Ctr. peptide) and a C5aRa on bacterial C3 and C4 opsonization was examined. The inhibitor concentrations used are described in the legends to Figs. 2 and 3. (A and B) *E. coli* (72×10^6 mL^{-1}), (C and D) *N. meningitidis* (72×10^6 mL^{-1}) with LPS (black bars) or the LPS-deficient 44/76*lpxA* mutant (white bars) were incubated for 10 min in lepirudin plasma (+Plasma) supplemented with PBS or complement inhibitors, as indicated. EDTA which efficiently inhibits complement activation and opsonization was included as control. (A and C) Bacterial C3 and (B and D) C4 opsonization was analyzed using flow cytometry; results are expressed as median fluorescence intensity (MFI). Results are presented as the means ± SD from independent experiments, using plasma from three different healthy donors. *$P < 0.05$, compared to bacteria in PBS alone, analyzed by one-way repeated measurements ANOVA followed by the Holm–Sidak post-test.

was not different from that in the isotype control mAb (data not shown).

3.5. Effect of the anti-CR1 blocking mAb 3D9 on phagocytosis, oxidative burst and the binding of E. coli to erythrocytes

The anti-CR1 blocking mAb 3D9 dose-dependently increased the number of free *E. coli* in plasma (Fig. 6A) and, simultaneously, efficiently blocked the binding of *E. coli* to erythrocytes (Fig. 6B). Interestingly, the anti-CR1 also effectively increased the granulocyte phagocytosis of the bacteria (Fig. 6C). Similar findings were obtained for monocyte phagocytosis (data not shown). This finding suggests that the binding of bacteria to erythrocytes inhibits leukocyte phagocytosis by reducing the number of bacteria available for leukocyte recognition. Microscopy of whole blood films also indicates that free bacteria, but not erythrocyte-bound bacteria, are phagocytized by leukocytes (Fig. 1E and H).

The effect of anti-CR1 on *E. coli*-induced oxidative burst was then examined (Fig. 6D). The anti-CR1 blocking mAb significantly ($P < 0.05$) increased *E. coli*-induced oxidative burst in granulocytes, indicating that bacterial binding to erythrocytes protects against leukocyte oxidative burst in whole blood. In comparison, a control mAb had no such effect.

3.6. Effect of anti-CR1 mAb 3D9 on the binding of N. meningitidis with or without LPS to erythrocytes

To examine whether LPS is involved in the binding of bacteria to erythrocyte CR1, we examined the effect of the CR1 blocking mAb 3D9 on the binding of *E. coli*, wild-type *N. meningitidis* 44/76 with LPS and the LPS-deficient 44/76*lpxA* mutant to erythrocytes. All three bacteria efficiently and similarly bound to erythrocytes after 10 min incubation, and the anti-CR1 blocking mAb 3D9 completely inhibited the binding (Fig. 7). In contrast, an isotype control
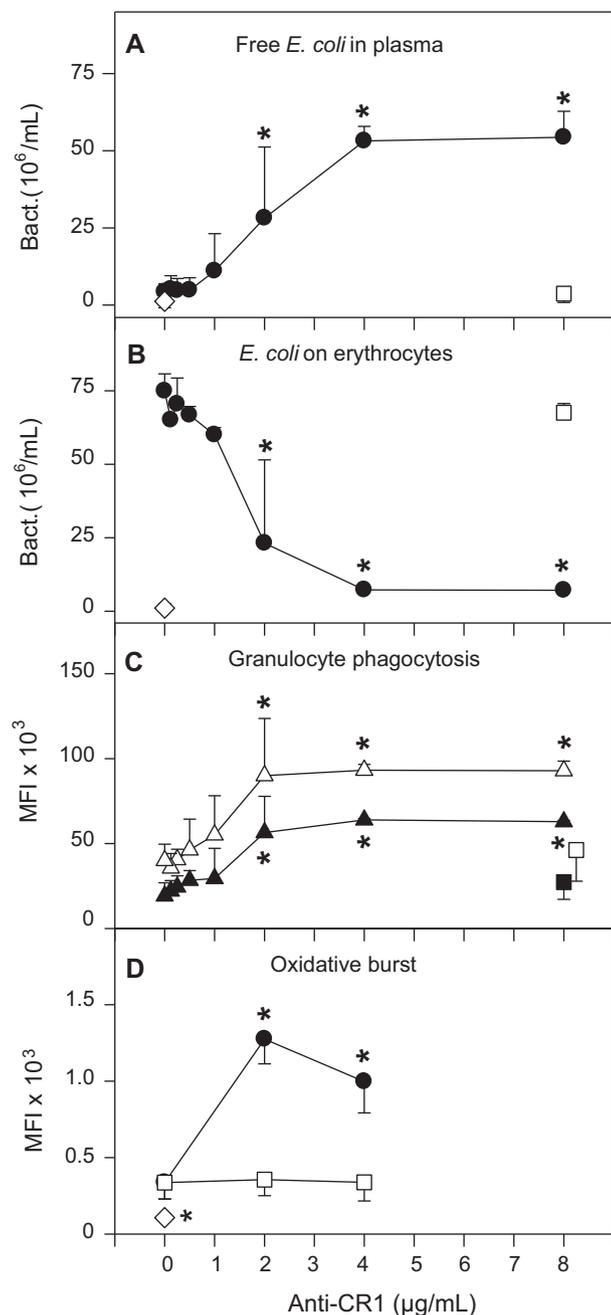
Fig. 6. Effect of the anti-CR1 blocking mAb 3D9 on free *E. coli* in plasma, *E. coli* binding to erythrocytes, phagocytosis and oxidative burst. The anti-CR1 blocking mAb 3D9 at increasing concentrations was added to whole blood with (black circles) or without (open diamonds) 72×10^6 *E. coli*/mL. (A) Free *E. coli* in plasma and (B) erythrocyte-bound bacteria were analyzed using flow cytometry; results are expressed as 10^6 bacteria/mL (Bact. 10^6 mL^{-1}). (C) Phagocytosis with (filled symbols) or without quench (open symbols) was analyzed in granulocytes (open and filled triangles). The effect of an isotype matched control mAb G3-519 (open and filled squares) is indicated. (D) Granulocyte oxidative burst in the absence of *E. coli* (open diamond) and after stimulation with *E. coli* in the presence of anti-CR1 (filled circles) or a control mAb (open squares) analyzed using flow cytometry; results are expressed as median fluorescence intensity (MFI). Data are presented as the means ± SD (N = 3). *P < 0.05, compared to the *E. coli* plus PBS control, analyzed by one-way repeated measurements ANOVA followed by the Holm–Sidak post-test.

