Key role of the number of complement receptor 1 on erythrocytes for binding of *Escherichia coli* to erythrocytes and for leukocyte phagocytosis and oxidative burst in human whole blood

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Abstract

Aim: To study the role of complement receptor 1 (CR1) for binding of *Escherichia coli* (*E. coli*) to erythrocytes, for leukocyte phagocytosis, oxidative burst and complement activation in human whole blood from a CR1 deficient (CR1D) patient and healthy controls with low, medium and high CR1 numbers.

Methods: Alexa-labelled bacteria were used to quantify erythrocyte-bound bacteria, free bacteria in plasma and phagocytosis using flow cytometry. Complement activation in plasma was measured by enzyme-linked immunosorbent assay. The CR1 numbers as well as C3bc and C4bc deposition on erythrocytes were measured by flow cytometry. Cytokines were measured using multiplex technology, and bacterial growth was measured by colony forming units. CR1 was blocked using the anti-CR1 blocking mAb 3D9.

Results: Approximately 85% of *E. coli* bound to erythrocytes after 15 minutes incubation in donor blood with high and medium CR1 numbers, 50% in the person with low CR1 numbers and virtually no detectable binding in the CR1D (r^2=0.87, P<0.0007). The number of free bacteria in plasma was inversely related to erythrocyte CR1 numbers (r^2=0.98, P<0.0001). *E. coli*-induced phagocytosis and oxidative burst were significantly enhanced by the anti-CR1 mAb 3D9 and in the CR1D and the donor with low CR1 numbers. *E. coli*-induced complement activation in plasma, C3bc and C4bc deposition on erythrocytes, and bacterial growth were similar in all four cases. Conclusions: CR1D and low CR1 numbers prevented *E. coli* binding to erythrocytes, increased free bacteria in plasma, phagocytosis and oxidative burst, but did not affect plasma or surface complement activation and bacterial growth.

1. Introduction

Despite the wide use of antibiotics, Gram-negative sepsis still exhibits high lethality in Western countries (Gaieski, Edwards et al. 2013). Improved knowledge concerning the basal mechanisms and cells involved in sepsis-induced inflammation and organ damage is needed, as well as the role of the complement system, which is rapidly activated during sepsis (Ward 2016).

Gram-negative bacteria are rapidly opsonized by complement C3b, iC3b and C4b due to activation of the alternative, classical or lectin pathways (Newman and Mikus 1985, Cooper 1969, Ricklin, Hajishengallis et al. 2010). This opsonization of bacteria by complement fragments facilitates binding of the bacteria to complement receptors, including CR1 (CD35) on red cells, a process named immune adherence (Birmingham and Hebert 2001). The binding of ligands to CR1 induces clustering of the receptors on the erythrocyte surface in complex with the scaffolding protein FAP-1 (Ghiran, Glodek et al. 2008). In the bloodstream, this immune adherence allows the transport of bacteria, immune complexes and opsonized cellular fragments on red cells to the sinusoids of liver and spleen (Benacerraf, Sebestyen et al. 1959, Schifferli, Ng et al. 1988, Birmingham and Hebert 2001). A complement receptor of the immunoglobulin family on liver Kupffer cells is involved in phagocytosis of the bacteria (Helmy, Katschke et al. 2006). The red blood cells then return to the circulation.

CR1 is a glycoprotein receptor that is located on human erythrocytes, most leukocytes, B cells and tissue phagocytes (Birmingham and Hebert 2001). Both Gram-positive (Nelson 1953), Gram-negative bacteria (Brekke, Hellerud et al. 2011) and mannose-binding lectin (MBL) (Ghiran, Barbashov et al. 2000) may bind to erythrocyte CR1 in human whole blood. Notably, the concentration of erythrocytes is approximately 700-fold higher than the concentration

leukocytes in human whole blood and thus, the main pool of CR1 receptors in blood is on the erythrocytes. Importantly, the erythrocyte CR1 numbers vary between individuals due to genetic polymorphisms (Wong, Cahill et al. 1989) or diseases, like systemic lupus erythematosus (SLE) where CR1 is decreased (Iida, Mornaghi et al. 1982). Interestingly, the clearance of *Streptococcus pneumonia in vivo* in transgenic mice expressing human CR1 is enhanced compared with wild type mice lacking CR1 (Li, Wang et al. 2010). The numbers of CR1 on erythrocytes may therefore influence the degree of bacterial clearance in infections. Furthermore, malaria parasites invade red blood cells by binding to CR1 (Spadafora, Awandare et al. 2010). CR1 is also a regulator of complement activation, acting as co-factor for factor I in cleavage of C3b to iC3b and further to C3c and C3dg (Iida and Nussenzweig 1981).

Phagocytosis of bacteria is facilitated by contact between the complement-opsonized bacterial surface and complement receptor 3 (CD11b/CD18) on leukocytes (Mollnes, Brekke et al. 2002, Brekke, Christiansen et al. 2007). CR1 on leukocytes, although not being a phagocytic receptor per se, is involved in the phagocytosis of bacteria, probably by participating in the initial binding of the bacteria to the leukocyte surface (Birmingham and Hebert 2001, Brekke, Hellerud et al. 2011). Bacterial structures acting as pathogen-associated molecular patterns (PAMPS) are recognized by leukocyte pattern recognition receptors (PRRs) on the cell surface and in phagosomes, including extra- and intra-cellular Toll-like receptors (TLRs), leading to cytokine release (Kawai and Akira 2010). Macrophage phagocytosis of several bacteria is also mediated by complement receptors, including CR1, and is closely linked to an enhanced interferon gamma response, which inhibits complement receptor function (Schlesinger and Horwitz 1991). Enhanced interferon gamma release has been observed in mononuclear cells from patients with SLE compared with healthy controls (Csiszar, Nagy et al. 2000).

The aim of the present study was to examine the role of erythrocyte CR1 numbers in the binding of Gram-negative *E. coli* bacteria to erythrocytes, and its consequences for free bacteria in plasma, as well as for phagocytosis, oxidative burst and cytokine release. Finally, the effect on complement activation was studied. For this purpose we took advantage of blood donors with different numbers of erythrocyte C1 numbers using a human whole blood model anticoagulated with lepirudin, since this has no adverse effects on complement activation (Mollnes, Brekke et al. 2002).

2. Materials and methods

2.1 Equipment and reagents

All equipment, including polypropylene tubes (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 Sigma-Aldrich (MO, USA). Lepirudin (Refludan®) was obtained from Celgene (Windsor, Great Britain). The Protein G Spin Kit columns (0.2 mL) for antibody purification were obtained from Thermo Fisher Scientific (Pierce, Rockford, IL). The Burst- and Phago-test kits were both obtained from ORPEGEN Pharma (Heidelberg, Germany). LDS-751, Alexa 488, a BacLight green kit for direct fluorescent staining of unlabelled bacteria, and dimethyl sulfoxide (DMSO) were obtained from Invitrogen Molecular Probes (Eugene, OR). Zymosan A, EDTA and bovine serum albumin were all obtained from Sigma-Aldrich (St. Louis, MO). The mouse anti human CR1 blocking (clone 3D9) has been previously characterized (O'Shea, Siwik et al. 1985), and used in whole blood (Brekke, Hellerud et al. 2011), was a kind gift from Ronald P. Taylor, Univ. of Virginia, US. The isotype-matched mouse anti-human IgG_1 control mAb (clone BH1) was obtained from Diatec Monoclonals AS (Oslo, Norway).

2.2 Flow cytometry of CR1 on erythrocytes

The number of CR1 antigenic sites on erythrocytes was analysed using flow cytometry as previously described (Pham, Kisserli et al. 2010). The CR1D blood donor had a very low CR1 expression, i.e. less than 50 CR1 antigenic sites per erythrocyte (Kiss, Csipo et al. 1996), while the low, medium and high blood donors had 200, 500 and 800 antigenic sites per erythrocyte, respectively.

2.3 Bacteria

Escherichia coli (*E. coli*) LE392 strain (ATCC 33572) was obtained from the American Type Culture Collection (Manassas, VA) and was grown, washed and counted using flow cytometry as previously described (Brekke, Christiansen et al. 2007). The bacteria were harvested and washed once using Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) by centrifugation for 10 min at 3220 x *g* (4°C). Subsequently, the bacteria were aliquoted, heat-inactivated for 1 hour at 60°C and stored at -80°C. A frozen ampoule was thawed at ambient temperature, washed six times with PBS and centrifuged for 10 min at 3220 x *g* (4°C) to remove extracellular lipopolysaccharides. Bacteria for Alexa 488 staining were separated, whereas the remainder were washed three more times. For counting, the bacteria were stained for 5 min using Syto BC (Invitrogen Molecular Probes) and counted in Truecount tubes (Becton-Dickinson) using a FACScalibur or a LSRII flow cytometer with FACSDiva software (Becton-Dickinson).

2.4 Whole blood model of bacterial inflammation

Whole blood was obtained using a standard venipuncture technique and lepirudin (50 mg/L) as anticoagulant (Mollnes, Brekke et al. 2002). The whole blood (5 volume parts) was preincubated for 5 min at 37°C in PBS (1 part) and thereafter incubated as indicated with PBS (control), cobra venom factor (CVF), heat-aggregated immunoglobulin G (HAIGG) or *E. coli* bacteria in PBS (1 volume part). The experiments with blood from the different blood donors were repeated on separate days.

2.5 Quantitation of bacteria bound to erythrocytes and free in plasma

Alexa 488-labelled *E. coli* bacteria bound to erythrocytes and free bacteria in plasma were quantified by flow cytometry as previously described (Brekke, Hellerud et al. 2011). Whole

blood cells were fixed with 0.25% paraformaldehyde for 4 min at 37°C. To avoid coincidences of erythrocytes not carrying bacteria, samples were diluted at 1:320 using PBS and counted using Truecount tubes. Whole blood with EDTA (10 mM) served as a control since EDTA completely blocked complement activation and bacterial opsonisation (Brekke, Hellerud et al. 2011). Samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson), with forward scatter (FSC) and side scatter (SCC) in log mode. Threshold was set on the Alexa FLUOR® 488 channel whereby only beads, free bacteria, erythrocytes with bound bacteria and leucocytes having bound or phagocytozed bacteria were acquired. Gates were established around the beads, the erythrocytes and the free bacteria for analysis. The number of events in the erythrocyte gate and the gate for free bacteria were related to the number of beads giving an absolute count. In general RBCs binding bacteria had only one bacteria attached to the surface and this was confirmed by immunofluorescence microscopy of blood smears. To confirm that bacteria/erythrocyte conjugates were not formed due to the presence of paraformaldehyde, we performed control experiments without paraformaldehyde and obtained similar results.

