Complement activation on platelet-leukocyte complexes and microparticles in enterohemorrhagic *Escherichia coli*-induced hemolytic uremic syndrome

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Abstract

Hemolytic uremic syndrome (HUS) is commonly associated with Shiga toxin (Stx)-producing *Escherichia coli* O157:H7 infection. This study examined patient samples for complement activation on leukocyte-platelet complexes and microparticles as well as donor samples for Stx and lipopolysaccharide (O157LPS)-induced complement activation on platelet-leukocyte complexes and microparticles. Results, analyzed by flow cytometry, showed that whole blood from a child with HUS had surface-bound C3 on 30% of platelet-monocyte complexes compared to 14% after recovery and 12% in pediatric controls. Plasma samples from 12 HUS patients were analyzed for the presence of microparticles derived from platelets, monocytes and neutrophils. Acute phase samples exhibited high levels of platelet microparticles and, to a lesser extent, monocyte microparticles, both bearing C3 and C9. Levels decreased significantly at recovery. Stx or O157LPS incubated with donor whole blood increased the population of platelet-monocyte and platelet-neutrophil complexes with surface-bound C3 and C9, an effect enhanced by co-stimulation with Stx and O157LPS together. Both Stx and O157LPS induced the release of C3 and C9-bearing microparticles from platelets and monocytes. Released microparticles were phagocytosed by neutrophils. The presence of complement on platelet-leukocyte complexes, and microparticles derived from these cells, suggests a role in the inflammatory and thrombogenic events occurring during HUS.

Introduction

Typical hemolytic uremic syndrome (HUS) is associated with gastrointestinal infections caused by Shiga toxin (Stx)-producing bacteria such as enterohemorrhagic *Escherichia coli* (EHEC), of which *E. coli* O157:H7 is the most common clinical isolate.[1] HUS is characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. The renal glomerular lesion, termed thrombotic microangiopathy, features microthrombi in the capillaries leading to their occlusion and profound endothelial injury.[2] Thrombocytopenia is presumably secondary to platelet consumption in microthrombi along the damaged endothelial wall.[3,4]

Gastrointestinal infection with EHEC may result in diarrhea or hemorrhagic colitis followed in severe cases by HUS.[1] Although the bacteria are non-invasive[5] virulence factors gain access to the circulation after passing the intestinal barrier, and are thus presumed to reach the kidney during HUS.[6] Both Stx and *E. coli* O157:H7 lipopolysaccharide (O157LPS) have been detected in the circulation bound to platelets, monocytes and neutrophils.[7,8]

Stx is a bipartite molecule composed of a pentamer of B subunits which bind to the globotriaosylceramide (Gb3) receptor and an enzymatically active A subunit. The A subunit inhibits eukaryotic cell protein synthesis.[9] Both Stx and O157LPS have been shown to activate platelets, monocytes and neutrophils.[7] O157LPS binds to and activates platelets directly via toll-like receptor 4 (TLR-4) in complex with CD62 (P-selectin)[10] and Stx binds to pre-activated platelets.[3,11] Binding of the B subunit of Stx1 (Stx1B) alone induces platelet activation.[3]

Stx and O157LPS induce the formation of platelet-leukocyte complexes in whole blood.[7] These complexes formed mainly between platelets and monocytes and expressed tissue factor, particularly under conditions of high shear stress. Tissue factor was also detected on microparticles released from these blood cells.[7] These in vitro findings were confirmed in whole blood from HUS patients[7] and suggested that platelet-leukocyte complexes, and microparticles derived thereof, could contribute to the prothrombotic state occurring during HUS.

In the current study we investigated if complement was activated on blood cell complexes and on the surface of blood cell-derived microparticles in Stx-associated HUS. Activation of platelets, neutrophils and monocytes is accompanied by complement deposition.[12-14] Complement activation on the surface of platelets and leukocytes is followed by the formation of the membrane-attack complex (MAC) C5b-9.[15,16] In addition to its lytic capacity, the MAC may induce cell activation and proliferation, as previously reviewed.[17] Deposition of C5b-9 on platelets leads to release of microparticles.[18] Microparticles, released from platelets and monocytes during complement deposition, may partake in a prothrombotic state when they are coated with tissue factor as previously shown in atypical HUS mediated by factor H mutations.[15]

The aim of this study was to investigate if complement was deposited on platelet-leukocyte complexes and microparticles-derived from these cells during Stx-associated HUS and if Stx and O157LPS could induce complement deposition contributing to the inflammatory and prothrombotic mechanisms of this disease.

Materials and methods

Subjects

Blood samples were available from 12 patients (Table 1 diagnosed with EHEC-associated HUS and treated at the Department of Pediatrics, Lund University Hospital. There were 8 boys and 4 girls aged 1-11 years (median age 3 years). Patient samples were taken within three days (median 4 h) of admission while the children still had clinical signs of HUS. HUS was defined as hemolytic anemia (hemoglobin levels <100 g/L), thrombocytopenia (platelet counts <140 x 10^9/L), and acute renal failure. Blood was also obtained from the children two to nine months after recovery. EHEC infection was detected as previously described [19]. Blood samples were also available from six pediatric controls, 3 girls and 3 boys, aged 1-15 years (median 9.5 years). These children were seen at the out-patient clinic of the Department of Pediatrics, Lund University Hospital, for unrelated conditions such as reflux nephropathy, immune deficiency or metabolic disorders and did not have a history of HUS or diarrhea at the time of sampling. Whole blood was available from five of these pediatric controls and plasma was available from all. Blood samples were also obtained from three patients with acute renal failure without any history of HUS or diarrhea at the time of blood sampling. These patients were seen at the Department of Pediatrics, Lund University Hospital, for acute myeloid leukemia (a boy 13 years old) or at the Department of Nephrology, Lund University Hospital for acute renal failure associated with hypertension and sepsis (an adult woman and man). Blood was also obtained from 12 healthy adult volunteers (7 women, 5 men) not using any medications. Samples from patients, controls and healthy donors were taken with the informed written consent of the subjects or their parents in accordance with the Declaration of Helsinki. The study was performed with the approval of the Ethics Committee of the Medical Faculty, Lund University.

Blood collection

Whole blood from patients and controls was drawn by venipuncture into vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing 0.5 mL of 0.129 M sodium citrate and platelet-poor-plasma obtained by centrifugation at 2600 x g for 15 min. Whole blood used for *in vitro* stimulation experiments was drawn by venipuncture via a butterfly needle (Plasti Medical S.p.A, Villamarzana, Italy) into plastic tubes (Becton Dickinson, Plymouth, UK, to prevent spontaneous formation of platelet-leukocyte complexes) containing 0.27 mL 0.109M sodium citrate and immediately diluted (1:2) in sterile LPS-free RPMI-1640 (Invitrogen, Paisley, UK) containing Gly-Pro-Arg-Asp (GPRP, 10μM, Sigma-Aldrich, St. Louis, MO). Alternatively, whole blood was centrifuged at 200 x g for 20 min to obtain platelet-rich-plasma and diluted (1:2) in sterile LPS-free RPMI-1640. Whole blood samples were used for determination of platelet-leukocyte complexes by flow cytometry. Platelet-poor plasma samples were further processed to quantify and assay microparticles (by flow cytometry and immunoblotting) or used for analysis of C3a(desArg) or terminal complement complex (TCC) levels by ELISA. Platelet-rich-plasma was used to determine C3 or C9 on platelets by flow cytometry. Whole blood or platelets were used within 2 hours of sampling and plasma samples were stored at -80˚C until assayed.

Stimulation of whole blood by Stx2, Stx1, Stx1B or LPS

Whole blood was stimulated with Stx2, Stx1, Stx1B and/or LPS according to a previously described method [7] with modification of the stimulation time which was reduced to 40 min as well as the inclusion of Stx1 and Stx1B experiments. Stx2 (a gift from T.G. Obrig, Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore) , Stx1 and Stx1B (a gift from J. Brunton, Department of Microbiology, Toronto General Hospital, Toronto, Canada) were all used at a final concentration of 2.8×10^{-9} M

diluted in LPS-free phosphate buffered saline (PBS, PAA Laboratories GmbH, Pasching, Austria). The LPS content of Stx2 and Stx1 preparations were assayed by the limulus amebocyte assay (Coatex, Gothenburg, Sweden) and found to be less than 50 pg/mL (the detection limit). Likewise, whole blood was incubated with O157LPS (a gift from P.I. Tarr, Department of Pediatrics, Division of Gastroenterology and Nutrition, Washington University, Saint Louis, O157LPS was purified by phenol extraction)[20] or non-EHEC-LPS O111:B4 (Sigma-Aldrich, St. Louis, MO) which were used at a final concentration of 0.5 μg/mL determined by the limulus amebocyte assay.

Detection of platelet-leukocyte complexes

Platelet-leukocyte complexes were detected as previously described [7]. Briefly, whole blood was incubated with mouse anti-human CD42b:Allophycocyanin (APC, 1:8, to detect platelets) and mouse anti-human CD38:Phycoerytrin (RPE, 1:10, to detect monocytes), simultaneously, to detect platelet-monocyte complexes. Similarly, whole blood was incubated with mouse anti-human CD42b:APC and mouse anti-human CD66:RPE (1:10, to detect neutrophils) simultaneously, to detect platelet-neutrophil complexes. Mouse IgG_1:APC and mouse IgG_1:RPE were used as isotype controls (all antibodies from BD Biosciences, San Diego, CA).

The experimental conditions are described in the supplementary data and in Supplementary Figure 1. These conditions were the basis for further experiments used for detection of complement on complexes.

Detection of Stx2 or O157LPS binding to platelet-leukocyte complexes

Binding of Stx2 or O157LPS to platelet-leukocyte complexes was detected as previously described.[7,10] Briefly, whole blood was incubated with mouse monoclonal anti-Stx2 IgG_1 antibody (11E10, 200ng/ml a gift from T.G. Obrig) diluted in 0.1% Saponin (Sigma-Aldrich)/PBS or mouse anti-O157LPS (10µg/mL, a gift from R. Johnson, Public Health Agency of Canada, Guelph, Canada) diluted in PBS. Mouse IgG_1 (DAKO, Glostrup, Denmark) was used as negative control for both antibodies and rabbit anti-mouse:RPE $F(ab´)_2$ (1:1000, DAKO) was the secondary antibody.

Detection of surface-bound C3 or C9 on platelet-leukocyte complexes

C3 or C9 deposition on platelet-leukocyte complexes was detected by incubation of whole blood with an antibody mixture of chicken anti-human C3:fluorescein isothiocyanate (FITC, 1:600, Diapensia, Linköping, Sweden) or mouse anti-human C9:FITC (1:50, Hycult Biotech, Uden, Netherlands), CD42b:APC and mouse anti-human CD38:RPE or mouse anti-human CD66:RPE. Chicken anti-human insulin:FITC (1:600, Diapensia) or mouse IgG_1:FITC (1:50, Hycult Biotech) were used as irrelevant antibodies.

Inhibition of complement deposition on platelet-leukocyte complexes

Whole blood was diluted 1:1 in a combination of RPMI and 25mM ethylenediaminetetraacetic acid (EDTA)-Veronal-buffered saline (VBS) solution (145mM NaCl, 1,8mM Na-diethyl-barbiturate, 3mM 5.5´-diethyl barbiturate acid, pH 7.3) or RPMI and 10mM ethylene glycol tetraacetic acid (EGTA)-VBS solution. Ca^{2+} and Mg^{2+} concentrations were adjusted to 0.1 mg/mL to resemble the concentrations in RPMI alone before incubation with Stx2 and/or O157LPS for 40 min at 37°C. Alternatively, whole blood was incubated with rat anti-globotriaosylceramide (Gb3, 20µg/mL, Beckman Coulter Inc,

Brea, CA, to block the Stx receptor), rabbit anti-human toll-like receptor 4 (TLR4, 20 µg/mL, eBiosciences, San Diego, CA, to block the LPS receptor) or a combination of both antibodies for 1 hour at rt before incubation with Stx2 and/or O157LPS for 40 min at 37°C. Rat IgM (Beckman Coulter Inc) or rabbit IgG (DAKO) were used as irrelevant antibodies. C3 or C9 deposition were detected as described above using chicken anti-human C3:FITC and mouse anti-human C9:FITC.