mAb had no such effect. EDTA which efficiently inhibits complement activation and opsonization (Section 3.4), completely blocked the binding of all three bacteria to erythrocytes. As a control for the bacterial count, we also added the same concentration of all three bacteria to blood-free PBS buffer (right hatched columns) and found
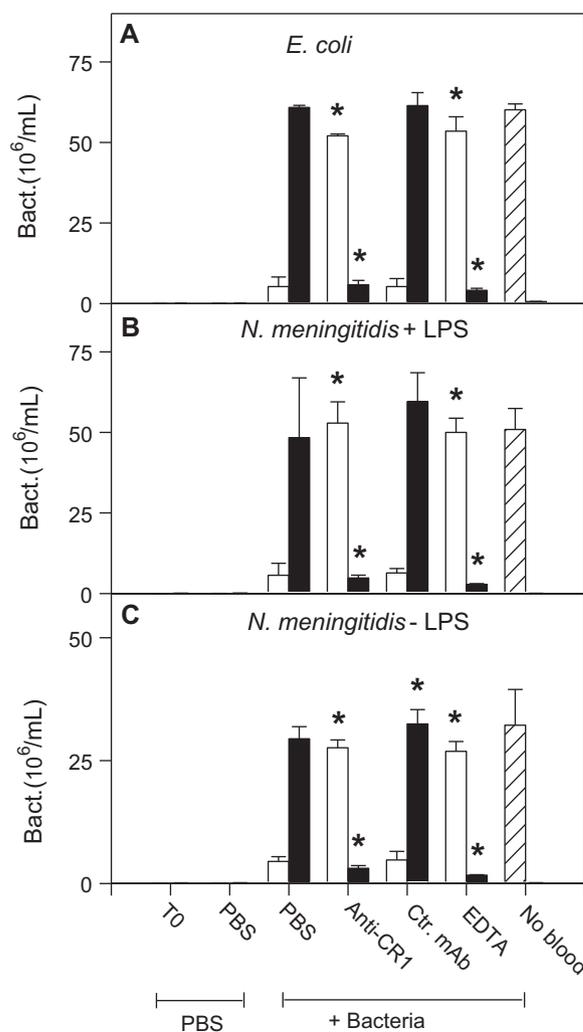