2.6 Phagocytosis

For the phagocytosis assay, heat-inactivated *E. coli* (6×10^9) were washed six times as described above, and the supernatant was discarded. Subsequently, $NaHCO_3$ (0.2 M, 600 µL, pH 8.35, sterile-filtered and heat-inactivated for 1 hour at 60°C) was added together with 6 µL of Alexa FLUOR® 488 carboxylic acid succinimidyl ester (10 g/L) in dimethyl sulfoxide (DMSO). The tube was packed in tinfoil and rotated for 1 hour at ambient temperature. Bacteria were washed three times with PBS, centrifuged for 5 min at 8000 x *g*, resuspended in PBS and counted as described above. Phagocytosis of Alexa-labelled heat-inactivated *E. coli* bacteria was analysed by flow cytometry and expressed as the median fluorescence intensity (MFI) or percent (%)

positive cells. In brief, Alexa-labelled bacteria were added to human whole blood and further incubated for 20 min. at 37°C.

2.8 ELISA for complement proteins and activation products

Plasma C4bc, C1rs-C1-inhibitor complexes, C3bBbP, C3bc and the terminal complement complex (TCC) were analysed by ELISA as previously described (Bergseth, Ludviksen et al. 2013). A sample of pooled human sera from healthy donors activated with zymosan (10 mg/mL) was used as the standard and set to 1000 arbitrary units (AU/mL). The functional activity of the classical pathway (CP) and alternative pathway (AP) in serum were analysed by Wielisa® (Life Science AB, Malmø, Sweden). Total C3 and C4 in serum were analysed on a Siemens BN ProSpec® nephelometer using reagents from Siemens (Siemens Healthcare Diagnostics Ltd, Camberley, UK).

2.9 Flow cytometric analysis of IgG, C3bc and C4bc opsonization

HAIGG or CVF was added to whole blood and incubated for 30 min (37ºC). Unopsonized *E. coli* were added to lepirudin plasma obtained from whole blood after centrifugation for 15 min at 3220 x *g* (4ºC). Five µL of blood (HAIGG or CVF) or plasma with *E. coli* bacteria were then washed twice with PBS and centrifuged for 15 min at 3220 x *g* (4ºC) and resuspended in PBS containing 0.1% (w/v) BSA. IgG, C3bc and C4bc opsonization was analysed using rabbit anti-human IgG (Dako F0056) and isotype control (Dako X0929), FITC-conjugated rabbit anti-human C3c which also recognize C3b (Dako F0201) and rabbit anti-human C4c mAbs, respectively (Brekke, Christiansen et al. 2007). FITC-conjugated rabbit anti-mouse Ig (Dako F0261) was used as a control. The results are expressed as the median fluorescence intensity (MFI).

2.10 Bacterial growth

Bacterial growth in whole blood was measured using standard microbiological techniques. In brief, live *E. coli* bacteria were added to fresh human whole blood in PBS, or in the presence of ant-CR1 blocking mAb 3D9 (4 µg/mL) or an isotype control mAb (4 µg/mL) and incubated for the times indicated. At the time points indicated, an aliquot of blood was redrawn and diluted in PBS. The blood (1 µL) was then spread on lactose agar plates, and bacterial growth was estimated after an overnight incubation in an air incubator at 37°C. Bacterial growth is presented as colony forming units (CFU/mL).

2.11 Cytokine analysis

Cytokines in EDTA plasma were analysed using the Bio-Plex human 27-plex kit from Bio-Rad Laboratories (Hercules, CA). The following cytokines were analysed: interleukin (IL)-1 beta (IL-1β), IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (C-X-C motif chemokine ligand 8; CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, monocyte chemoattractant protein 1 (MCP-1 or CCL2), macrophage inflammatory protein-1-alpha (MIP-1α or CCL3), macrophage inflammatory protein-1-beta (MIP-1β or CCL4), eotaxin-1 (C-C motif chemokine ligand 11; CCL11), interferon-γ-inducing protein 10 or IP-10 (CXCL10), basic fibroblast growth factor (FGF-basic), granulocyte colony stimulating factor (G-CSF), granulocyte–macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), platelet-derived growth factor-BB (PDGF-BB), RANTES (CCL5), tumour necrosis factor (TNF) and vascular endothelial growth factor (VEGF). The samples were analysed using a Bio-Plex 200 instrument from Bio-Rad.

2.12 Statistical analysis

The experiments were performed with each blood donor on two separate days, and the results are expressed as the means ± range. Non-linear and linear regression analysis on the means of data from the CR1D, the person with low, medium and high erythrocyte CR1 numbers (n=4) of duplicate measurements were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). The CFU results after adding the anti-CR1 blocking mAb were analyzed using Student`s T-test.

2.13 Ethics

The study was approved by the Regional ethics committee in the Northern Health Region of Norway and written informed consent was obtained from the blood donors.

3 Results

3.1 Effect of erythrocyte CR1 numbers on the binding of *E. coli* bacteria to erythrocytes and free bacteria in plasma in fresh human whole blood

The role of erythrocyte CR1 numbers for binding of *E. coli* to erythrocytes and the number of free bacteria in plasma were examined in fresh human whole blood (Fig. 1). The CR1D blood donor showed barely any binding of bacteria to erythrocytes (Fig 1A), whereas this donor showed very high numbers of free bacteria in plasma (Fig. 1D). In the blood donor with low CR1 numbers, 42% of the bacteria were bound to erythrocytes at the highest bacterial concentration assessed (10^8 *E. coli*/mL) (Fig. 1A), and 40% of the bacteria were free in plasma (Fig. 1D). In the blood donors with medium and high CR1 erythrocyte numbers, 80-90% of the bacteria were bound to erythrocytes (Fig. 1A). In these blood donors, less than 5% of the bacteria were free in plasma (Fig. 1D). EDTA, chelating calcium and magnesium and efficiently blocking complement activation, completely abolished the binding of *E. coli* bacteria to erythrocytes in all donors (Fig. 1A) and virtually all bacteria were detected free in plasma (Fig. 1D). The numbers of *E. coli* bacteria bound to erythrocytes were significantly and positively correlated (r^2= 0.87, P<0.0007) with the log10 of erythrocyte CR1 numbers when analysed using linear regression (Fig. 1C). Similarly, the numbers of *E. coli* bacteria free in plasma were significantly and negatively correlated (r^2=0.98, P<0.0001) with the log10 of erythrocyte CR1 numbers when analysed using linear regression (Fig. 1F).

3.2 Effect of erythrocyte CR1 numbers on *E. coli*-induced phagocytosis in fresh human whole blood

The role of erythrocyte CR1 numbers in granulocyte and monocyte phagocytosis of *E. coli* bacteria was then examined (Fig. 2). Phagocytosis of *E. coli* was analysed by flow cytometry

and presented as the median fluorescence intensity (MFI, Fig. 2). The blood donor with low erythrocyte CR1 numbers and CR1D on erythrocytes had the highest granulocyte phagocytosis measured as MFI (Fig. 2A), and monocyte phagocytosis measured as MFI (Fig. 2D). In comparison, the blood donor with medium and high CR1 numbers had the lowest phagocytosis in granulocytes (Fig. 2A) and monocytes (Fig. 2D). The granulocyte phagocytosis were negatively and significantly correlated (r^2= 0.49, P<0.05) with the log10 of the erythrocyte CR1 numbers when analysed using linear regression (Fig. 2C). The phagocytosis in monocytes were similarly negatively and significantly correlated (r^2= 0.51, P=0.046) with the log10 of erythrocyte CR1 numbers (Fig. 2F).

3.3 Effect of erythrocyte CR1 numbers on *E. coli*-induced oxidative burst in fresh human whole blood

We next examined the granulocyte and monocyte oxidative burst using flow cytometry (Fig. 3). The *E. coli*-induced oxidative burst in granulocytes (Fig. 3A) and monocytes (Fig. 3D) was most enhanced in blood from the CR1D and the donor with low erythrocyte CR1 numbers. The blood donors with medium and high erythrocyte CR1 numbers exhibited similar and lower oxidative burst (Fig. 3). The *E. coli*-induced granulocyte oxidative burst were negatively and significantly correlated (r^2= 0.73, P = 0.0066) with the log10 of erythrocyte CR1 numbers when analysed using linear regression (Fig. 3C). The oxidative burst in monocytes were similarly negatively and significantly correlated (r^2= 0.72, P = 0.008) with the log10 of erythrocyte CR1 numbers (Fig. 3F).

3.4 The effect of erythrocyte CR1 numbers on *E. coli*-induced complement activation in the fluid phase

To examine the role of erythrocyte CR1 numbers on complement activation in the fluid phase, we incubated fresh human whole blood with *E. coli* and measured plasma complement activation products in the four blood donors. As expected, *E. coli* activated complement as detected by C4bc (classical and lectin pathway), C3bBbP (alternative pathway), C3bc (all pathways) and TCC (sC5b-9; terminal pathway) in the fluid phase (Fig. 4A-D). Although the blood donor with low CR1 numbers had the highest complement activation in the fluid phase for all activation products, and particularly for C4bc, CR1D showed complement activation in the fluid phase close to the donors with medium and high erythrocyte CR1 numbers. These findings indicated that the *E. coli*-induced complement activation in the fluid phase was not significantly affected by erythrocyte CR1 numbers.

3.5 Effect of erythrocyte CR1 numbers on cobra venom factor and heat-aggregated immunoglobulin G (HAIGG)-induced complement activation

No correlations were found between plasma C4bc, C3bc, C3bBbP and TCC levels and different erythrocyte CR1 numbers after activating whole blood with CVF and HAIGG (Fig. 5). The functional activity of the CP and AP in serum from the different blood donors were within normal limits (Fig. 5E,F). However, the donor with low CR1 numbers had the highest CP and AP activity. Similarly, the erythrocyte deposition of IgG, C3bc and C4bc were similar after activating whole blood from donors with different CR1 numbers with CVF or HAIGG (Supplementary Fig. 1). The positive control *E. coli* bacteria showed significant opsonization with IgG, C4c and C3c as expected (Supplementary Fig. 1). The total C3 and C4 levels in plasma were similar in all four blood donors.