Isolation of microparticles

Microparticles were isolated from patient whole blood or from whole blood stimulated with or without agonists (Stx2, Stx1, Stx1B or LPS). Platelet-free plasma was obtained from whole blood by an initial centrifugation step at 2600 x g for 15 min followed by a 5 min centrifugation at 9900 x g at rt to remove larger apoptotic bodies. Microparticles were washed twice in Hank's Balanced Salt Solution without Ca^{2+}/Mg^{2+} (PAA Laboratories), centrifuged at 20800 x g for 5 min and resuspended in Annexin V binding-buffer (BD Biosciences). All buffers and cell media were filtered to exclude larger particles using a 0.2 µm filter (Schleicher-Schuell, Dassel, Germany) before use. The numbers of microparticles/mL plasma were calculated as previously described.[15] Briefly, a defined quantity of 6.0 µm non-fluorescent calibration beads (BD Biosciences) was added to each sample tube and run concurrently with the microparticles, thus allowing for quantitative determination of microparticles. The analysis was terminated when 10000 beads were counted and the absolute number of microparticles determined by the formula N = (microparticles/beads counted) x (beads added/µl plasma) x the dilution factor.

Labeling of microparticles with cell-specific antibodies

Microparticle subpopulations were determined by incubation with antibodies directed against membrane-specific antigens on platelets, monocytes and neutrophils. This was achieved by using a combination of mouse anti-human CD42b:RPE (1:20, BD Biosciences), mouse anti-human CD38:Peridinin Chlorophyll Protein-Cyanin 5.5 (PerCP-Cy5.5, 1:20, BD Biosciences) or mouse anti-human CD66:RPE, respectively. Surface-bound C3 or C9 on microparticles were detected by incubation with chicken anti-human C3:FITC, mouse anti-human C9:FITC or mouse anti-human C5b-9 (1:1000, Quidel, San Diego, CA). Non-specific binding was determined by subtraction of bound irrelevant antibodies. Irrelevant control antibodies with equivalent protein:fluorescein ratios were used for this purpose: mouse IgG_1:RPE, IgG_1:PerCP-Cy 5.5 (BD Biosciences) or IgG_1:FITC as well as chicken anti-human insulin or mouse IgG_1 (DAKO) followed by rabbit anti-mouse:FITC $F(ab')_2$ (1:500, DAKO).

Characterization of C3 or C9 on platelets and platelet-derived microparticles

Platelet-rich-plasma was obtained from whole-blood, diluted 1:2 in RPMI 1640 and incubated with Stx2 (2.8×10^{-9} M) or O157LPS (0.5 µg/mL) or left unstimulated for 40 min at 37ºC followed by centrifugation at 2000 x g for 10 min to isolate platelets which were washed once in PBS. Likewise, platelet-derived microparticles were prepared from whole blood stimulated with Stx2 or O157LPS and isolated as described above. Platelets or platelet-derived microparticles were incubated with a combination of mouse anti-human CD42b:APC (1:10) and mouse anti-human activated C3b (1:100 Hycult Biotech), mouse anti-human iC3b (1:100, Thermo Scientific, Rockford, IL) or mouse anti-human C3c (1:1000, Quidel, San Diego, CA), respectively, for 20 min. Mouse IgG_{2a}, IgG_{2b} or IgG_1 were used as negative control antibodies and rabbit anti-mouse:FITC $F(ab')_2$ (DAKO) was used as the secondary antibody.

Similarly, platelets or microparticles were incubated with a combination of mouse anti-human CD42b:APC and mouse anti-human C9 neoantigen (1:100, Hycult Biotech) or mouse anti-human C5b-9. Mouse IgG_1 was the negative control antibody and rabbit anti-mouse:FITC $F(ab')_2$ was the secondary antibody.

Detection of membrane-bound complement regulators on microparticles

Membrane-bound complement regulators on microparticles was determined by incubation of microparticles with mouse anti-human CD35:FITC (Complement receptor 1, CR1, 1:20), mouse anti-human CD55:APC (Decay accelerating factor, DAF, 1:40) or mouse anti-human CD46:RPE (membrane cofactor protein, MCP,1:50). Subpopulations of microparticles were identified by incubation with mouse anti-human CD42b:RPE or CD42b:APC, mouse anti-human CD38PerCP-Cy5.5 or mouse anti-human CD66:FITC (1:20) or CD66:RPE, respectively. Mouse IgG_1:FITC, IgG_{2a}:APC or IgG_1:FITC were used as irrelevant antibody controls (for anti-CR1, DAF and MCP antibodies). Mouse IgG_1:RPE, IgG_1:APC, IgG_1:PerCP-Cy 5.5 and IgG_1:FITC were used as irrelevant control antibodies for identification of microparticle populations. All antibodies were from BD Biosciences.

Isolation of neutrophils

Neutrophils were isolated under LPS-free conditions from citrated peripheral blood collected from healthy donors using one-step density gradient centrifugation with Polymorphprep® (Nycomed. Oslo, Norway), followed by hypotonic lysis of erythrocytes. Lysis was stopped by addition of x10 PBS (6:1). Neutrophils were washed twice in PBS and resuspended in RPMI-1640 with GlutaMax (Invitrogen), resulting in a suspension consisting of ~96% neutrophils as determined by flow cytometry using mouse anti-human CD66:RPE antibody.

Detection of microparticle phagocytosis by neutrophils

Microparticles were isolated from Stx2- or O157LPS-stimulated or unstimulated whole blood, washed twice in PBS and stained with 0.1µM 2′.7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxy-methylester (BCECF-AM, Invitrogen) for 30 min at 37ºC, washed in PBS and resuspended in a buffer of RPMI 1640 with GlutaMax. BCECF-AM is non-fluorescent when extracellular but becomes fluorescent when converted to BCECF by intracellular esterases.

Neutrophils ($1x10^6$/mL) were incubated with microparticles ($2.5x10^6$/mL, stained with BCECF-AM) and 20% (v/v) plasma from ABO-matched individuals for 1h at 37ºC, with end-over-end rotation. Samples were washed twice and kept on ice until assayed. Phagocytosis of microparticles was detected as described below.

Flow cytometry acquisition and interpretation of data

Whole blood samples were lyzed with FACSLyse (Dako). Flow cytometry was performed using a FACSCanto^{TM}II instrument with FACSDiva software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Platelet-leukocyte complexes and microparticles were defined and detected as previously described [7]. Determination of C3 or C9 deposition on platelet-leukocyte complexes and microparticles was performed using three- or four-color analysis for simultaneous detection of platelet-leukocyte complexes or microparticles, and C3 or C9. In phagocytosis experiments, neutrophils were selectively identified by appropriate settings of forward- and side scatter and measured for green fluorescence from associated BCECF-labeled microparticles.

Immunoblotting to detect C3 and C9 on microparticles

Microparticles were isolated from 1 mL of Stx2- or O157LPS-stimulated or unstimulated whole blood. Samples were diluted (1:10) and subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis under reducing conditions. Proteins were transferred onto equilibrated polyvinylidine difluoride membranes and blocked with casein solution (Vector Laboratories Inc, Burlingame, CA). Immunoblotting was performed with rabbit anti-human C3c (DAKO) or mouse anti-human iC3b followed by goat anti-rabbit HRP or goat anti-mouse HRP (DAKO), respectively. Signal was detected by chemiluminescence using ECL plus (Amersham Biosciences, Uppsala, Sweden).

Detection of C3a(desArg) or terminal complement complex in plasma

C3a(desArg) and human terminal complement complex (TCC) in plasma from patients or controls was quantified using a C3a assay kit or TCC kit (both from Hycult Biotech), following the manufacturer's instructions.

Alternatively, citrated whole blood (diluted 1:2 in RPMI1640) was stimulated with Stx2 or O157LPS or left unstimulated for 40 min at 37ºC and platelet-free-plasma obtained by centrifugation at 2000 x g for 15 min. Samples were stored at -80ºC until analyzed using the C3a(desArg) and TCC kits.

Statistical analysis

Comparison of blood cells incubated with the various agonists or unstimulated blood cells, generation of microparticles in stimulated or unstimulated whole blood, microparticles in patient plasma or control plasma, as well as C3 or C9 on complexes or microparticles were analyzed by the Mann-Whitney U-test. Correlations between clinical manifestations and

microparticles were analyzed using the Spearman's rho test. P values ≤ 0.05 were considered significant. Statistical analyses were performed using PASW 18 (SPSS, Chicago, IL).

Results

C3 bound to platelet-leukocyte complexes in a patient with HUS

Stx2, O157LPS and C3 deposition on circulating platelet-monocyte or platelet-neutrophil complexes (Figure 1A-B) were measured in whole blood from one patient with *E. coli* O157:H7-associated HUS taken during the acute phase of disease (Patient 1 in Table 1) and compared to pediatric controls (n=5). C3 was detected by flow cytometry on 30% of platelet-monocyte complexes and 15% of platelet-neutrophil complexes during the acute phase of disease but decreased to 14% of platelet-monocyte complexes and 9% of platelet-neutrophil complexes after recovery, levels that were comparable with the pediatric controls (Figure 1A-B). C9 was not determined on the patient's platelet-leukocyte complexes. Stx2 was detected on 15% of platelet-monocyte complexes and on 30% of platelet-neutrophil complexes. Similarly, O157LPS was detected on 30% platelet-monocyte complexes and on 37% of platelet-neutrophil complexes. No binding of Stx2 or O157LPS was detected after recovery or in the pediatric controls.

Patients with HUS have increased levels of circulating microparticles with bound C3 and C9

Plasma levels of circulating microparticles with surface-bound C3 or C9 were measured by flow cytometry during the acute phase of disease and compared to pediatric controls (n=6) and patients with acute renal failure (n=3). Plasma taken during the acute phase showed significantly higher levels of circulating microparticles with surface-bound C3 and C9 (Table 2) compared to the controls. Mean fluorescent intensity (MFI) levels of C3, C9 and C5b-9 on platelet- and leukocyte-derived microparticles are presented in supplementary Figure 2. Microparticles originated primarily from platelets followed by monocytes. Levels at recovery were similar to those found in the controls.

Laboratory findings (hemoglobin, platelet counts, creatinine, urea, dialysis (+ or -), C3 and C4 levels and extra-renal complications (+ or -), during the acute phase of HUS (Table 1) were correlated to levels of C3 and C9 on platelet-derived microparticles (10^3/mL) and did not show a significant correlation (data not shown).

Patients with HUS had increased levels of C3a(desArg) and TCC in plasma

Plasma levels of C3a(desArg) and TCC were measured during the acute phase (n=10, patients 1-10) and after recovery and compared to pediatric controls (n=6). Levels were elevated during the acute phase of disease as presented in Table 3.

Stx and O157LPS induce C3 and C9 deposition on platelet-leukocyte complexes

Incubation of whole blood with Stx2, Stx1 or Stx1B induced a significant increase in platelet-monocyte and platelet-neutrophil complexes with surface-bound C3 (Figure 2A-B) compared to PBS-treated whole blood. Similarly, Stx2 or Stx1 induced a significant increase in platelet-monocyte and platelet-neutrophil complexes with surface-bound C9 (Figure 3A-B) while Stx1B only induced a significant increase in surface-bound C9 in the platelet-monocyte population. Stimulation with O157LPS also induced C3 and C9 deposition. Co-stimulation with Stx2 and O157LPS, simultaneously, increased the population of platelet-monocyte complexes with surface-bound C3 (Figure 2A-B) and the population of platelet-monocyte and platelet-neutrophil complexes with surface-bound C9 (Figure 3A-B) compared to PBS-treated samples or Stx2 or O157LPS alone. Both C3 (Figure 2C-D) and C9 (Figure 3C-D) bound to a greater extent to monocytes and neutrophils in complex with platelets than to platelet-free monocytes or neutrophils.