Fig. 7. Effect of the anti-CR1 blocking mAb 3D9 on the binding to erythrocytes of *E. coli* and *N. meningitidis* with or without LPS. Whole blood was incubated with 72×10^6 bacteria/mL in the presence of PBS, anti-CR1 mAb 3D9 (Anti-CR1; 4 mg/L), an isotype matched control mAb (Ctr. mAb, 4 mg/L) or the EDTA (10 mM). In addition, bacteria added to blood-free PBS were included as a control (hatched bar to the right). The numbers of free bacteria in plasma (white bars) and erythrocyte-bound bacteria (black bars) were analyzed using flow cytometry; results are expressed as 10^6 bacteria/mL (Bact. 10^6 mL^{-1}). Data are presented as the means ± SD from individual experiments, using samples from three different blood donors. *P < 0.05, analyzed by one-way repeated measurements ANOVA followed by the Holm–Sidak post-test and multiple comparisons versus the bacteria plus PBS as a control.

the same concentration of bacteria as that in the whole blood samples. The binding of *N. meningitidis* 44/76*lpxA* mutant to erythrocyte CR1 clearly indicates that this binding is LPS-independent.

3.7. In vivo experiments with Alexa-stained E. coli and N. meningitidis 44/76 in a porcine model of sepsis

Finally, we examined the association between erythrocyte binding and the fate of Gram-negative bacteria *in vivo* using a porcine model of Gram-negative sepsis because pig erythrocytes lack CR1. The peak concentrations of *E. coli* and *N. meningitidis* were 6×10^7 and 2×10^5 bacteria/mL whole blood, respectively (Fig. 8A and C). The numbers of bacteria in the erythrocyte fraction, plasma and the buffy coat were then examined by analyzing the bacterial genome using RT-qPCR and by calculating the percentage in each fraction (Fig. 8B and D). The percentage of *E. coli* bacteria in the erythrocyte fraction was only 24% 5 min after the bolus injection and decreased to 14%, 55 min later (Fig. 8B). During this time period, the percent-
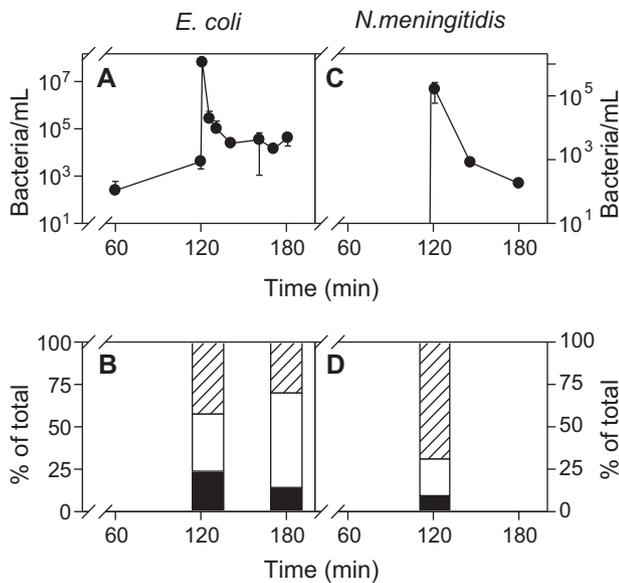
Fig. 8. Time course of bacterial genome concentrations in whole blood analyzed by RT-qPCR after intravenous infusion of bacteria in pigs. (A) *E. coli* and (C) *N. meningitidis* genomes in whole-blood samples were individually analyzed by RT-qPCR; results are expressed as bacteria/mL (log scale) and are presented as the means ± SD. (B and D) The percentages of the bacteria in erythrocyte (black bar), buffy coat (white bar) and plasma fractions (hatched bar) are expressed as % of the total bacteria concentration.

age of *E. coli* in plasma decreased from 42% to 30%, whereas the percentage in the buffy coat fraction increased from 34% to 56%. In contrast, only 6% of *N. meningitidis* was present in the erythrocyte fraction shortly after the bolus injection (Fig. 8D), indicating that pig erythrocytes bind Gram-negative bacteria to a much less extent than human erythrocytes. Notably, immunofluorescence histology of the lungs, spleen and liver of the pigs after 4 h showed that the Alexa-stained *E. coli* bacteria were mainly located in the lungs (supplemental Fig. 1A), and not in the liver (supplemental Fig. 1B) or spleen (supplemental Fig. 1C).

4. Discussion

The present study demonstrates that erythrocytes can rapidly bind the Gram-negative bacteria *E. coli* and *N. meningitidis* in human whole blood anticoagulated with lepirudin. This complement-dependent, LPS-independent binding occurs through CR1. Blocking CR1 led to the increased concentration of bacteria in plasma, followed by enhanced phagocytosis and the subsequent oxidative burst by leukocytes. Therefore, our data provide new insights into important mechanisms involved in the pathophysiology of Gram-negative sepsis in humans, suggesting that the initial binding to erythrocytes reduce intravascular phagocytosis and oxidative burst by blood leukocytes.