3.6 Effect of erythrocyte CR1 numbers on cobra venom factor and HAIGG-induced complement activation

The effect of erythrocyte CR1 numbers on PBS, CVF- and HAIGG-induced complement activation on the surface on red blood cells were then examined (Fig. 6). Erythrocyte-bound IgG, C3c and C4c were analysed using flow cytometry and expressed as MFI. Since the fluorescence of the isotype control was the same, no detectable IgG, C3c or C4c deposition on these RBCs were detected. CR1D patient showed the same binding of IgG, C3c and C4c on erythrocytes after addition of PBS, CVF and HAIGG as the donors with low, medium and high erythrocyte CR1 numbers (Fig. 6). As expected, the positive control *E. coli* bacteria showed enhanced IgG, C3c and C4c deposition after 30 min incubation.

3.7 Effect of erythrocyte CR1 numbers on bacterial growth in human whole blood

We then examined the effect of erythrocyte CR1 numbers on bacterial growth in human whole blood (Fig. 7A). Live *E. coli* bacteria were added to fresh human whole blood from blood donors with high and absent erythrocyte CR1 and bacterial growth measured as colony forming units (CFU/mL). When the *E. coli* bacteria were added to a PBS control, the CFU count was stable at all time points (Fig. 7A). In comparison, the CFU decreased with increasing incubation time when the bacteria were added to human blood with high CR1 numbers. However, the absence of erythrocyte CR1 in the CR1D had no influence on *E. coli* growth in whole blood. Similarly, when the anti-CR1 blocking mAb 3D9 was added to increasing concentrations of live *E. coli* (1×10^6, 1×10^7/mL and 1×10^8/mL) in donors with high CR1 numbers, no effect on bacterial growth was found (Fig. 7B).

3.8 Effect of the anti-CR1 blocking mAb on the binding of *E. coli* to erythrocytes, free bacteria in plasma, phagocytosis and oxidative burst

The anti-CR1 blocking mAb 3D9 efficiently reduced the binding of *E. coli* to erythrocytes, increased the number of free bacteria in plasma, phagocytosis and oxidative burst in monocytes (Fig. 8). In comparison, an isotype matched control mAb had no effect (Fig. 8), in line with previous results (Brekke, Hellerud et al. 2011).

Discussion

The present results indicate that erythrocyte CR1 numbers significantly affect the binding of *E. coli* bacteria to erythrocytes and strongly affects the amount of free bacteria in plasma, with subsequent consequences for phagocytosis and bacteria-induced oxidative burst. Collectively these results support a relation between phagocytosis and oxidative burst. The erythrocytes from the CR1D donor, nature's own human knock-out, bound only very small amounts of *E. coli* bacteria. Since this clonal CR1D in erythrocytes is extremely rare, these "lessons from nature" experiments should be interpreted with caution. Despite only one CR1 deficient individual could be examined, the addition of donors with different amounts of CR1 on their erythrocytes gave substantial support to our conclusions, since linear regression analyses between the readouts and the number of erythrocyte CR1 molecules could be calculated.

Leukocyte phagocytosis of *E. coli* bacteria was increased in CR1D compared with the other donors. Since the binding of bacteria to erythrocytes was significantly lower in CR1D and in the donor with low CR1 numbers, we speculate that this reduced binding of *E. coli* to erythrocytes, leading to a marked increase in free bacteria in plasma, increased the availability of bacteria for leukocyte phagocytosis. This was supported by the effect of the anti-CR1 blocking mAb which decreased the number of *E. coli* on erythrocytes, increased free bacteria in plasma, phagocytosis and oxidative burst in monocytes. This hypothesis has been confirmed in a previous study by addition of a blocking mAb to CR1, which resulted in an increased number of free bacteria in plasma and enhanced phagocytosis and oxidative burst in granulocytes (Brekke, Hellerud et al. 2011). Binding of *E. coli* to erythrocyte CR1 therefore restricts the uptake of bacteria in leukocytes similarly to the reduced uptake of immune complexes mediated by erythrocyte CR1 binding (Nielsen, Matthiesen et al. 1997). *E. coli*-induced phagocytosis and oxidative burst in human whole blood are closely related events

involving complement C5a formation and uptake through complement receptor 3 (Mollnes, Brekke et al. 2002). The number of erythrocytes in human whole blood exceeds the number of leukocytes by approximately 700 times. Thus, C3b and C4b-opsonized bacteria will initially mainly bind to erythrocyte CR1 (Nelson 1953, Brekke, Hellerud et al. 2011). Similarly, the number of CR1 molecules on each erythrocyte in human blood is approximately four-fold higher than those on a leukocyte, further increasing the total amount of erythrocyte CR1 as compared to leukocyte population (Fearon 1980). The number of erythrocyte CR1 molecules in Caucasians vary from about 150 to 1200 per erythrocytes due to genetic polymorphisms (Pham, Kisserli et al. 2010).

Thus, our data support the notion that CR1 has an important role in the initial binding of bacteria to erythrocytes, and that this binding probably affects the distribution of bacteria and other iC3b opsonized molecules *in vivo* (Atkinson, Chan et al. 1988). Previous data have indicated that CR1 binding may affect the distribution of bound immune complexes from spleen to the liver (Atkinson, Chan et al. 1988, Klein, Zhadkewich et al. 1994). To explore these mechanisms in detail in the future, experiments with Gram-negative bacteria could be performed in a CR1 knock-in model with human CR1 on mouse erythrocytes (Li, Wang et al. 2010). In particular, the distribution of different bacteria between organs could be interesting to examine in such a knock-in model. However, a limitation of mouse models is the occurrence of another different complement receptor encoded by the complement receptor-related gene Y (CrrY), which has complement inhibitory actions (Quigg, Kozono et al. 1998) and is not present in humans (Naik, Sharma et al. 2013).

This report indicates, for the first time, that CR1 erythrocyte numbers does not affect the degree of complement activation induced in the fluid phase by various complement activators

like *E. coli*, HAIGG and CVF. The donor with low CR1 numbers had the highest CP and AP activity, but the CP and AP activity in serum was as expected not related to erythrocyte CR1 numbers. Although CR1 is a well-established complement regulator, acting as cofactor for cleavage of C4 and C4 controlling the complement activation at the level of C3 (Iida and Nussenzweig 1981), and soluble CR1 is an efficient inhibitor of C3 activation (Atkinson, Chan et al.) (Fearon 1979), our data indicate that the number of CR1 on erythrocytes does not affect the degree of fluid phase complement activation. Thus, deficiency of CR1 on erythrocytes does not lead to an uncontrolled complement activation, neither at basal level nor when activation is induced by external activators.

No detectable IgG, C3c or C4c deposition on the erythrocytes were detected after stimulation with CVF and HAIGG. The lack of an effect of CR1 numbers on C3c, C4c and IgG deposition on the erythrocyte cell surface probably reflects that the erythrocyte prevents complement attack by other regulators, including the decay-accelerating factor (DAF) on the erythrocyte surface (Hourcade, Liszewski et al. 2000). Furthermore, HAIGG-induced complement activation probably occurs on the surface of HAIGG particles, i.e., on particles a certain distance away from erythrocyte CR1. Furthermore, since only a few *E. coli* bacteria initially bound to CR1D erythrocytes, complement activation on the *E. col*i surface probably did not occur in close proximity to the few CR1 receptors still present on CR1D erythrocytes.

Next, we examined the effect of erythrocyte CR1 binding on bacterial growth in whole blood. We used a complement-sensitive strain *E. coli* strain, explaining the rapid decrease in CFU after addition of live *E coli* to fresh human whole blood. However, bacterial growth did not differ in blood from CR1D compared with blood from a person with high CR1 numbers. This result was further confirmed using a blocking mAb against human CR1 at several bacterial concentrations.

The results indicated that the growth of *E. coli* was not affected by the number of CR1 on the erythrocytes.

The *E. coli*-induced release of inflammatory cytokines were not affected by the number of erythrocyte CR1. The hypothesis for the cytokine assays was that since the CR1 erythrocyte numbers significantly affected the number of free bacteria in plasma and leucocyte phagocytosis, the release of cytokines could be affected. These data may indicate that although the initial phagocytosis was increased in CR1D, bacterial pathogen associated molecular patterns (PAMPs), such as lipopolysaccharides, that participate in cytokine release are probably released and bind to Toll-like receptor 4 (TLR4) on leukocytes both when bacteria are bound to CR1 and when they are free in plasma. The TLR4 co-receptor CD14 and complement activation play an important role in the *E. coli*-induced cytokine release *in vivo* (Thorgersen, Pharo et al. 2009). However, the levels of several cytokines in the baseline sample were increased substantially increased in CR1D. This was particularly pronounced for IL-1RA and IFN-γ. These data should be interpreted with great caution and might not be due to an direct effect of low erythrocyte CR numbers, but rather due to a generally increased cytokine release caused by the SLE-like inflammatory disease in this individual, in agreement with a previous report (Csiszar, Nagy et al. 2000).

The human whole blood model of sepsis used in this study has some limitations in comparison to the *in vivo* situation, including a lack of endothelial cells. In addition, the model lacks physiological blood flow, although the tubes are gently rotated. However, this model of bacterial-induced complement activation and inflammation is currently to our knowledge the best *in vitro* model to study the role of complement in whole blood since lepirudin do not affect complement activation (Mollnes, Brekke et al. 2002).

In conclusion, the CR1 numbers on erythrocytes significantly affect the binding of *E. coli* to erythrocytes and the number of free bacteria in plasma, with subsequent consequences for leukocyte phagocytosis and oxidative burst in fresh human whole blood. The results indicate that individuals with low erythrocyte CR1 numbers may be predisposed to an enhanced initial leukocyte response after exposure to bacteria in blood. Whether this phenomenon plays a role in the development of human diseases remains to be investigated.

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Legends to figures

Fig. 1.