Stx2- and/or O157LPS-stimulated whole blood induced complement activation via the alternative pathway

Whole blood diluted in RPMI/EDTA-VBS and stimulated with Stx2 and/or O157LPS exhibited marked reduction of surface-bound C3 or C9 on platelet-monocyte complexes (Table 4). Whole blood diluted in RPMI/EGTA-VBS and stimulated with Stx2 and/or O157LPS exhibited slightly reduced surface-bound C3 and C9 on platelet-monocyte complexes compared to unstimulated whole blood diluted in RPMI alone. Inhibition experiments were not performed for platelet-neutrophil complexes. The results indicate that Stx2 and/or O157LPS activated complement mostly via the alternative pathway.

Blocking of Stx2 and/or O157LPS binding reduced platelet-monocyte complex formation and surface-bound C3 and C9

Incubation of whole blood with anti-Gb3 before incubation with Stx2 reduced platelet-monocyte complex formation significantly by 67%. Similarly, incubation of whole blood with anti-TLR4 before incubation with O157LPS reduced platelet-monocyte complex formation by 76% (Figure 4A). An even more prominent reduction by 80% was noted when whole blood was preincubated with both α-Gb3 and α-TLR4, together, before addition of Stx2 and O157LPS, simultaneously (Figure 4A). Likewise, pre-incubation with anti-Gb3 and/or anti-TLR4 reduced surface-bound C3 (Figure 4B) or C9 (Figure 4C) in Stx2 and/or O157LPS-stimulated samples. The most pronounced reduction of complex formation and surface-bound C3 or C9 was noted in whole blood pre-incubated with a combination of anti-Gb3 and anti-TLR4 before incubation with Stx2 and O157LPS, simultaneously. The irrelevant antibodies used, rabbit IgG or rat IgM, did not inhibit complex formation as well as C3 and C9 deposition (data not shown). Inhibition experiments were not performed for platelet-neutrophil complexes.

Stx and O157LPS induce release of microparticles with surface-bound C3 and C9

Levels of microparticles with surface-bound C3 or C9 were measured in whole blood stimulated with Stx2, Stx1, Stx1B, O157LPS or a combination of Stx2 and O157LPS. The agonists induced a significant increase in microparticles with surface-bound C3 or C9 compared to PBS-treated samples (Table 5). The highest levels of microparticles were noted in whole blood co-stimulated with Stx2 and O157LPS compared to Stx2 or O157LPS alone. The combination of Stx2 and O157LPS induced a significant increase in C3- and C9-positive microparticles derived from platelets, in comparison to each agonist alone.

Characterization of surface-bound C3 or C9 on platelets and platelet-derived microparticles

To further characterize surface-bound C3 on platelets and platelet-derived microparticles two monoclonal anti-C3 antibodies were used, one which binds to activated C3b and one which binds to iC3b. These antibodies specifically react with neoepitopes exposed in activated and conformationally altered C3 but not in native C3. Both antibodies bound to platelets and platelet-derived microparticles from Stx2- or O157LPS-stimulated whole blood (Figure 5A and B) indicating that surface-bound C3 was activated C3b which was, to some extent, further processed to iC3b. The presence of C3 and iC3b on microparticles stimulated with Stx and O157LPS was further confirmed by immunblotting. The results are summarized in the supplementary data and supplementary Figure 3.

Similarly, surface-bound C9 on platelets were characterized by use of two antibodies specifically reacting with the neoepitope in C9 (C9neo) or neoepitopes in the C5b-9 complex. Both antibodies bound to the surface of platelets and platelet-derived microparticles (Figure 5A and B) indicating formation of the membrane attack complex (MAC) on the surface of

platelets and microparticles. O157LPS appeared to induce more deposition of C3b on platelets and platelet-derived microparticles than Stx2, although the difference was non-significant. Both stimulants induced significant C3 and C9 deposition compared to unstimulated platelets.

Microparticles express complement regulators on their surface

Expression of surface-bound complement regulators was examined on microparticles from O157LPS-stimulated whole blood. CR1 (Figure 6A), DAF (Figure 6B) and MCP (Figure 6C) were detected on microparticles from platelets, monocytes and neutrophils and there was no significant difference between stimulated and unstimulated microparticles.

Microparticles released in Stx2- or O157LPS-stimulated whole blood were phagocytosed by neutrophils

Microparticles were isolated from Stx2- or O157LPS-stimulated whole blood, labeled with a fluorescent dye (BCECF-AM) and incubated with neutrophils. Microparticles from Stx2- or O157LPS-stimulated samples were found to be phagocytosed by neutrophils as increased fluorescence indicated intracellular localization of the microparticles (Figure 7). Microparticles from unstimulated samples were taken up by neutrophils to a lesser extent (Figure 7). As equivalent concentrations of microparticles ($2.5x10^6$/mL) were incubated with neutrophils ($1x10^6$/mL) these results indicate that Stx2 or O157LPS-stimulated were preferentially phagocytosed.

Stx and O157LPS induce C3a(desArg) and TCC release in whole blood

Levels of C3a(desArg) and TCC were measured in Stx2- or O157LPS-stimulated whole blood. Both Stx2 and O157LPS induced a significant increase in C3a(desArg) and TCC concentrations (Table 6) compared to unstimulated samples.

Discussion

This study demonstrates the presence of C3 on platelet-leukocyte complexes in a patient with EHEC-associated HUS. Activated leukocytes and platelets release microparticles during HUS,[7] and these blood cell-derived microparticles, particularly those originating from platelets, were shown here to have C3, C9 and C5b-9 deposition on their surfaces. Patients also exhibited elevated levels of C3a and TCC in plasma. Stimulation of blood cells with Stx2, Stx1, Stx1B or O157LPS, in vitro, induced the formation of platelet-leukocyte complexes bearing C3 and C9. Stx and/or O157LPS induced the release of platelet and leukocyte-derived microparticles bearing C3 and C9 on their surfaces. In addition, Stx and O157LPS induced the release of C3a and TCC in whole blood. This study provides evidence for complement activation on circulating platelets and leukocytes, as well as microparticles derived from these cells, during EHEC-associated HUS, and suggests that Stx and O157LPS contribute to this process. Complement activation on these blood cells could further enhance the inflammatory and thrombotic process.

C3b binds to platelets via P selectin and to neutrophils and monocytes via complement receptor 1 (CR1).[12,21] The opsonin iC3b binds to these cells via CR3 (the integrin receptor CD11b/CD18).[22,23] We have shown the presence of C3c, iC3b, C3b, C9 and C5b-9 on the platelet surface, particularly after stimulation with Stx2 and O157LPS. Microparticles released from cells during apoptosis and activation [24] may bind complement components such as C1q, C3 and C4.[25] When stimulated with Stx and O157LPS platelet-derived microparticles were also demonstrated to have C3c, iC3b, C3b, C9 and C5b-9 on their surfaces. Complement activation on platelets, platelet-leukocyte complexes and on platelet-derived microparticles may be a mechanism of physiological clearance of unwanted or apoptotic cells and cell blebs[26] but may also activate the cells.[15,27,28]

Platelet- and leukocyte-derived microparticles, which were coated with C3 and C9, underwent neutrophil phagocytosis. Complement-labeling is a well-studied phenomenon whereby opsonized bacteria undergo phagocytosis by professional phagocytes such as neutrophils, monocytes, macrophages and, to a lesser extent, dendritic cells.[29] iC3b is an important opsonin in this process.[30] Complement-coated microspheres undergo neutrophil phagocytosis. [31] The same process appears to occur when complement-coated microparticles are incubated with neutrophils, as shown here. This may represent a protective mechanism by which neutrophils ingest pro-coagulant microparticles to prevent excessive thrombosis.

The formation of the C5b-9 complex induces cytolysis, when present in high amounts, but in sublytic amounts cell activation is induced resulting in secretion of cytokines, prostanoids and free radicals.[17,32-34] C5b-9 assembly on platelets promotes the formation of the prothrombinase complex allowing generation of thrombin.[35] Thrombin and C5b-9 induce the exposure of negatively-charged phospholipids serving as a procoagulant surface on the platelet membrane[36] thus enabling clot formation. Interestingly, thrombin generation precedes the renal injury in HUS.[37] In the absence of C3, thrombin can function as a C5 convertase cleaving C5[38] and has therefore been suggested to induce C3-independent complement activation.[39] Thus, there appears to be cross-talk between the complement and thrombotic systems and C5b-9 deposition on blood cells may promote thrombus formation. This is further supported by the finding that complement C3 and C9 deposition on platelets induced their activation and release of tissue factor-bearing microparticles.[15] In vitro stimulation of blood cells with Stx and O157LPS lead to formation of blood-cell complexes bearing complement C3 and C9 suggesting that complement activation could occur on the cell surface even before membrane blebbing. We propose that the observed complement activation on

blood cells may partake in the inflammatory process by enhanced opsonization and chemotaxis[40] as well as induce a prothrombotic state by enhancing platelet aggregation.[13]

Stx is cytotoxic for most cells, but not for blood cells.[3,41,42] On the contrary, the life-span of neutrophils is extended by Stx.[43] The toxin has a stimulatory effect on platelets, monocytes and neutrophils.[3,41,44] Stx and O157LPS induced C3 and C9 deposition on platelets, monocytes and, to a lesser extent, neutrophils and even Stx1B alone induced complement deposition, indicating that toxin binding to its receptor was sufficient to induce C3 and C9 deposition. Stx and O157LPS may, however, not be the only underlying causes of complement deposition on blood cells during HUS as complement activation has been documented in other conditions associated with acute renal failure, most probably due to complement activation on dialysis membranes.[45] Most of the patients studied here did not, however, undergo hemodialysis. All the same we cannot exclude that Stx and O157LPS were not the only factors promoting complement activation and that inflammatory mediators and uremic toxins may have also contributed to the process.

Previous studies have shown that complement may be activated via the alternative pathway during EHEC-associated HUS. Patients were shown to have low levels of C3,[46] and elevated levels of complement factor Bb and sC5b-9.[47] Stx was reported to activate complement via the alternative pathway in the fluid phase,[48] and induce C3 deposition on human microvascular endothelial cells promoting thrombus formation.[4] Taken together with the results presented here, these studies indicate that complement may be activated via the alternative pathway during EHEC-associated HUS, which may promote disease-activity by further chemotaxis, tissue damage and platelet activation. The findings may have implications for future treatments because complement-inhibitors, such as the humanized anti-C5 antibody

Eculizumab, have been effective in complement-mediated diseases, such as atypical HUS.[49] Further studies are, however, required as the use of complement inhibitors during active infectious disease may pose a risk and postpone intestinal bacterial clearance.

In summary, this study provides evidence for complement activation on blood cells and blood cell-derived microparticles during EHEC-associated HUS, a process which could be related to contact between Stx and/or O157LPS and the blood cells as shown in vitro. This suggests that complement activation occurs in the circulation during HUS. Although complement activation is not the primary event occurring during EHEC infection, we speculate that it may contribute to blood cell activation and, via this cell activation and the release of microparticles, free radicals and cytokines, even to end-organ damage of the kidney in HUS.

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Authorship

A-lS designed and performed the research, analyzed data and wrote the paper. LS performed preliminary experiments, analyzed data and assisted in writing the paper. DK designed the research, analyzed data and wrote the paper.

Conflict-of-interest disclosure

Diana Karpman was the national coordinator in Sweden of the multi-center trial of Eculizumab (Alexion Pharmaceuticals) in patients with atypical hemolytic uremic syndrome.