Because human erythrocytes express complement receptor CR1, and leukocytes express CR1 and CR3 (Birmingham and Hebert, 2001), we speculated that both cell types might bind complement-opsonized bacteria. The finding that approximately 80–90% of the Gram-negative bacteria initially bind to erythrocytes is most likely due to the approximately 400–1500-fold higher concentration of erythrocytes, compared to that of leukocytes in human whole blood. In addition, erythrocyte CR1 may bind bacteria with a higher avidity than leukocyte CR1, as shown by the binding of ICs (Paccaud et al., 1990). This explains why less than 5% of the bacteria in our study were phagocytized after 10 min incubation (data not shown). The reported ligands for human CR1 are C3b, iC3b, C4b, MBL and C1q (Birmingham and Hebert, 2001). In this study, C3 and C4, but

not C1q (data not shown), were detected on the bacterial surfaces. Complement factors seem to be the most important opsonins involved in the binding of Gram-negative bacteria to erythrocytes because complement activation inhibitors at the C3 level significantly reduced this binding. The increased C4 opsonization found after incubation with the C3 inhibitor compstatin is probably due to the increased space for C4 deposition on the bacterial surfaces when C3 deposition was reduced. Such C4b deposition probably explains why some bacterial binding to erythrocytes was still observed when complement was inhibited. In contrast, EDTA abolished both C3 and C4 opsonization and almost completely blocked the binding of bacteria to erythrocytes. The anti-CR1 mAb 3D9 that specifically inhibits the binding of C3b/C4b to CR1 (Krych et al., 1991; O'Shea et al., 1985) also completely blocked the bacterial binding to erythrocytes. This finding further indicates that C3b and C4b are the most important opsonins in the binding of bacteria to erythrocytes and that this binding occurs solely through CR1. As expected, the C5aRa had no effect on the binding to erythrocytes, but significantly inhibited phagocytosis (Mollnes et al., 2002). The almost linear decrease in the number of detectable erythrocyte-bound bacteria with time suggests that the initial rate of bacterial removal from erythrocytes is relatively constant. The removal of bacteria from erythrocytes may involve release of bacteria into plasma or degradation following phagocytosis. The release process may be due to a time-dependent change in the number of C3b molecules converted by factor I to the lower affinity CR1 ligand iC3b on the bacterial surface (Medof et al., 1982; Newman and Mikus, 1985; Turner et al., 1986). The further proteolysis of bound C3b then produces C3c and C3dg fragments which are recognized by different macrophage receptors. Therefore, the time-dependant increase in the leukocyte phagocytosis of bacteria may reduce the number of bacteria available for erythrocyte binding. However, the exact mechanism involved in the removal of bacteria from erythrocytes needs further examination.

The binding of bacteria to erythrocyte CR1 was LPS-independent because the *N. meningitidis* LPS-deficient mutant bound to the erythrocytes and was found in plasma when CR1 was blocked as efficiently as the wild-type strain. The levels of neisserial DNA in blood samples from humans with fatal fulminant neisserial septicemia analyzed using RT-qPCR was in the range of 1.4×10^5 to 5.4×10^8 mL^{-1} (Ovstebo et al., 2004), *i.e.*, similar to the neisseria concentrations used in this study. Both the wild-type and the LPS-deficient *N. meningitidis* mutant used in this study are encapsulated. However, the opsonization probably also occurs under the capsule surface because a previous study has shown that the opacity protein in the subcapsular outer membrane and in other neisseria structures binds C3b and C4b (Lewis et al., 2008). The observation that both LPS-containing and LPS-deficient *N. meningitidis* initially bind to erythrocyte CR1 further indicates that some bacterial structures other than LPS bind C3b and C4b. However, the detailed interaction between human erythrocytes and *N. meningitidis* remains to be elucidated.