Effect of erythrocyte complement receptor 1 (CR1) numbers on *E. coli* binding to erythrocytes (A,B,C) and free bacteria in plasma (D,E,F). Increasing concentrations of heat inactivated *E. coli* were added to fresh human whole blood from the CR1 deficient (CRID) donor (< 50 CR1/erythrocyte: open triangle up) and from donors with low (200 CR1/erythrocyte: open circle), medium (500 CR1/erythrocytes: open square) and high (800/CR1 /erythrocyte: open triangle down) CR1 numbers. The legends to symbols are indicated in panel A, and are the same in Fig. 1-4. As a control, EDTA (10 mM final concentration) was added to the same blood donors (filled symbols). The number of erythrocytes with bound *E. coli* (A,B,C), and bacteria free in plasma (D,E,F) were analysed by flow cytometry after 10 min incubation at 37°C. The results are expressed as 10^8 bacteria/mL and are presented as the means ± range. Correlation between erythrocyte CR1 numbers and (B) the mean number of erythrocytes with bound *E. coli* against erythrocyte CR1 numbers using non-linear regression analysis and the log10 of erythrocyte CR1 numbers using linear regression analysis (C) were performed. The mean number of bacteria bound on erythrocytes were non-linearly correlated with erythrocyte CR1 numbers (B), following the equation $Y = -0.02479 + 0.002758X - 0.000001955X^2$ (r^2=0.88) when analysed using polynomial second order non-linear regression. The mean number of bacteria on erythrocytes were linearly correlated with the log10 of erythrocyte CR1 numbers (C) ($r^2 = 0.87$, $P < 0.0007$). The number of free bacteria in plasma was non-linearly correlated with erythrocyte CR1 numbers analysed using non-linear regression analysis (E), and following the equation $Y = 0.8581 - 0.002651X + 0.000002013X^2$ (r^2=0.999) when analysed using polynomial second order non-linear regression. The log10 of erythrocyte CR1 numbers was

linearly correlated with the mean number of free *E. coli* (F) in plasma ($r^2 = 0.98$, $P < 0.0001$) analysed using linear regression analysis in GraphPad Prism (n=4).

Fig. 2.

Effect of erythrocyte CR1 numbers on *E. coli* phagocytosis in granulocytes (A,B,C) and monocytes (E,F,G). Increasing concentrations of Alexa-labelled heat-inactivated *E. coli* were added to fresh human whole blood from the CR1 deficient donor (CR1D: triangle up), and from donors with low (circle), medium (square) and high CR1 numbers (triangle down). Phagocytosis in granulocytes and monocytes was analysed after a 20-min incubation at 37°C using flow cytometry. The results are expressed as the median fluorescence intensity (MFI) and presented as the means ± range. The granulocyte (B) and monocyte phagocytosis (E) was non-linearly correlated with erythrocyte CR1 numbers analysed using non-linear regression analysis (B,E). The polynomial second order non-linear regression in panel B followed the equation $Y = 84794 – 29.98X + 0.04115X^2$ (r^2=0.75). The log10 of CR1 numbers were significantly and linearly related with the (C) granulocyte phagocytosis ($r^2 = 0.49$, $P = 0.05$) and (F) monocyte phagocytosis ($r^2 = 0.51$, $P = 0.046$) analysed using linear regression analysis in GraphPad Prizm (n=4). The polynomial second order non-linear regression of the data in panel E followed the equation $Y = 73554 – 75.24X + 0.02921\ X^2$ (r^2=0.65).

Fig. 3.

Effect of erythrocyte CR1 numbers on *E. coli*-induced oxidative burst in granulocytes (A) and monocytes (C). Increasing concentrations of heat-inactivated *E. coli* were added to fresh human whole blood from the donor with CR1 deficiency (CR1D, triangle up), and from donors with

low (circle), medium (square) and high (triangle down) CR1 numbers. The oxidative burst in granulocytes and monocytes was analysed after a 10-min incubation at 37°C using flow cytometry and expressed as the median fluorescence intensity (MFI). The results are presented as the means ± range (n=4, in duplicates). The polynomial second order non-linear regression of the data in panel B followed the equation $Y = 13920 – 36.17X + 0.03086 X^2$ (r^2=0.79). The log10 of erythrocyte CR1 numbers were significantly and linearly correlated with the mean (C) granulocyte oxidative burst ($r^2 = 0.73$, $P = 0.0066$) and (F) monocyte oxidative burst ($r^2 = 0.72$, $P = 0.008$) analysed using linear regression analysis in GraphPad Prizm (n=4). The polynomial second order non-linear regression analysis of the data in panel E followed the equation $Y = 4670 – 13.13X + 0.01146 X^2$ (r^2=0.75).

Fig. 4.

Effect of erythrocyte CR1 numbers on *E. coli*-induced complement activation in plasma**.** Increasing concentrations of *E. coli* were added to whole blood from the CR1 deficient donor (open triangle up) and from donors with low (open circle), medium (open square) and high (open triangle down) CR1 numbers, and further incubated for 30 min at 37°C. As a control, EDTA (10 mM final concentration) was added to the same blood donors (filled symbols). The following complement activation products were measured in plasma using ELISA: (A) C4bc, (B) C3bc, (C) C3bBbP and (D) the soluble terminal complement complex (TCC). The results are expressed as arbitrary units/mL (AU/mL) and presented as the means ± range.

Fig. 5.

Effect of erythrocyte CR1 numbers on complement activation in the fluid phase in fresh human whole blood after stimulation with cobra venom factor (CVF) and heat-aggregated immunoglobulin G (HAIGG). Phosphate-buffered saline (PBS), CVF (2.5 U/mL) and HAIGG

(0.1 and 1.0 g/L) were added to fresh human blood from the donor with CR1 deficiency (CR1D) (open bars) and from donors with low (grey bars), medium (hatched bars) and high (black bars) CR1 numbers, and further incubated for 30 min at 37ºC. (A) C4bc, (B) C3bBbP, (C) C3bc and (D) the terminal complement complex (TCC) were analysed by ELISA. The baseline sample (T0) at time zero contained EDTA and indicates basal levels. The results are expressed as arbitrary units/mL (AU/mL) and presented as the means ± range. (E) The functional activity of the classical pathway (CP) and alternative pathway (AP) were analysed by Wielisa® and expressed as percent. The dashed lines in panels E and F indicates the upper reference ranges of the assay.

Fig. 6.

Effect of erythrocyte CR1 numbers on (A) IgG, (B) C3c and (C) C4c deposition on erythrocytes in human whole blood analysed using flow cytometry. Phosphate-buffered saline (PBS), cobra venom factor (CVF, 2.5 U/mL) and heat-aggregated immunoglobulin (HAIGG, 0.1 and 1.0 g/L) were added to fresh human blood from the donor with CR1 deficiency (CR1D) (open bars) and from the donors with low (grey bars), medium (hatched bars) and high (black bars) CR1 numbers, and further incubated for 30 min at 37°C. *E. coli* (1 x 10^8/mL) was added as a positive control. The results are expressed as the median fluorescence intensity (MFI). Data are presented as the means ± range.

Fig. 7.

Effect of erythrocyte CR1 numbers on bacterial growth in human whole blood. (A) Live *E. coli* were added to human whole blood (1 x 10^8/mL) from the donor with CR1 deficiency (CR1D) (triangle up) and from the donor with high (triangle down) erythrocyte CR1 numbers, and further incubated up to 30 min at 37°C. As a control, live bacteria were added to PBS only

(open circle). Bacterial growth was analysed using a standard bacterial culture technique and expressed as colony forming units (CFU/mL). The results are presented as the means ± range. The anti-CR1 blocking mAb 3D9 (4 µg/mL) was added to increasing concentrations of live *E. coli* (1 x 10^6, 1 x 10^7/mL and 1 x 10^8/mL) in donors with high CR1 numbers and compared with the isotype matched control mAb (Fig. 6B). The results are presented as the means ± SD. The results in Fig. 6B was analyzed using Student`s T-test.

Fig. 8.

Effect of the anti-CR1 blocking mAb 3D9 on *E. coli* binding to erythrocytes (A), free *E. coli* in plasma (B), monocyte phagocytosis (C) and monocyte oxidative burst (D). The number of erythrocytes with bound *E. coli* (A), and bacteria free in plasma (B) were analyzed by flow cytometry after 10 min incubation at 37°C. Phagocytosis were analysed by flow cytometry and expressed as median fluorescence intensity (MFI). Oxidative burst was analysed by flow cytometry and expressed as MFI (percent of the *E. coli* + PBS control which was set to 100%). The anti-CR1 blocking mAb 3D9 (4 µg/mL) was added without or with *E. coli* (0.72 x 10^8/mL) and compared with the isotype matched control mAb. The results are from independent experiments with healthy blood donors (n=3-6). **P*<0.05 analyzed by repeated-measures ANOVA and using the sample with *E. coli* + PBS as control.

Supplementary Results and Figures:

"Effect of erythrocyte CR1 numbers on the plasma cytokine levels

We then examined the basal plasma levels of 27 cytokines and their response to *E. coli* incubation in fresh whole blood. Twenty of the cytokines either had detectable basal levels and/or responded with increased release when incubated with *E. coli* (Supplementary Fig. 1, 2,3,4). In general, there was no differences in responses to *E. coli* dependent on the erythrocyte CR1 numbers, whereas the following 10 cytokines were remarkably higher in the baseline plasma sample in the CR1D donor than in the other three donors: IL-1RA, IL-2, IL-4, IL-9, IL-12 (p70), IL-13, IL-15, eotaxin, GM-CSF and IFN-γ (Supplementary Fig. 1,2,3,4). Quantitatively, IL-1RA (Suppl. Fig. 1B) and IFN-γ (Suppl. Fig. 2F) were the two most prominent, reaching ng/mL levels in the baseline sample in CR1D whereas hardly detectable in the other donors."

Legends to supplementary Fig.:

Supplementary Fig. 1

"Effect of erythrocyte complement receptor 1 (CR1) numbers on *E. coli*-induced (A) Interleukin (IL-1)β, (B) IL-1RA, (C) IL-6, (D) IL-8, (E) tumor necrosis factor (TNF) and (F) IL-10 levels. The cytokine levels in the baseline sample are indicated as T0. Phosphate buffered saline (T2) and different concentrations of heat-inactivated *E. coli* were added to fresh human whole blood from the donor with CR1 deficiency (CR1D) (open bars) and from donors with low (grey bars), medium (hatched bars) and high (black bars) CR1 numbers, and further incubated for 2 hours (T2) at 37°C. Thereafter, plasma was harvested, and the cytokines were analysed using multiplex technology. The results are expressed as pg/mL and presented as the means ± range."