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Table 1. **Characteristics of HUS patients included in this study**

Patient	Sex	Age at diagnosis	Prodromal diarrhea	Laboratory findings							Extrarenal symptoms	*E. coli* serotype[d]
				Hemolytic anemia[a] Hemoglobin (lowest) g/l	Platelet count x10^9 (lowest) Ref 140-400	Renal failure			Complement levels			
						Creatinine (Highest) µmol/l[b]	Urea (Highest) mmol/l[c]	Duration of peritoneal dialysis	C3 (g/l) Ref (0.77-1.38)	C4 (g/l) Ref (0.12-0.33)		
1	M	3	+ [e]	62	21	103	15.6		NA	NA	-	O157
2	M	3	+ [e]	72	44	48	5.1		NA	NA	-	O157
3	M	7	+	56	18	453	27.8	17 days	1.11	0.16	-	O157
4	M	1	+	58	27	88	16.3		0.96	0.22	-	O26
5	F	3	+ [e]	63	35	286	21.1	21 days[f]	0.79	0.05[g]	Rectal prolapse	O157
6	F	2	+	43	10	96	16.7		1.21	0.16	Rectal prolapse	O157
7	M	2	+ [e]	63	31	174	17.3		1.01	0.18	-	O145
8	M	10	+	49	35	468	37.5	10 days	1.02	0.21	-	O157
9	F	2	+ [e]	57	<10	64	10.9		NA	NA	-	O157
10	F	11	+ [e]	55	27	280	28.2		1.26	0.14	-	O157
11	M	10	+ [e]	41	30	379	28.3	15 days	1.08	0.15	Coma, hemiplegia, seizures	O157
12	M	5	+ [e]	66	<10	188	20.6	4 days	0.95	0.06[g]	-	O157

[a]; Hemolytic anemia was defined as hemoglobin levels <100 g/L, elevated reticulocyte counts, elevated lactic dehydrogenase and unconjugated bilirubin. All patients had a negative direct antiglobulin test. [b]; Ref value < 5 years: 14-37 µmol/l, 5-15 years: 25-68 µmol/l. [c]; Ref value < 5 years: 1.7-5.0 mmol/l, 5-16 years: 1.7-7.3 mmol/l. [d]; All strains produced Stx2. [e]; Bloody diarrhea. [f]; Due to peritonitis also treated with hemodialysis. [g]; C4 level normalized after 10 days. NA, not available.

Table 2. Numbers and cellular origin of microparticles with surface-bound C3 or C9 in plasma from HUS patients

	CD42b-positive microparticles (10^3/mL)		CD38-positive microparticles (10^3/mL)		CD66-positive microparticles (10^3/mL)	
	C3-positive	**C9-positive**	**C3-positive**	**C9-positive**	**C3-positive**	**C9-positive**
Acute phase (n=12)	818*** (552 – 1001)	780*** (654 – 1031)	83** (48 – 128)	84** (50 – 124)	1.8*** (1.3 – 2.5)	1.5*** (1.1 – 2.4)
Recovery (n=12)	64 (15 – 342)	28 (11 – 258)	35 (8 – 285)	36 (10 – 304)	0.2 (0.1 – 0.4)	0.2 (0.1 – 0.6)
Pediatric controls (n=6)	73 (54 – 271)	69 (55 – 284)	48 (17 – 216)	38 (15 – 202)	0.3 (0.1 – 0.6)	0.4 (0.2 – 0.7)
Renal failure controls (n=3)	65 (35 – 132)	52 (10 – 109)	41 (18 – 198)	38 (7 – 124)	5 (2 – 133)	2.7 (2 – 93)

Data are expressed as median and (range) of circulating microparticles positive for each membrane-specific marker/mL. *** denotes *P* value <0.001 and ** *P* <0.01 when comparing microparticles in plasma from patients with pediatric controls.

Table 3. C3a(desArg) and TCC levels in plasma from HUS patients

	n	C3a(desArg) ng/mL Median (range)	TCC AU/mL Median (range)
Acute phase	10[a]	465** (280 – 690)	5.8* (4.0 – 7.3)
Recovery	10[a]	109 (72 – 180)	1.4 (0.7 – 2.4)
Pediatric controls	6	90 (52 – 124)	0.98 (0.68 – 2.5)

[a] Plasma samples analyzed from patients 1-10 (Table 1). ** denotes P <0.01 and * P <0.05 comparing plasma taken during the acute phase with pediatric controls. There was no significant difference between plasma taken after recovery and pediatric controls.

Table 4. Surface-bound C3 or C9 on platelet-monocyte complexes in whole blood

	RPMI		EDTA-VBS/RPMI		EGTA-VBS/RPMI	
Agonist	**C3-positive**	**C9-positive**	**C3-positive**	**C9-positive**	**C3-positive**	**C9-positive**
Unstimulated	7 (5 – 8)	5 (2 – 9)	9 (8 – 11)	5 (1 – 13)	10 (7 – 12)	4 (2 – 13)
Stx2	44 (10 – 82)	15 (15 – 21)	15 (7 – 27)	7 (3 – 15)	34 (10 – 40)	17 (2 – 28)
O157LPS	58 (35 – 81)	31 (20 -46)	20 (16 – 44)	8 (7 – 16)	47 (10 – 53)	21 (14 – 25)
Stx2/O157LPS	60 (46 – 87)	28 (15 – 40)	22 (12 – 43)	10 (8 – 21)	55 (14 – 67)	25 (5 – 31)

Data are expressed as (%) of platelet-monocyte complexes positive for surface-bound C3 or C9 and shown as median and range from three different experiments. VBS: Veronal-buffered saline

Table 5. Numbers and cellular origin of microparticles and microparticles with surface-bound C3 and/or C9

	Total amount of microparticles (10^3/mL)	CD42b-positive microparticles (10^3/mL)		CD38-positive microparticles (10^3/mL)		CD66-positive microparticles (10^3/mL)	
Agonist		**C3-positive**	**C9-positive**	**C3-positive**	**C9-positive**	**C3-positive**	**C9-positive**
Stx1	1992*** (1068 – 2395)	702*** (259 – 871)	717*** (268 – 870)	336*** (245 – 528)	312*** (236 – 598)	0.3 NS (0.2 – 0.4)	0.2 NS (0.1 – 0.4)
Stx1B	1301*** (967 – 2364)	766*** (505 – 864)	721*** (478 – 819)	264*** (136 – 479)	351*** (116 – 519)	0.1 NS (0.1 – 0.2)	0.2 NS (0.1 – 0.3)
Stx2	1341*** (1145 -2003)	845*** (745 – 991)	839*** (776 – 990)	421*** (155 – 638)	455*** (170 – 685)	0.3 NS (0.1 – 0.5)	0.2 NS (0.1 – 0.3)
O157LPS	1749*** (1487 – 2128)	859*** (528 – 1047)	822*** (512 – 1055)	487*** (300 – 739)	499*** (281 – 732)	1.4*** (1.0 – 1.7)	1.5*** (0.9 – 1.8)
O111:B4LPS	1351*** (525 – 2970)	805*** (499 – 1003)	831*** (514 – 1019)	415*** (178 – 880)	453*** (185 – 848)	1.4*** (0.5 – 1.8)	1.5*** (0.5 – 1.7)
Stx2/O157LPS	2015*** (1632– 2785)	1466*** (811 – 1895)[a]	1414*** (817 – 1861) [a]	312*** (269 – 735)	328*** (275 - 761)	1.9*** (1.5 -2.2)	1.8*** (1.4 – 2.3)
Stx2/O111:B4LPS	1430*** (931 – 2623)	994*** (725 – 1468)	1218*** (802 – 1865)	286*** (251 – 626)	295*** (268 – 640)	1.7*** (1.5 – 1.9)	1.6*** (1.2 – 1.9)
PBS	240 (153 – 565)	99 (54 – 202)	85 (48 – 212)	67 (34 – 154)	63 (28 – 151)	0.3 (0.1 – 0.3)	0.2 (0.1 – 0.3)

Data are expressed as median and (range) of circulating microparticles positive for each membrane-specific marker/mL. Values were derived from six different experiments. *** denotes *P* value <0.001 comparing microparticles released in whole blood stimulated with Stx2, Stx 1, Stx1B, LPS or Stx/LPS with PBS-treated whole blood. [a], Co-stimulation of whole blood with Stx2 and O157LPS induced an increase in platelet microparticles with surface-bound C3 or C9 compared to Stx2 ($P < 0.001$) or O157LPS ($P < 0.001$), alone while no significant increase was noted on monocyte microparticles in whole blood co-stimulated with Stx2 and O157LPS compared to each agonist alone.

Table 6. C3a(desArg) and TCC levels in plasma

Agonist	C3a(desArg) ng/mL median (range)	TCC AU/mL median (range)
Stx2	235 (175 – 350) *	6.0 (4.0 – 10.0) *
O157LPS	385 (200 – 430) *	5.8 (3.8 – 7.0) *
Unstimulated	90 (58 – 129)	1.4 (1.2 – 2.3)

Data were generated from five different experiments. *denotes P <0.05 when comparing Stx2- or O157LPS-stimulated samples with unstimulated samples.

Figure legends

Figure 1. Circulating platelet-leukocyte complexes and surface-bound C3 in a patient with EHEC-associated HUS. A pediatric HUS patient had increased levels of circulating platelet-monocyte (A) and platelet-neutrophil complexes (B) during the acute phase of disease compared to levels obtained after recovery and to pediatric controls (n=5). Levels of platelet-monocyte (A) and platelet-neutrophil complexes (B) with surface-bound C3 were elevated during the acute phase of disease and decreased at recovery to levels similar to those seen in the pediatric controls.

Figure 2. Surface-bound C3 on platelet-leukocyte complexes induced by Stx2, Stx1, Stx1B and/or O157LPS

Incubation of whole blood with Stx2, Stx1, Stx1B or O157LPS induced C3 deposition on (A) platelet-monocyte and (B) platelet-neutrophil complexes and a lesser increase of C3 on (C) unbound monocytes and (D) unbound neutrophils. Data are expressed as box-plots depicting the median and range regarding the percentage of the platelet-monocyte or platelet-neutrophil population or the percentage of unbound monocytes or neutrophils that were positive for C3 (n = 12 experiments). The horizontal line within each box represents the median. The upper and lower limits of the box represent the interquartile range. The lower and upper limits represent the range. *** denotes $P<0.001$, ** $P<0.01$ and * $P<0.05$, when comparing C3 deposition on complexes or on unbound monocytes or neutrophils in whole blood incubated with an agonist and PBS-treated whole blood. NS; indicates not significant.

Figure 3. Surface-bound C9 on platelet-leukocyte complexes induced by Stx2, Stx1, Stx1B and/or O157LPS

Incubation of whole blood with Stx2, Stx1, Stx1B or O157LPS induced C9 deposition on (A) platelet-monocyte and on (B) platelet-neutrophil complexes and to a lesser extent on (C) unbound monocytes and (D) unbound neutrophils. Data are expressed as box-plots depicting the median and range regarding the percentage of the platelet-monocyte or platelet-neutrophil population or the percentage of unbound monocytes or neutrophils that were positive for C9 (n = 12 experiments). *** denotes $P<0.001$, ** $P<0.01$ and * $P<0.05$, when comparing C9 deposition on complexes or unbound monocytes or neutrophils in whole blood incubated with an agonist and PBS-treated whole blood. NS; indicates not significant.

Figure 4. Inhibition of platelet-monocyte complex formation and complement deposition

(A) Blocking of Stx2 and/or O157LPS binding by pre-incubation of whole blood with anti-Gb3 and/or anti-TLR4 before incubation with Stx2 and/or O157LPS reduced platelet-monocyte complex formation significantly. C3 (B) and C9 (C) deposition on the blood cell complexes was similarly reduced by anti-Gb3 and anti-TLR4 in these experiments. Data are expressed as box-plots depicting median and range (n = 4 experiments), * denotes $P<0.05$ when comparing complex formation, C3 or C9 deposition on complexes in whole blood incubated with an agonist, with whole blood pre-incubated with anti-Gb3 and/or anti-TLR4 before addition of the agonist.