The rapid binding of bacteria to human erythrocytes may affect and delay the reactions of peripheral leukocytes to bacteria in the circulation, as indicated by our finding that blocking CR1 increased the *E. coli*-induced phagocytosis and oxidative burst in monocytes and neutrophils. This observation is in agreement with previous studies using ICs, showing that the binding of ICs to erythrocyte CR1 inhibited IC-mediated neutrophil activation (Beynon et al., 1994; Nielsen et al., 1994). However, Nelson's first study of the immune adherence phenomenon in 1953 showed that the phagocytosis of pneumococci by guinea pig macrophages increased in the presence of human erythrocytes (Nelson, Jr., 1953). This discrepancy indicates that guinea pig macrophages behave differently from the human whole blood monocytes and granulocytes examined in this study. Subsequent studies have confirmed that the mononuclear

phagocyte system of the liver and spleen is particularly important for the removal of circulating ICs and bacteria and that, apparently, only a minor proportion of bacteria in the blood are phagocytized by circulating leukocytes (Benacerraf et al., 1959; Hirakata et al., 1991; Klein et al., 1994). These findings are in line with a recently published study, demonstrating a functional role of erythrocyte CR1 in the clearance of pneumococci from the circulation by facilitating the transfer of pneumococci to liver macrophages (Li et al., 2010).

Interestingly, in the *in vivo* studies using the porcine model of sepsis, the proportion of bacteria associated with the erythrocyte fraction was substantially lower than in our *in vitro* experiments with human whole blood. The bacterial numbers in the erythrocyte fraction in porcine blood may also have been overestimated in this study due to some remaining free bacteria in the small plasma volume between the erythrocytes after centrifugation. Notably, pig erythrocytes do not express a CR1 receptor because these molecules are restricted to primates (Birmingham and Hebert, 2001). We speculate that the relatively low proportion of bacteria bound to erythrocytes in the pigs could explain why the majority of the bacteria were removed from the circulation by the lungs, but not by the spleen and liver (Thorgersen et al., 2010). In addition, capillaries in porcine lungs are lined with macrophages; therefore, they are probably involved in the removal of bacteria from the circulation. We speculate that the binding of complement-opsonized bacteria to erythrocyte CR1 can reduce phagocytosis and oxidative burst in the blood stream in humans by specifically directing the bacteria to the liver and spleen where they can be phagocytized by the mononuclear phagocyte system with less systemic inflammatory responses. This suggests a supplementary role of complement to its established primary functions of mediating phagocytosis and serum bactericidal activity. This may also have implications for the potential use of complement inhibition as adjuvant therapy in sepsis (Mollnes and Kirschfink, 2006).

The effects of specific complement inhibitors may have several possible implications on the bacterial binding to erythrocytes. The use of the complement inhibitor acting early in the complement cascade, *i.e.*, at the C3 level or upstream steps, can decrease the bacterial opsonization, therefore reducing bacterial binding to erythrocytes and consequently increasing the number of free bacteria in plasma, as shown in this study. Decreased opsonization and decreased CR3 up-regulation (Brekke et al., 2007; Mollnes et al., 2002) can also lead to reduced phagocytosis by peripheral leukocytes and probably by tissue macrophages as well, further increasing the number of free bacteria in plasma. Decreased phagocytosis may attenuate the parts of the septic inflammatory responses associated with neutrophil activation and oxidative burst, *i.e.*, the release of reactive oxygen metabolites and other products important in some pathophysiological changes of sepsis, particularly capillary leakage (DiStasi and Ley, 2009). However, increased concentrations of circulating pathogens, even if they are dead due to adequate antibiotic treatment, may also increase the exposure of pathogen-associated molecular patterns to their respective receptors, including Toll-like receptors, therefore increasing the inflammatory response (Kawai and Akira, 2010). Also, inflammatory cells in organs more susceptible to inflammation than the liver and spleen, such as the lungs, may be more activated. An alternative strategy is to inhibit complement at a later step, for example, blocking the potent anaphylatoxin C5a by blocking C5aR, in which case the bacteria will still be fully opsonized and may bind to both erythrocyte and leukocyte CR1. According to the results in the present and previous studies (Mollnes et al., 2002), C5aR inhibition can substantially decrease granulocyte phagocytosis by diminishing CR3 up-regulation. However, according to our results, phagocytosis by monocytes and possibly by tissue macrophages in the liver and spleen is better protected from C5aR inhibition than from the inhibition of upstream steps that decrease complement opsonization. Therefore, C5aR inhibition may be an attractive complement inhibition strategy for treating sepsis because it does not affect the binding of bacteria to erythrocytes.

In conclusion, the present data provide new and important insights into the initial interaction of Gram-negative bacteria with erythrocytes and leukocytes in human whole blood and shed new light on how the bacterial binding to erythrocyte CR1 affects the response to bacterial infection. The mechanisms described supports the important role of complement in sepsis and may be of clinical importance especially in the development of complement inhibitors as potential therapeutic agents for sepsis.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2011.07.011.

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