Supplementary Fig. 2

"Effect of erythrocyte complement receptor 1 (CR1) numbers on *E. coli*-induced (A) FGF-basic, (B) eotaxin, (C) G-CSF, (D) GM-CSF, (E) VEGF and (F) IFN-γ. The cytokine levels in the baseline sample are indicated as T0. Phosphate buffered saline (T2) and different concentrations of heat-inactivated *E. coli* were added to fresh human whole blood from the donor with CR1 deficiency (CR1D) (open bars) and from the donors with low (grey bars), medium (hatched bars) and high (black bars) CR1 numbers, and further incubated for 2 hours (T2) at 37°C. Thereafter, plasma was harvested, and the cytokines were analysed using multiplex technology. The results are expressed as pg/mL and presented as the means ± range."

Supplementary Fig. 3

Effect of erythrocyte complement receptor 1 (CR1) numbers on *E. coli*-induced (A) Interleukin (IL)-2, (B) IL-5, (C) IL-9, (D) IL-12 (p70), (E) IL-13 and (F) IL-15 levels. The cytokine levels in the baseline sample are indicated as T0. Phosphate buffered saline (T2) and different concentrations of heat-inactivated *E. coli* were added to fresh human whole blood from the donor with CR1 deficiency (CR1D) (open bars) and from donors with low (grey bars), medium (hatched bars) and high (black bars) CR1 numbers, and further incubated for 2 hours at 37°C. Thereafter, plasma was harvested, and the cytokines were analysed using multiplex technology. The results are expressed as pg/mL and presented as the means ± range.

Supplementary Fig. 4. Effect of erythrocyte complement receptor 1 (CR1) numbers on *E. coli*-induced (A) IP-10 and (B) Interleukin (IL)-17 levels. The cytokine levels in the baseline sample are indicated as T0. Phosphate buffered saline (T2) and different concentrations of heat-inactivated *E. coli* were added to fresh human whole blood from the donor with CR1 deficiency (CR1D) (open bars) and from donors with low (grey bars), medium (hatched bars) and high (black bars) CR1 numbers, and further incubated for 2 hours at 37°C. Thereafter, plasma was

harvested, and the cytokines were analysed using multiplex technology. The results are expressed as pg/mL and presented as the means ± range.

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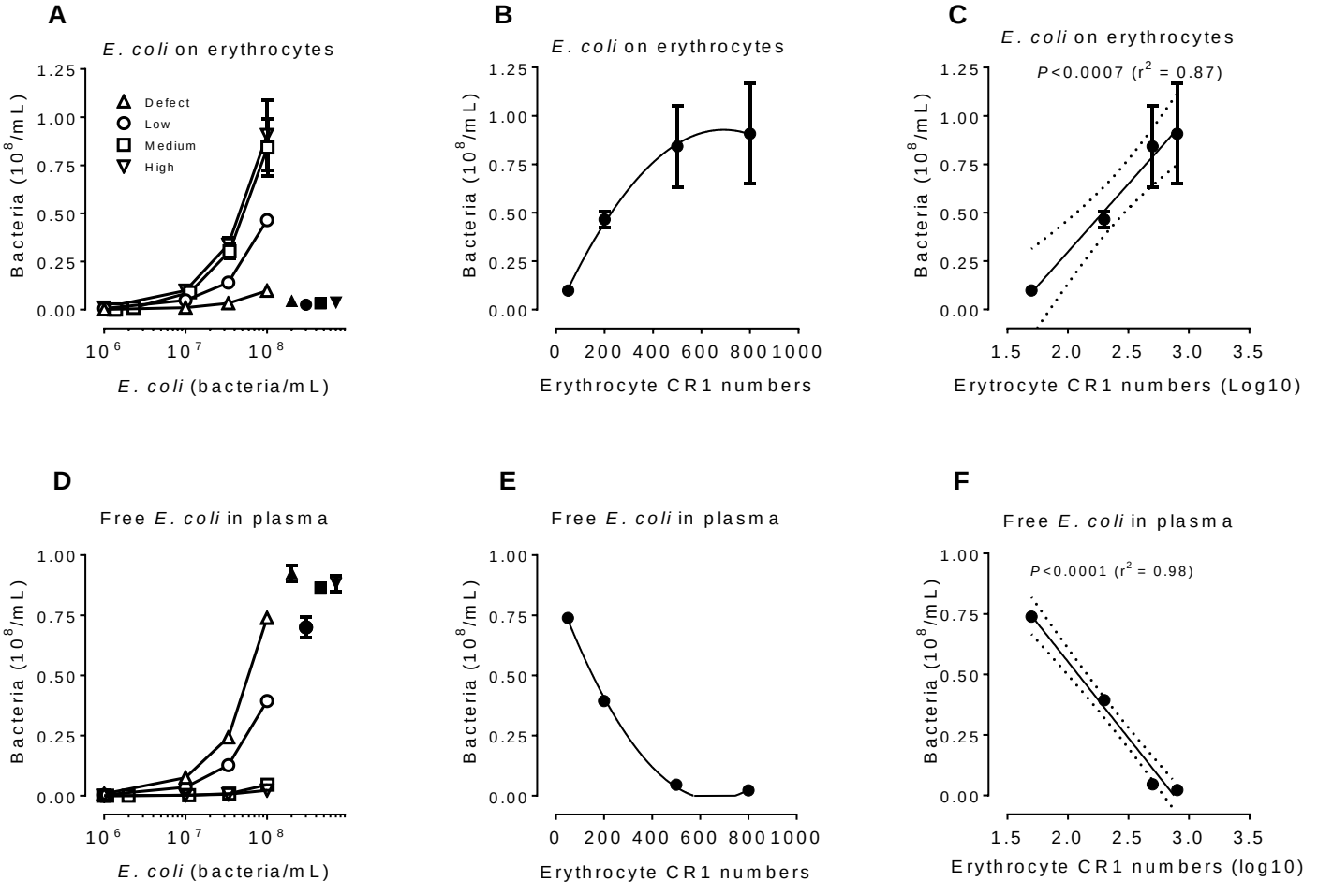
1

2

3

Fig. 1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E. coli on erythrocytes
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Erytrocyte CR1 numbers (Log10)
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Free E. coli in plasma
E. coli (bacteria/mL)
E
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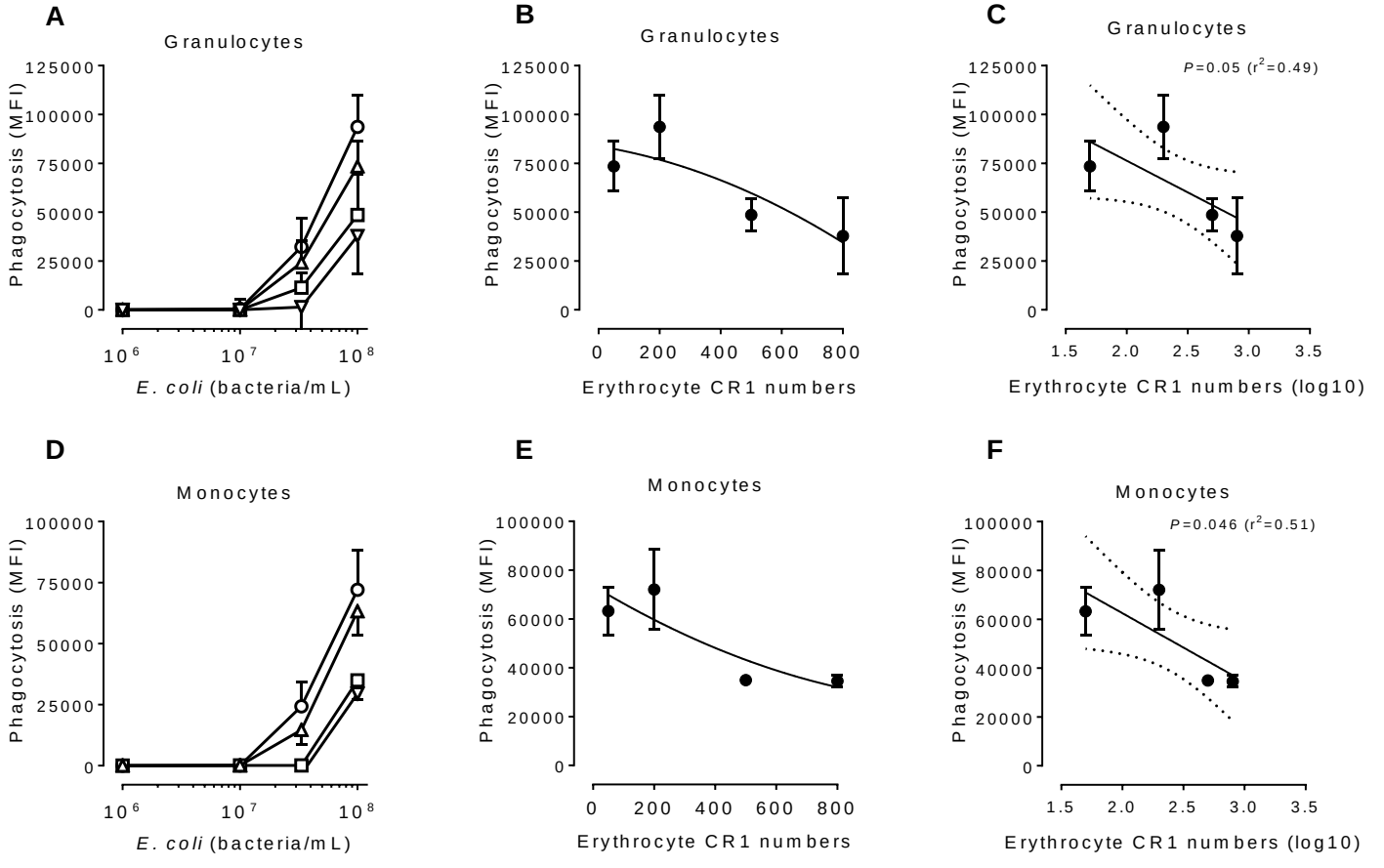
Erythrocyte CR1 numbers (log10)