Figure 5. Characterization of surface-bound C3 or C9 on platelets and on platelet-derived microparticles

Incubation of platelet-rich plasma (A) or whole blood (B) with Stx2 or O157LPS induced deposition of C3c, C3b, iC3b, C9neo or C5b-9 on the surface of platelets (A) and to a lesser

degree on platelet-derived microparticles (B). Data are expressed as median ± standard deviation (n = 5 experiments), ** denotes P<0.001 and * *P*<0.05, when comparing C3 or C9 deposition on platelets (in platelet-rich-plasma) or platelet microparticles (in whole blood) incubated with agonists with unstimulated samples . NS; indicates not significant.

Figure 6. Expression of complement regulators on microparticles.

Microparticles from O157LPS-stimulated whole blood were incubated with mouse anti-human CD35:FITC (CR1, A), CD55:APC (DAF, B) or CD46:RPE (MCP, C), respectively showing binding of the antibodies to platelet-, monocyte- and neutrophil-derived microparticles. Data are presented as MFI ± standard deviation from 5 independent experiments.

Figure 7. Phagocytic uptake of microparticles by neutrophils.

Incubation of neutrophils with microparticles isolated from Stx2- or O157LPS-stimulated whole blood induced phagocytic uptake of the microparticles compared to microparticles isolated from unstimulated whole blood. Data are presented as MFI ± standard deviation from 5 independent experiments. ** denotes *P*<0.01 when comparing uptake of microparticles from Stx2- or O157LPS-stimulated whole blood with unstimulated whole blood.

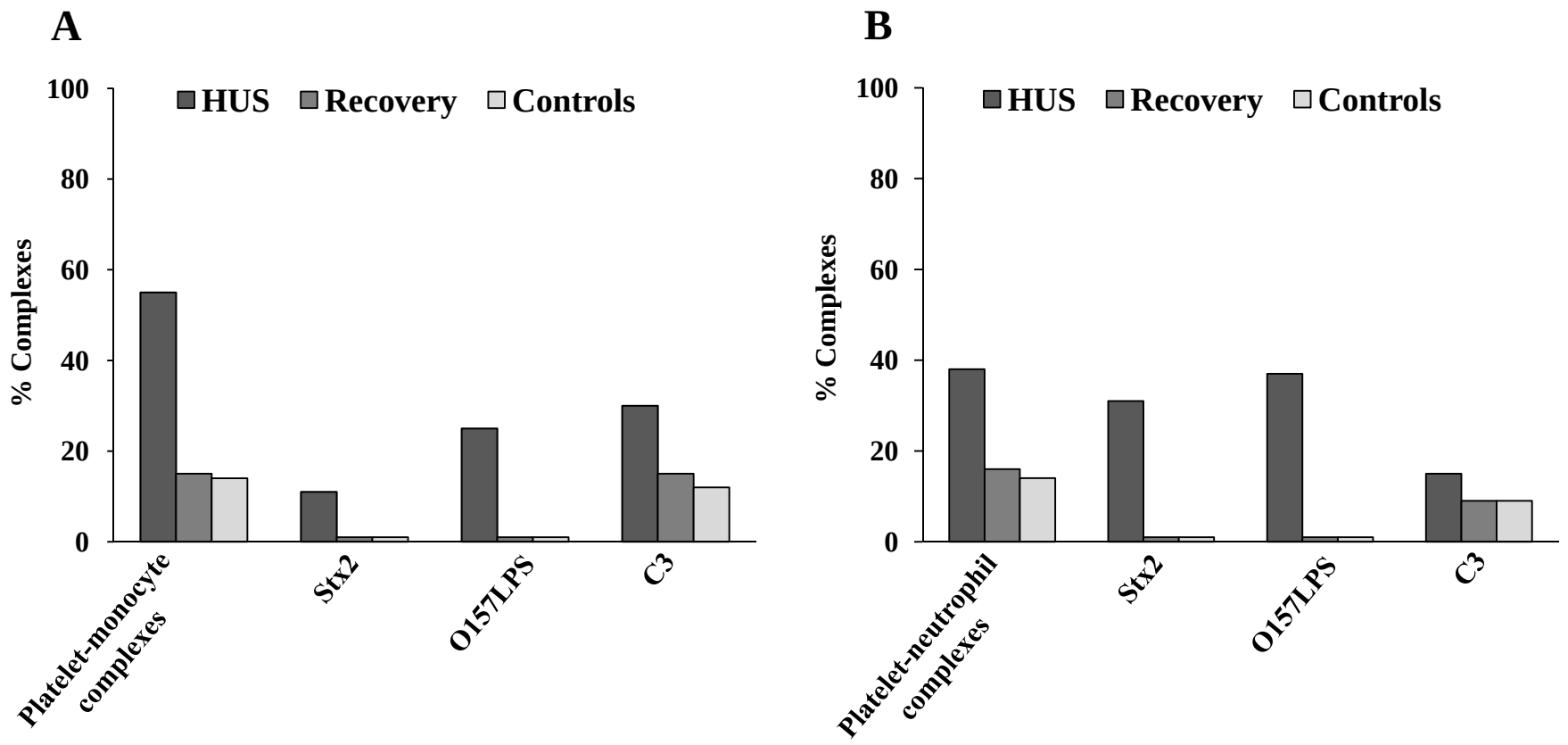

A
HUS
Recovery
Controls
100
80
60
40
20
0
% Complexes
Platelet-monocyte complexes
Stx2
O157LPS
C3
B
HUS
Recovery
Controls
100
80
60
40
20
0
% Complexes
Platelet-neutrophil complexes
Stx2
O157LPS
C3

Figure 1

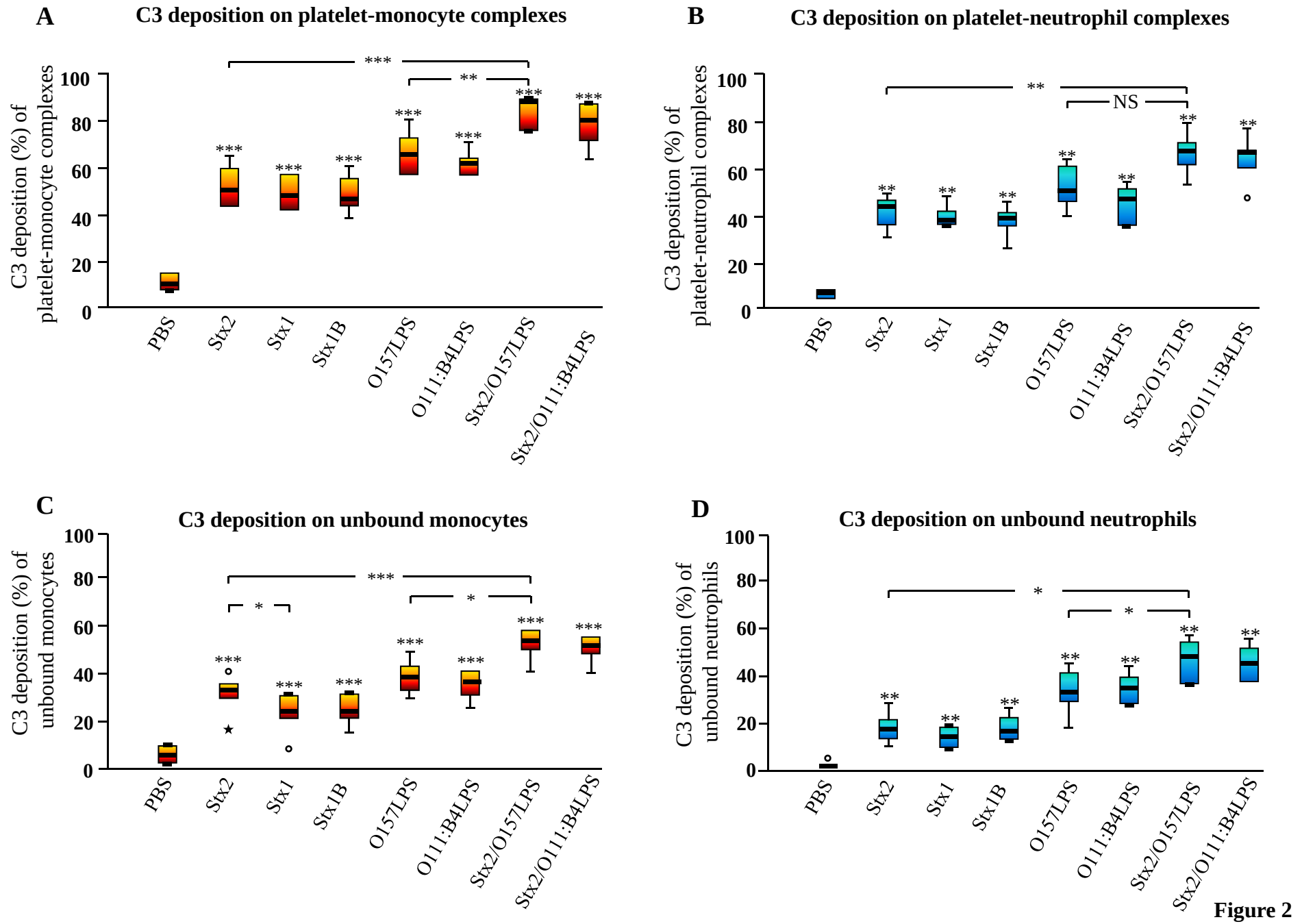

A
C3 deposition on platelet-monocyte complexes
C3 deposition (%) of platelet-monocyte complexes
B
C3 deposition on platelet-neutrophil complexes
C3 deposition (%) of platelet-neutrophil complexes
NS
C
C3 deposition on unbound monocytes
C3 deposition (%) of unbound monocytes
D
C3 deposition on unbound neutrophils
C3 deposition (%) of unbound neutrophils
PBS
Stx2
Stx1
Stx1B
O157LPS
O111:B4LPS
Stx2/O157LPS
Stx2/O111:B4LPS