Fig. 2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B
Granulocytes
Phagocytosis (MFI)
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100000
75000
50000
25000
0
0
200
400
600
800
Erythrocyte CR1 numbers
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Granulocytes
P=0.05 (r2=0.49)
Phagocytosis (MFI)
125000
100000
75000
50000
25000
0
1.5
2.0
2.5
3.0
3.5
Erythrocyte CR1 numbers (log10)
D
Monocytes
Phagocytosis (MFI)
100000
75000
50000
25000
0
10 6
10 7
10 8
E. coli (bacteria/mL)
E
Monocytes
Phagocytosis (MFI)
100000
80000
60000
40000
20000
0
0
200
400
600
800
Erythrocyte CR1 numbers
F
Monocytes
P=0.046 (r2=0.51)
Phagocytosis (MFI)
100000
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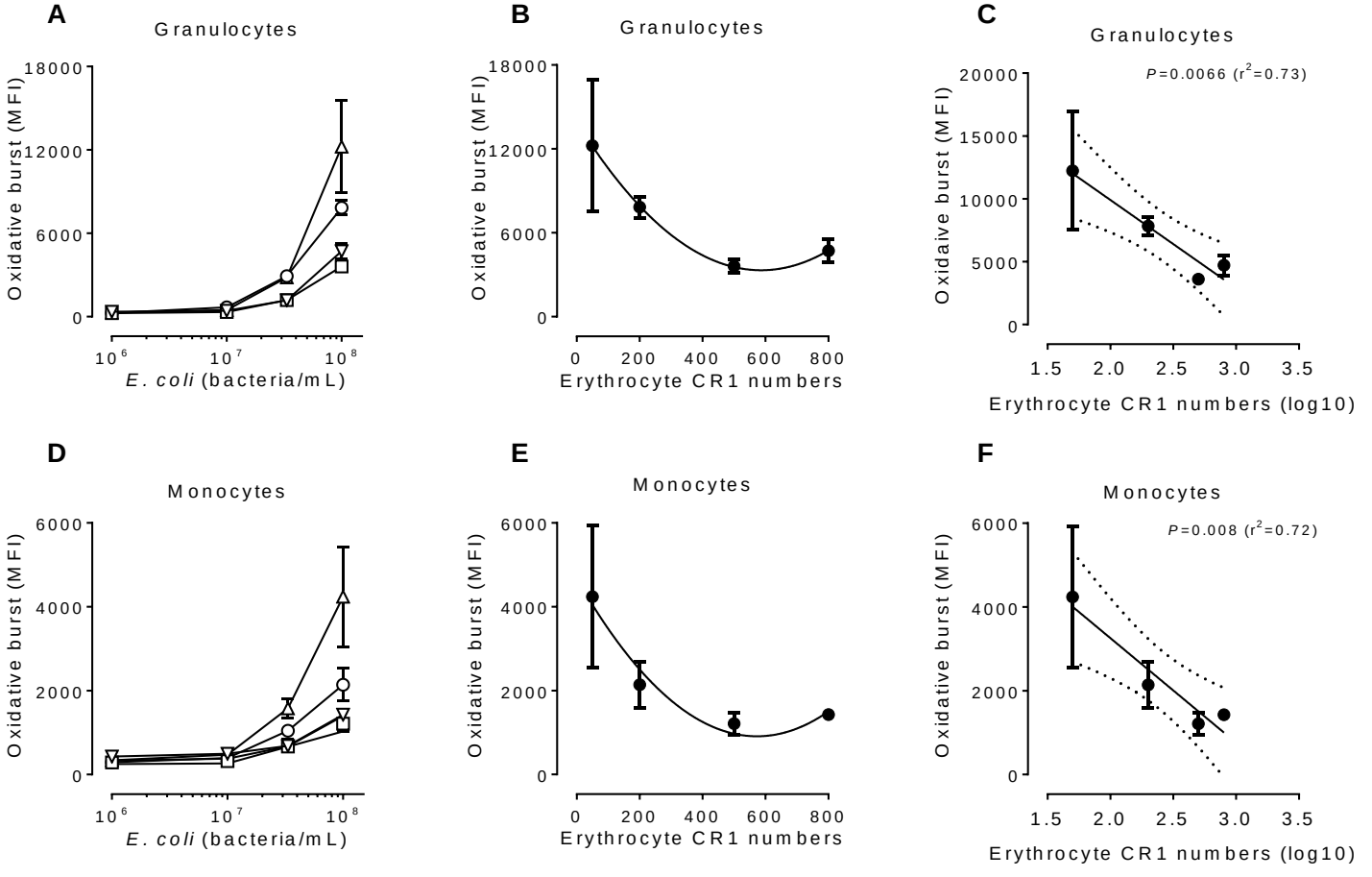
3.5
Erythrocyte CR1 numbers (log10)

Fig. 3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6000
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10^6
10^7
10^8
E. coli (bacteria/mL)
B
Granulocytes
Oxidative burst (MFI)
18000
12000
6000
0
0
200
400
600
800
Erythrocyte CR1 numbers
C
Granulocytes
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Oxidative burst (MFI)
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15000
10000
5000
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1.5
2.0
2.5
3.0
3.5
Erythrocyte CR1 numbers (log10)
D
Monocytes
Oxidative burst (MFI)
6000
4000
2000
0
10^6
10^7
10^8
E. coli (bacteria/mL)
E
Monocytes
Oxidative burst (MFI)
6000
4000
2000
0
0
200
400
600
800
Erythrocyte CR1 numbers
F
Monocytes
P=0.008 (r^2=0.72)
Oxidative burst (MFI)
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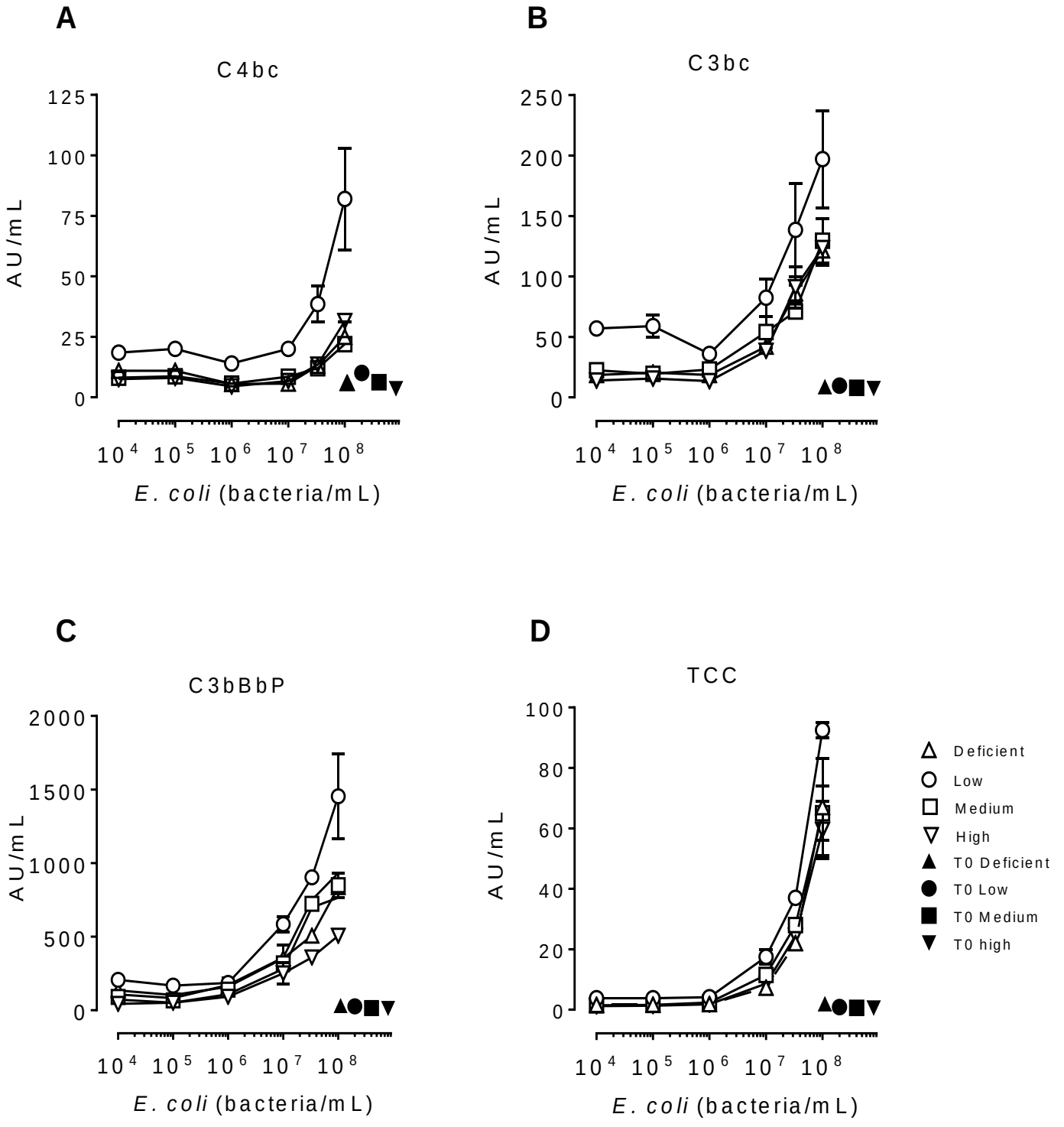
3.5
Erythrocyte CR1 numbers (log10)

Fig. 4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C4bc
AU/mL
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100
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E. coli (bacteria/mL)
B
C3bc
AU/mL
250
200
150
100
50
0
10^4 10^5 10^6 10^7 10^8
E. coli (bacteria/mL)
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AU/mL
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1500
1000
500
0
10^4 10^5 10^6 10^7 10^8
E. coli (bacteria/mL)
D
TCC
AU/mL
100
80
60
40
20
0
10^4 10^5 10^6 10^7 10^8
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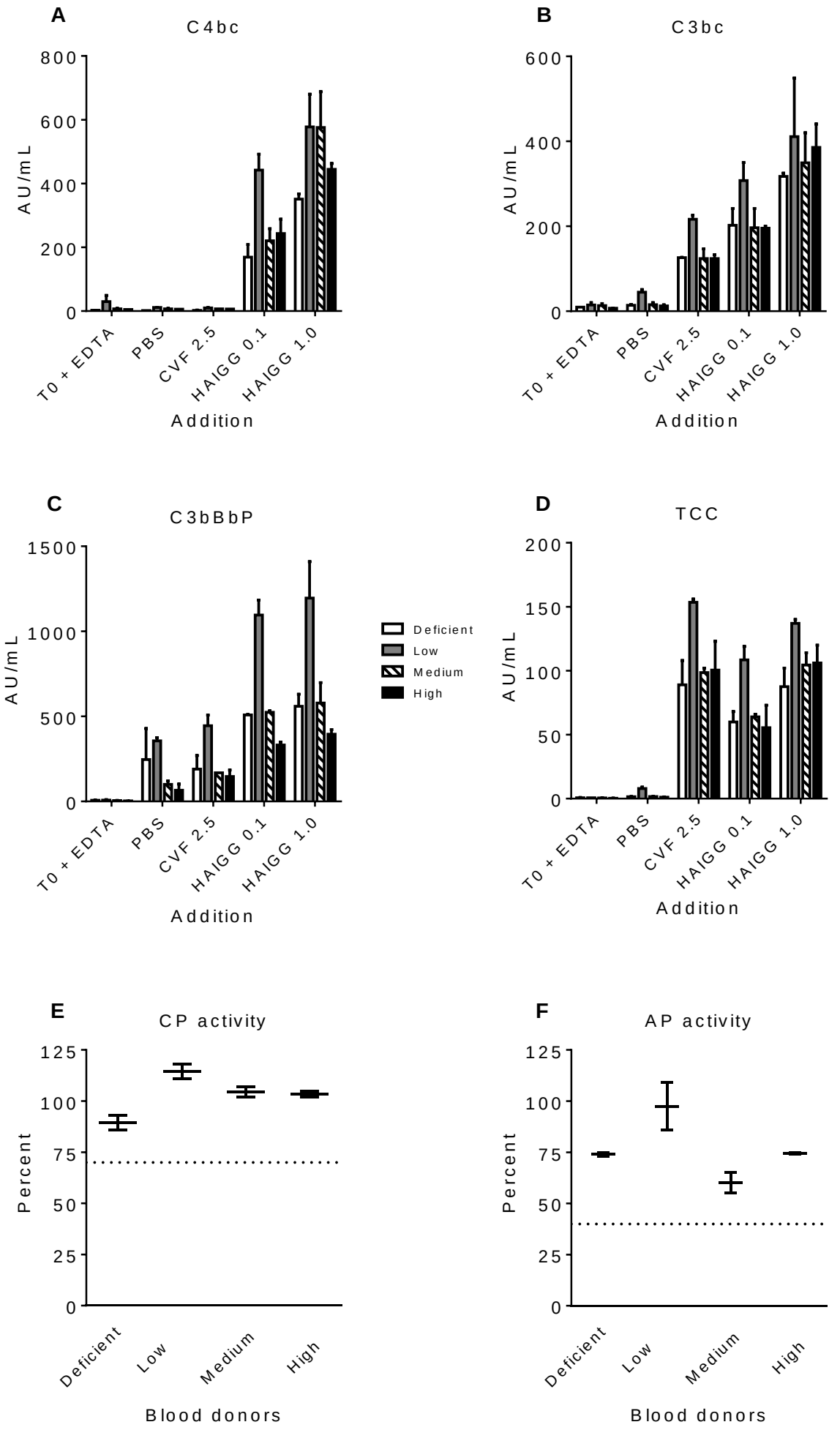
Deficient
Low
Medium
High
T0 Deficient
T0 Low
T0 Medium
T0 high

Fig. 5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CVF 2.5
HAIGG 0.1
HAIGG 1.0
Addition
B
C3bc
AU/mL
600
400
200
0
T0 + EDTA
PBS
CVF 2.5
HAIGG 0.1
HAIGG 1.0
Addition
C
C3bBbP
AU/mL
1500
1000
500
0
T0 + EDTA
PBS
CVF 2.5
HAIGG 0.1
HAIGG 1.0
Addition
Deficient
Low
Medium
High
D
TCC
AU/mL
200
150
100
50
0
T0 + EDTA
PBS
CVF 2.5
HAIGG 0.1
HAIGG 1.0
Addition
E
CP activity
Percent
125
100
75
50
25
0
Deficient
Low
Medium
High
Blood donors
F
AP activity
Percent
125
100
75
50
25
0
Deficient
Low
Medium
High
Blood donors

Fig. 6

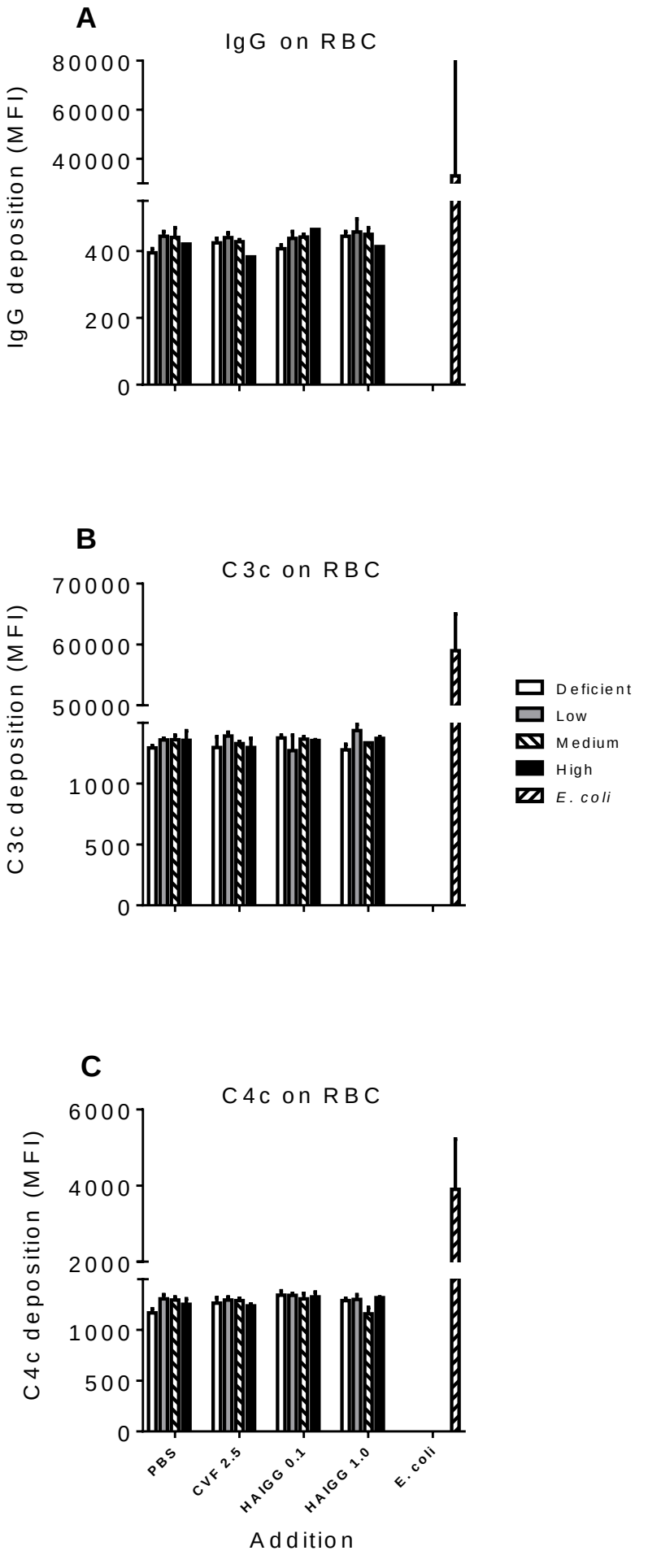

A
IgG on RBC
IgG deposition (MFI)
80000
60000
40000
400
200
0
B
C3c on RBC
C3c deposition (MFI)
70000
60000
50000
1000
500
0
Deficient
Low
Medium
High
E. coli
C
C4c on RBC
C4c deposition (MFI)
6000
4000
2000
1000
500
0
PBS
CVF 2.5
HAIGG 0.1
HAIGG 1.0
E. coli
Addition