Figure 2

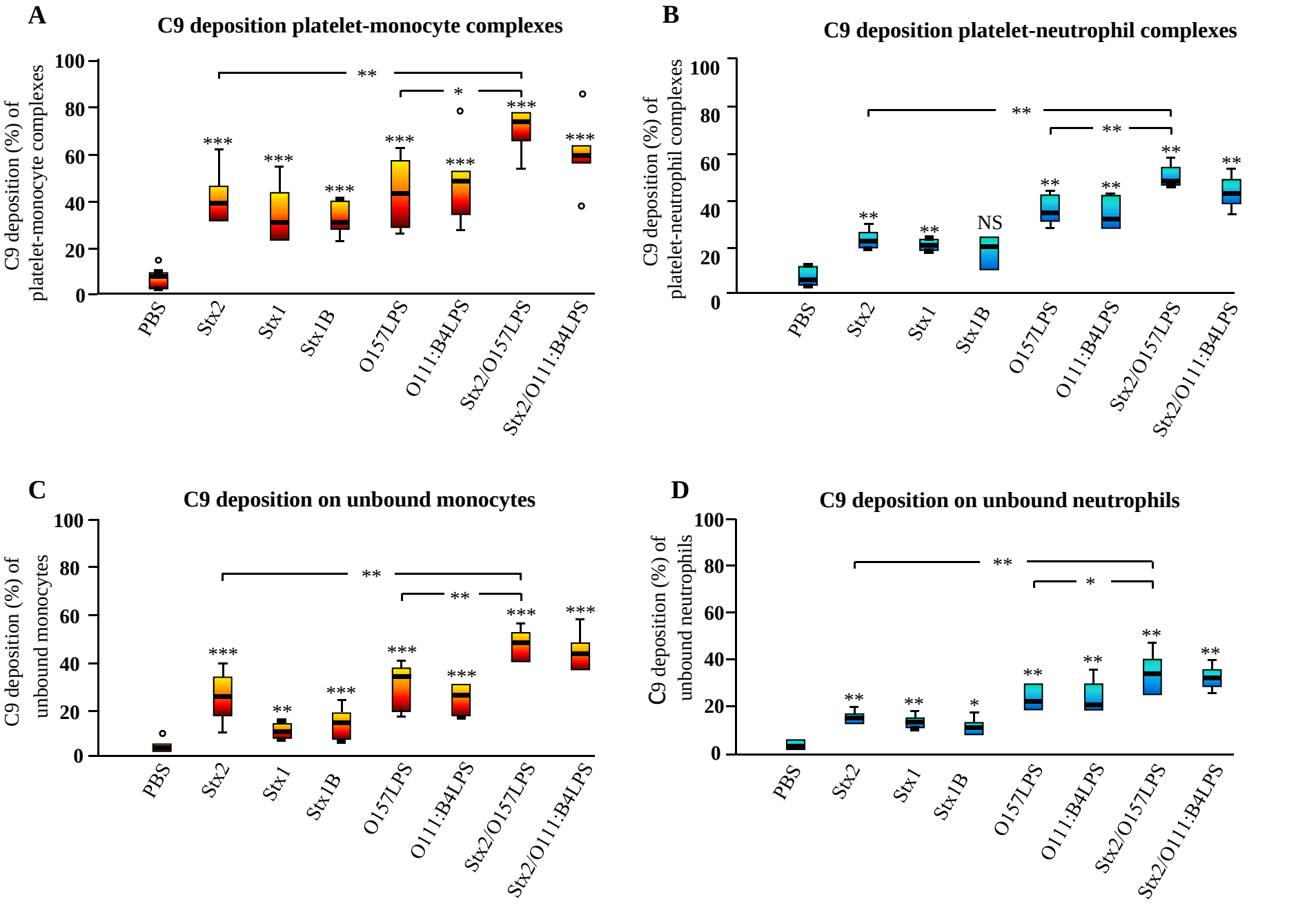

A
C9 deposition platelet-monocyte complexes
C9 deposition (%) of platelet-monocyte complexes
B
C9 deposition platelet-neutrophil complexes
C9 deposition (%) of platelet-neutrophil complexes
NS
C
C9 deposition on unbound monocytes
C9 deposition (%) of unbound monocytes
D
C9 deposition on unbound neutrophils
C9 deposition (%) of unbound neutrophils
PBS
Stx2
Stx1
Stx1B
O157LPS
O111:B4LPS
Stx2/O157LPS
Stx2/O111:B4LPS

Figure 3

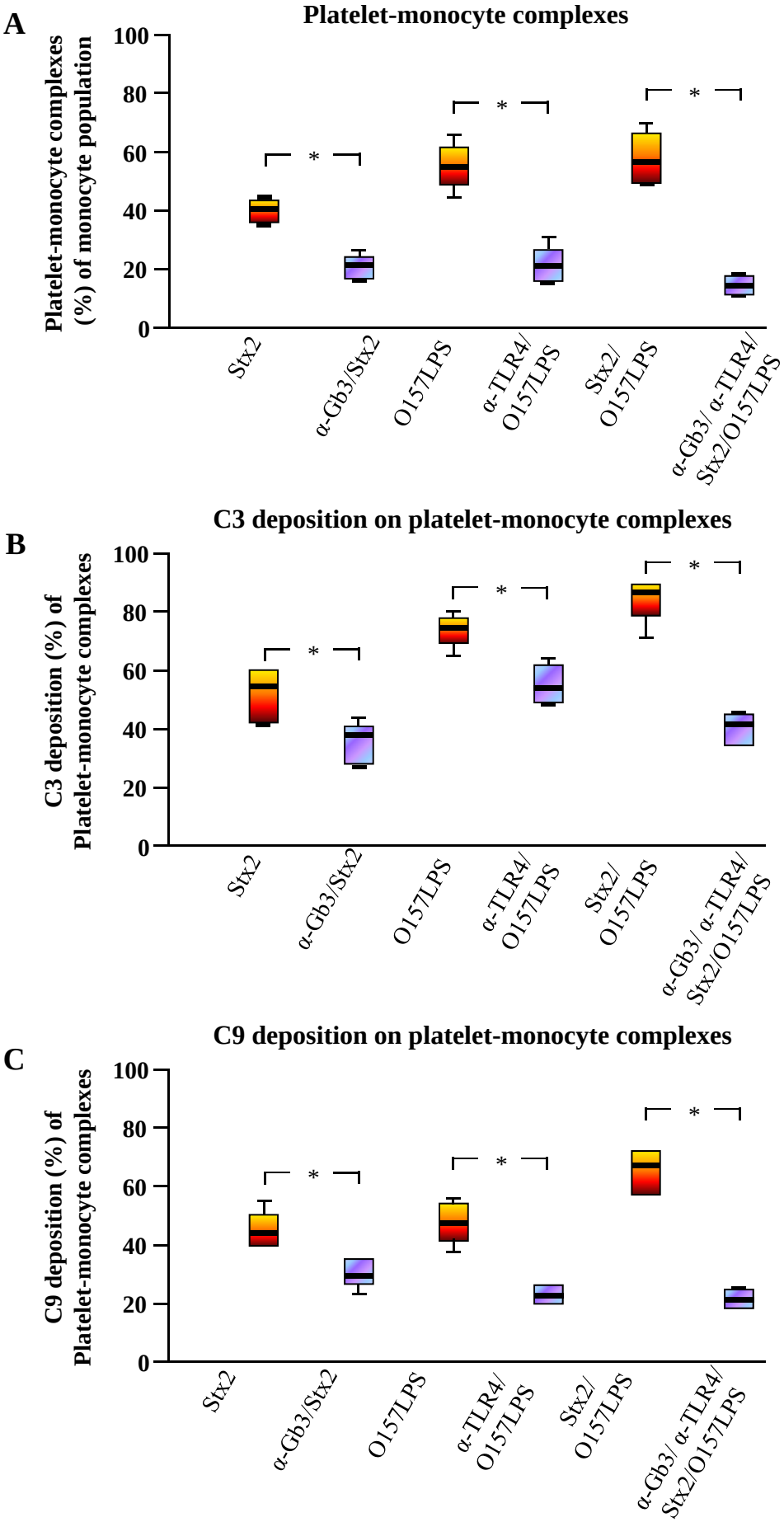
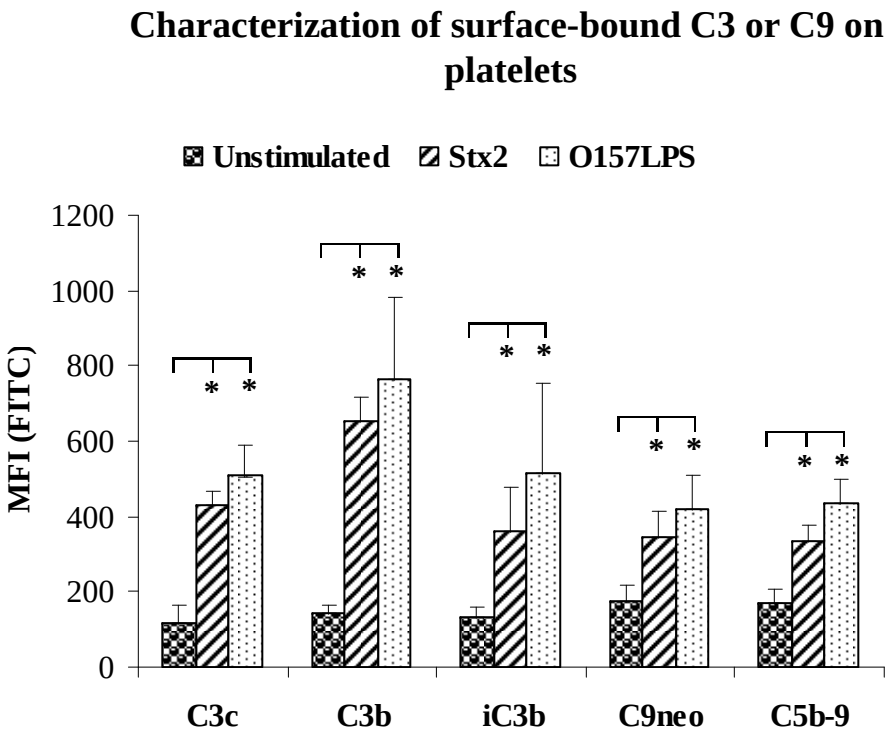

A
Platelet-monocyte complexes
Platelet-monocyte complexes (%) of monocyte population
100
80
60
40
20
0
*
*
*
Stx2
α-Gb3/Stx2
O157LPS
α-TLR4/ O157LPS
Stx2/ O157LPS
α-Gb3/ α-TLR4/ Stx2/O157LPS
B
C3 deposition on platelet-monocyte complexes
C3 deposition (%) of Platelet-monocyte complexes
100
80
60
40
20
0
*
*
*
Stx2
α-Gb3/Stx2
O157LPS
α-TLR4/ O157LPS
Stx2/ O157LPS
α-Gb3/ α-TLR4/ Stx2/O157LPS
C
C9 deposition on platelet-monocyte complexes
C9 deposition (%) of Platelet-monocyte complexes
100
80
60
40
20
0
*
*
*
Stx2
α-Gb3/Stx2
O157LPS
α-TLR4/ O157LPS
Stx2/ O157LPS
α-Gb3/ α-TLR4/ Stx2/O157LPS

Figure 4

A


Characterization of surface-bound C3 or C9 on platelets
Unstimulated
Stx2
O157LPS
MFI (FITC)
1200
1000
800
600
400
200
0
C3c
C3b
iC3b
C9neo
C5b-9

B

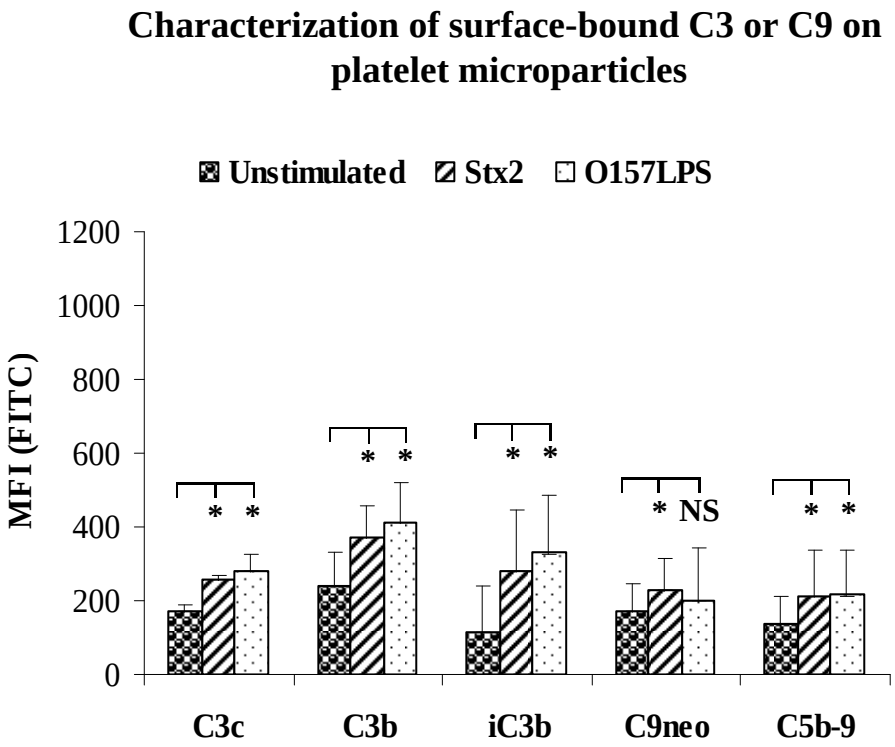

Characterization of surface-bound C3 or C9 on platelet microparticles
Unstimulated
Stx2
O157LPS
MFI (FITC)
1200
1000
800
600
400
200
0
NS
C3c
C3b
iC3b
C9neo
C5b-9

Figure 5

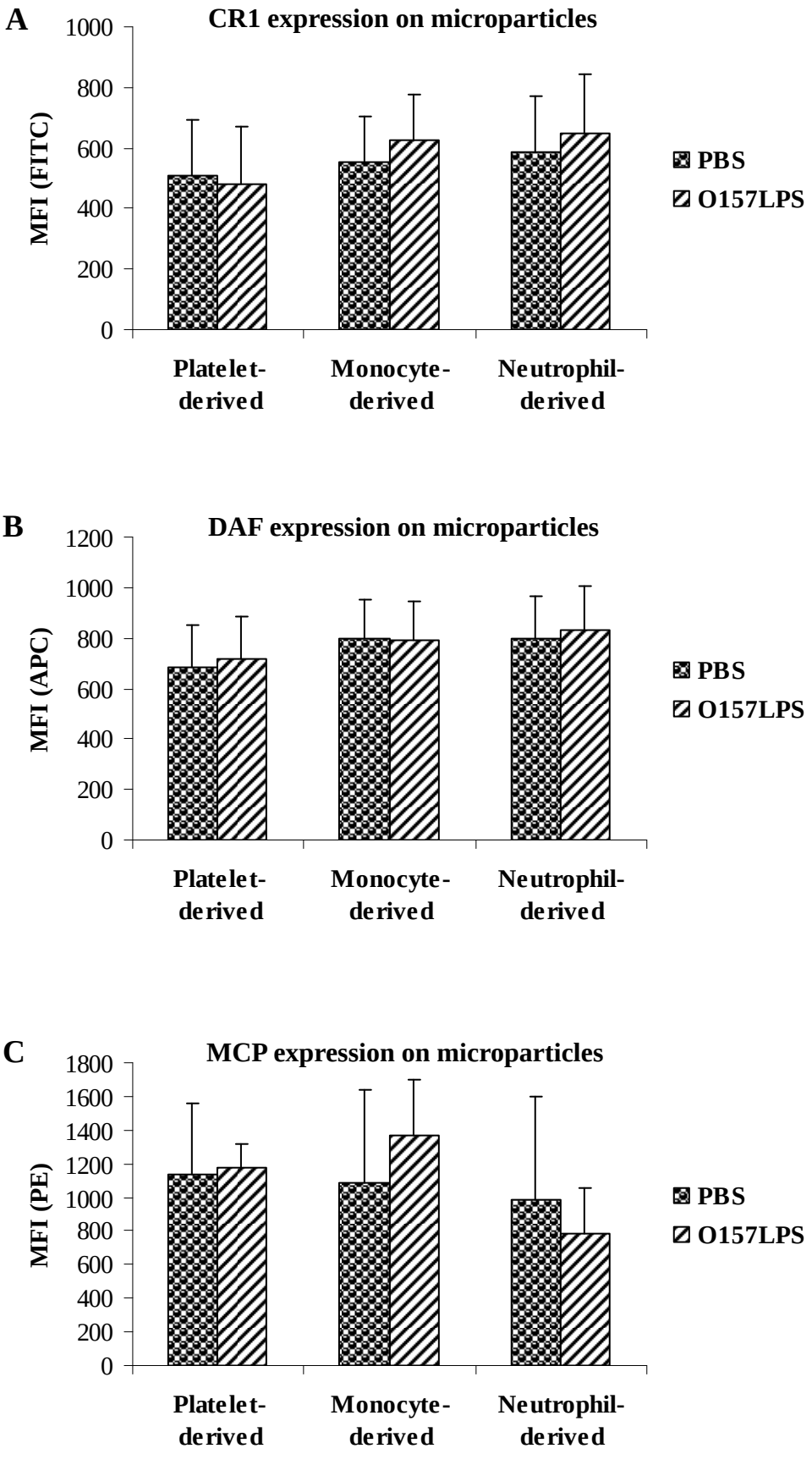

A
CR1 expression on microparticles
MFI (FITC)
1000
800
600
400
200
0
Platelet-derived
Monocyte-derived
Neutrophil-derived
PBS
O157LPS
B
DAF expression on microparticles
MFI (APC)
1200
1000
800
600
400
200
0
Platelet-derived
Monocyte-derived
Neutrophil-derived
PBS
O157LPS
C
MCP expression on microparticles
MFI (PE)
1800
1600
1400
1200
1000
800
600
400
200
0
Platelet-derived
Monocyte-derived
Neutrophil-derived
PBS
O157LPS

Figure 6

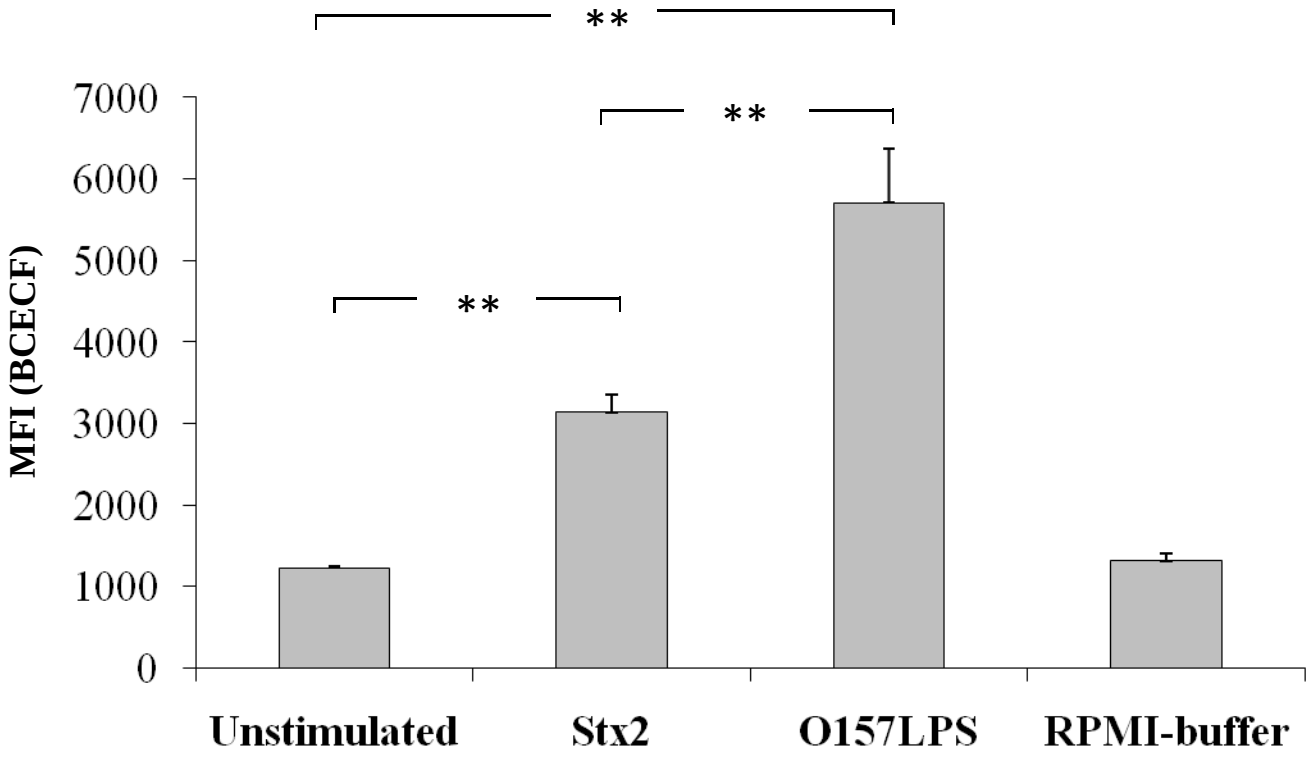

**
**
**
7000
6000
5000
4000
3000
2000
1000
0
MFI (BCECF)
Unstimulated
Stx2
O157LPS
RPMI-buffer

Figure 7

Complement activation on platelet-leukocyte complexes and microparticles in enterohemorrhagic *Escherichia coli*-induced hemolytic uremic syndrome

Anne-lie Ståhl, Lisa Sartz, Diana Karpman

Supplementary Material and Methods

Detection of platelet-leukocyte complexes

Stx2 and O157LPS induced both platelet-monocyte and platelet-neutrophil complex formation whereas Stx1 and Stx1B induced an increase in platelet-monocyte but not platelet-neutrophil complex formation in comparison to PBS-treated samples, as shown in supplementary Figure 1 A,B. Co-stimulation of whole blood with Stx2 and O157LPS, simultaneously, did not induce a significant increase in platelet-monocyte complex formation compared to Stx2 or LPS alone while it induced a significant increase in platelet-neutrophil complex formation compared to Stx2 alone but not compared to O157LPS alone (supplementary Figure 1 A-B). These conditions were the basis for further experiments

Immunoblotting to detect C3 and iC3b on microparticles

Microparticles were isolated from 1 mL of Stx2 or O157LPS stimulated or unstimulated whole blood. Samples were diluted (1:10) and subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were transferred onto equilibrated polyvinylidine difluoride membranes and blocked with casein solution (Vector Laboratories Inc, Burlingame, CA). Immunoblotting was performed with rabbit anti-human C3c (DAKO) or mouse anti-human iC3b followed by goat anti-rabbit HRP or goat anti-mouse HRP (DAKO), respectively. Signal was detected by chemiluminescence using ECL plus (Amersham Biosciences, Uppsala, Sweden).

Supplementary Results

Detection of C3 and iC3b on microparticles

The presence of C3 and iC3b was investigated by immunoblotting of microparticles isolated from whole blood incubated with Stx2 or O157LPS. Immunoblots (under reducing conditions) revealed a band at 110 kDa corresponding to the α-chain of C3 (supplementary figure 3) and two bands at 68 kDa and 41 kDa corresponding to the iC3b α_1 and α_2 fragments.[1] The 110 kDa band was also present in unstimulated samples while the two lower bands were not, indicating iC3b deposition in Stx2 and O157LPS-stimulated samples but not in the unstimulated samples. A band specifically corresponding to C3b was not visualized (it would presumably be directly under the 110 kDa band) but the presence of bands corresponding to iC3b indicates that C3b was formed in stimulated samples.

Supplementary Figure legends

Supplementary Figure 1: Platelet-monocyte and platelet-neutrophil complexes induced by Stx2, Stx1, Stx1B and/or LPS.

In vitro incubation of whole blood with Stx2 (2.8 x 10^{-9} M) or O157LPS (0.5μg/mL) induced a significant increase in both (A) platelet-monocyte and (B) platelet-neutrophil complexes while Stx1 and Stx1B (both at 2.8 x 10^{-9} M) induced a significant increase only in platelet-monocyte complex formation. Co-stimulation with Stx2 and O157LPS, simultaneously, induced a significant increase in platelet-neutrophil but not in platelet-monocyte complex formation compared to each agonist alone. Data are expressed as median ± range (n = 12 experiments), *** P<0.001 and ** P<0.01 when comparing complex formation in whole blood incubated with an agonist and PBS-treated whole blood. NS; indicates not significant. The horizontal line within each box represents the median. The upper and lower limits of the box represent the interquartile range. The lower and upper limits represent the range. Incubation of whole blood with PBS for 40 min at 37ºC did not induce an increase in platelet-monocyte or platelet-neutrophil complex formation compared to baseline levels (whole blood diluted in RPMI and lyzed). Baseline levels of platelet-monocyte complexes were 14% (range 7-25 %, 5 experiments) and platelet-neutrophil complexes 6% (range 2-8 %, 5 experiments).

Supplementary Figure 2: Surface-bound C3, C9 and C5b-9 on microparticles from patients shown as MFI values

Plasma levels of circulating microparticles with surface-bound C3, C9 or C5b-9 were measured by flow cytometry during the acute phase of disease and after recovery. Plasma taken during the acute phase showed significantly higher MFI levels of C3, C9 and C5b-9 on platelet-derived microparticles compared to levels after recovery. Levels of C3 and C9, but not C5b-9, were higher on monocyte-derived microparticles while there was no significant

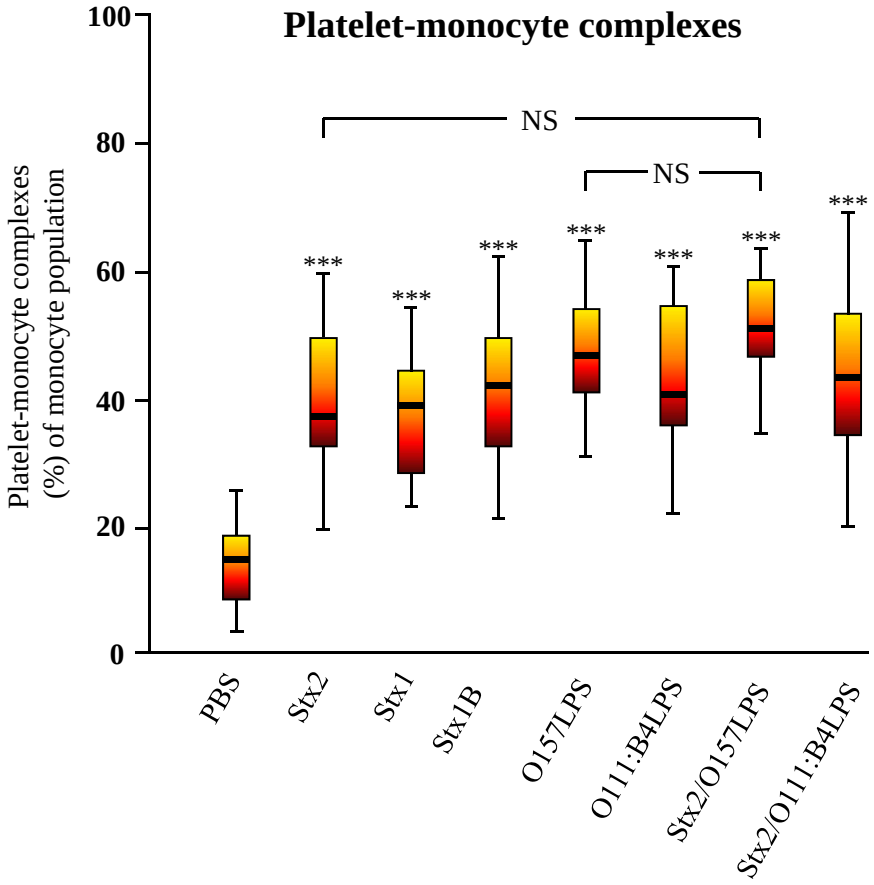
difference on neutrophil-derived microparticles. Data are expressed as median ± standard deviation. ** denotes $P<0.01$ and * $P<0.05$, when comparing C3, C9 or C5b-9 MFI-levels on microparticles in plasma from the acute phase to after recovery. NS; indicates not significant.

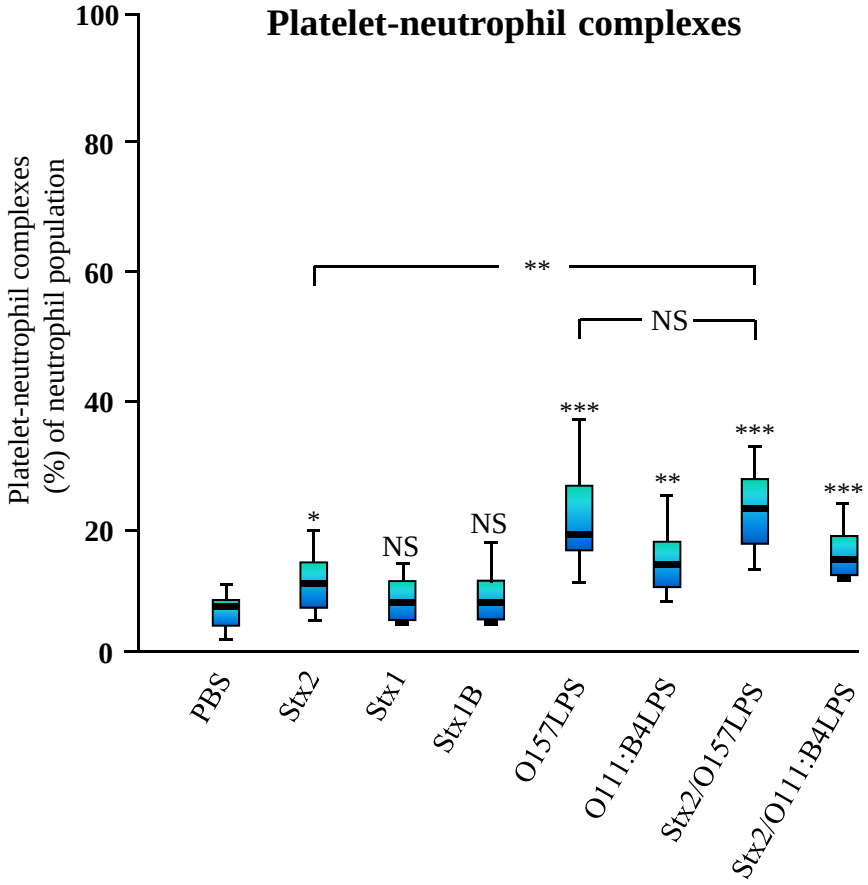
Supplementary Figure 3. C3 and iC3b on microparticles detected by immunoblotting

The presence of C3 and iC3b was investigated by immunoblotting of microparticles isolated from whole blood incubated with Stx2 or O157LPS. Immunoblots revealed a 110 kDa band corresponding to the α′-chain of C3 and two bands at 68 kDa and 41 kDa corresponding to the iC3b α_1 and α_2 fragments. The positions of the fragments are shown on the right. The molecular mass is shown on the left. The results are representative of three independent experiments.

References

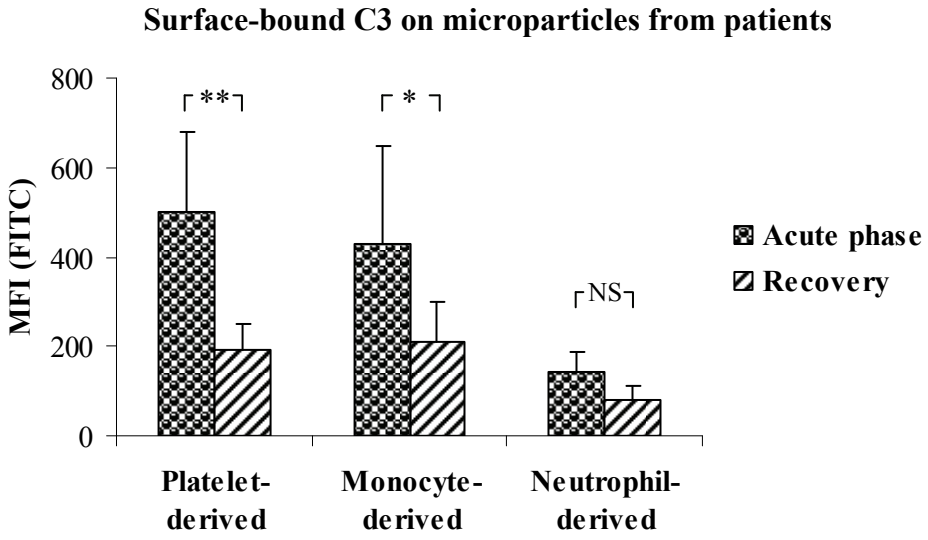
1. Barilla-LaBarca ML, Liszewski MK, Lambris JD, Hourcade D, Atkinson JP. Role of membrane cofactor protein (CD46) in regulation of C4b and C3b deposited on cells. *J Immunol*. 2002;168 (12):6298-6304.





Supplementary Figure 1

A



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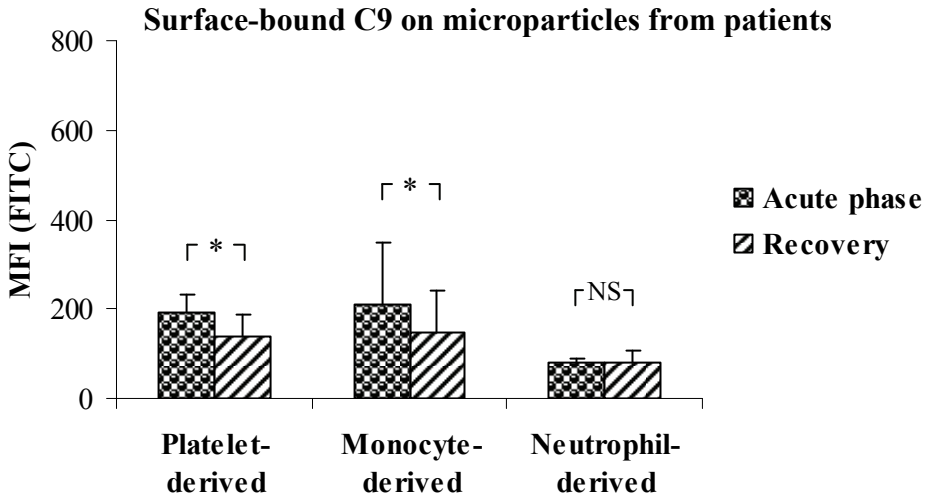
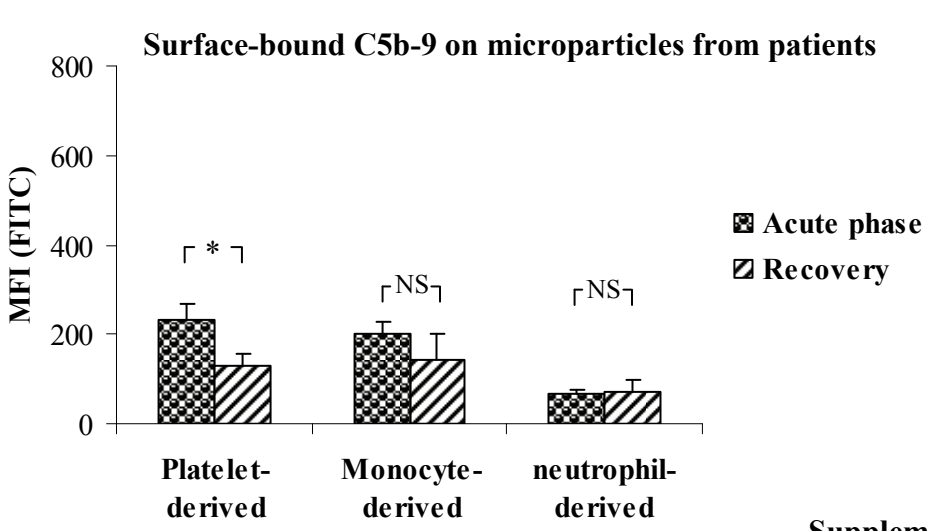
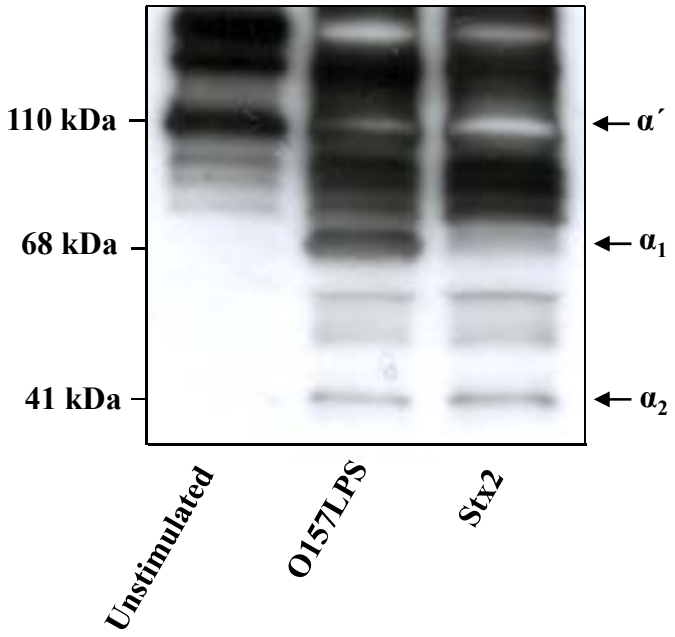


C



Supplementary Figure 2



Supplementary Figure 3