Fig. 7

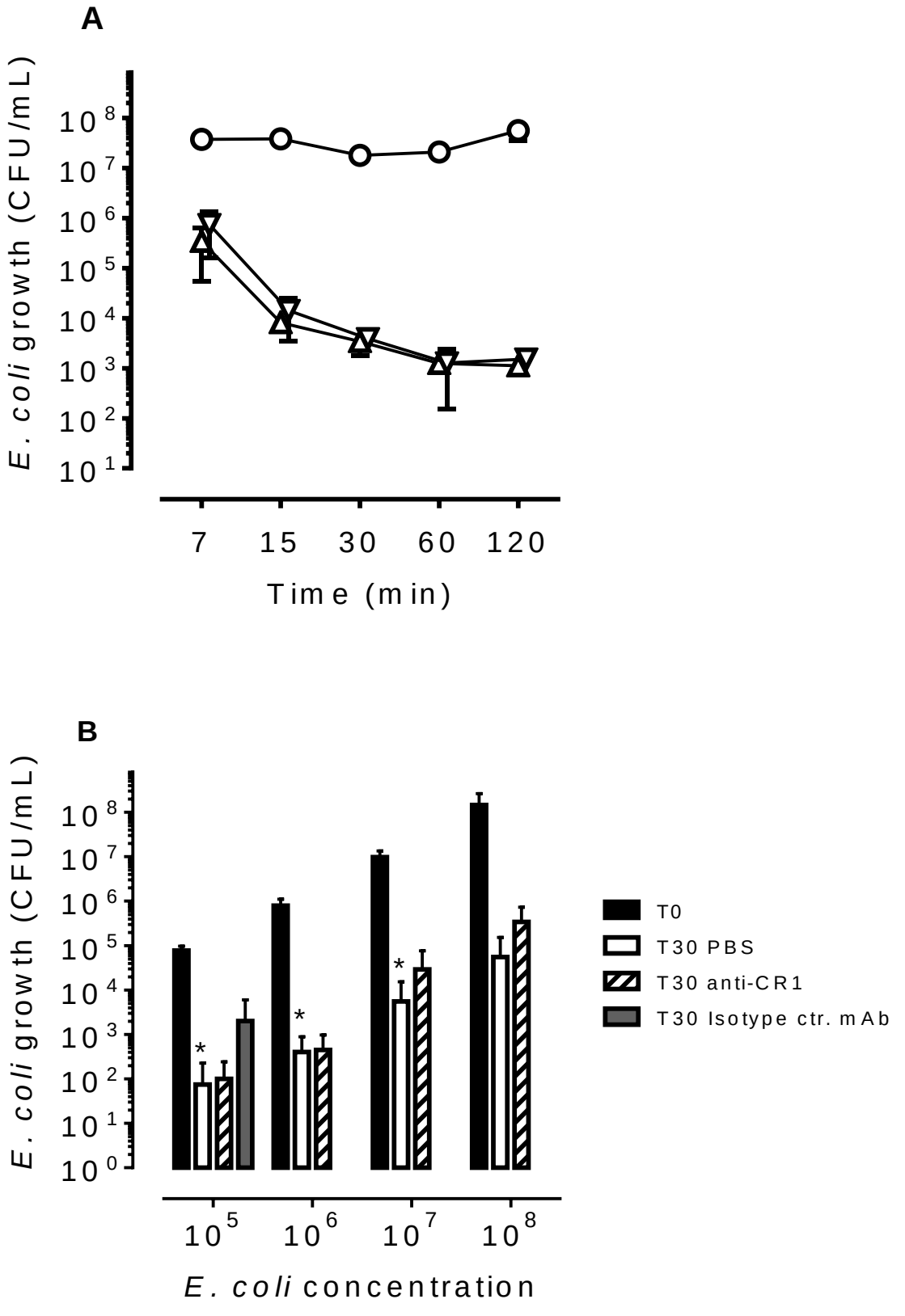
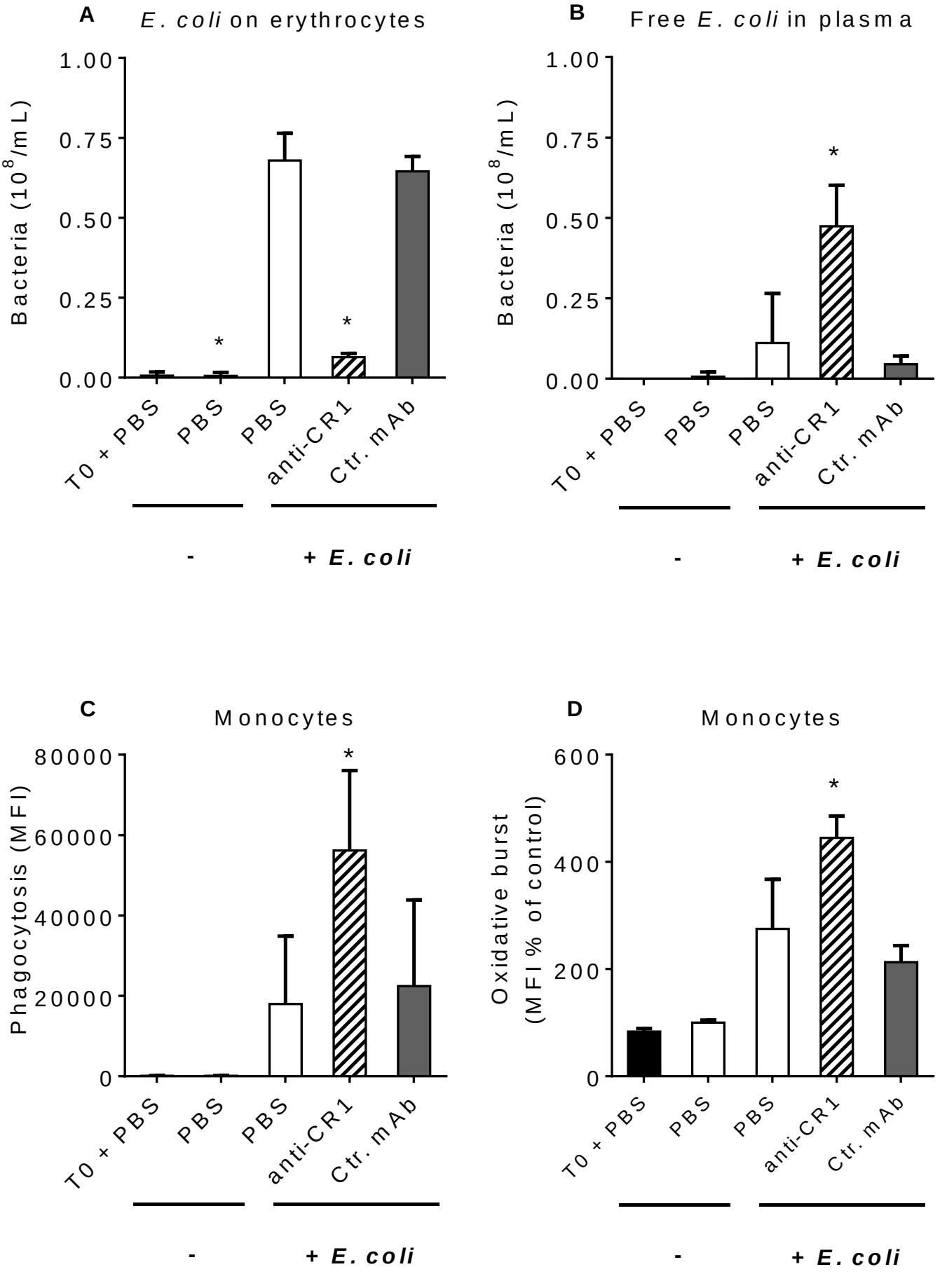
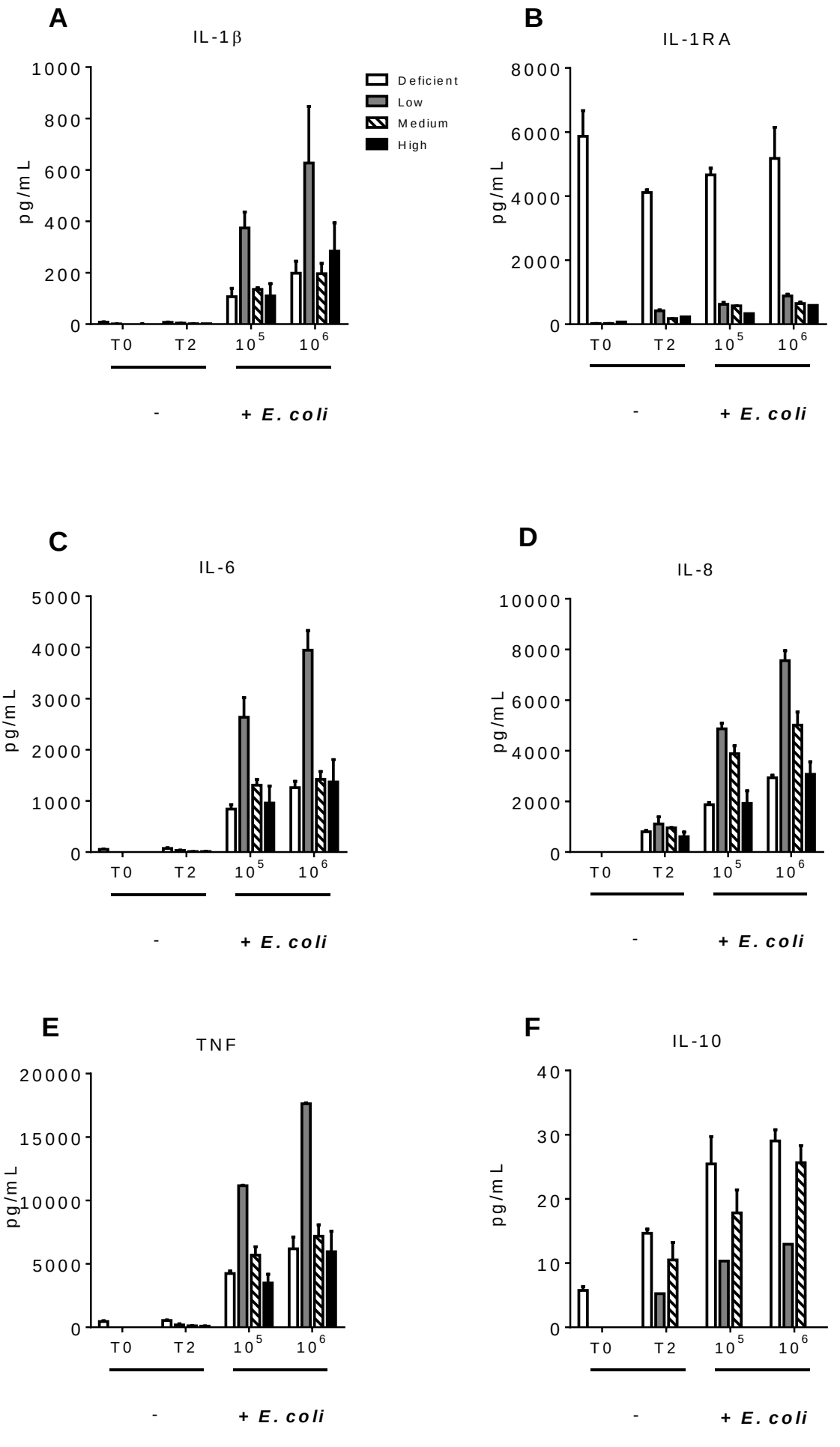

A
E. coli growth (CFU/mL)
10 8
10 7
10 6
10 5
10 4
10 3
10 2
10 1
7
15
30
60
120
Time (min)
B
E. coli growth (CFU/mL)
10 8
10 7
10 6
10 5
10 4
10 3
10 2
10 1
10 0
*
*
*
10 5
10 6
10 7
10 8
E. coli concentration
T0
T30 PBS
T30 anti-CR1
T30 Isotype ctr. mAb

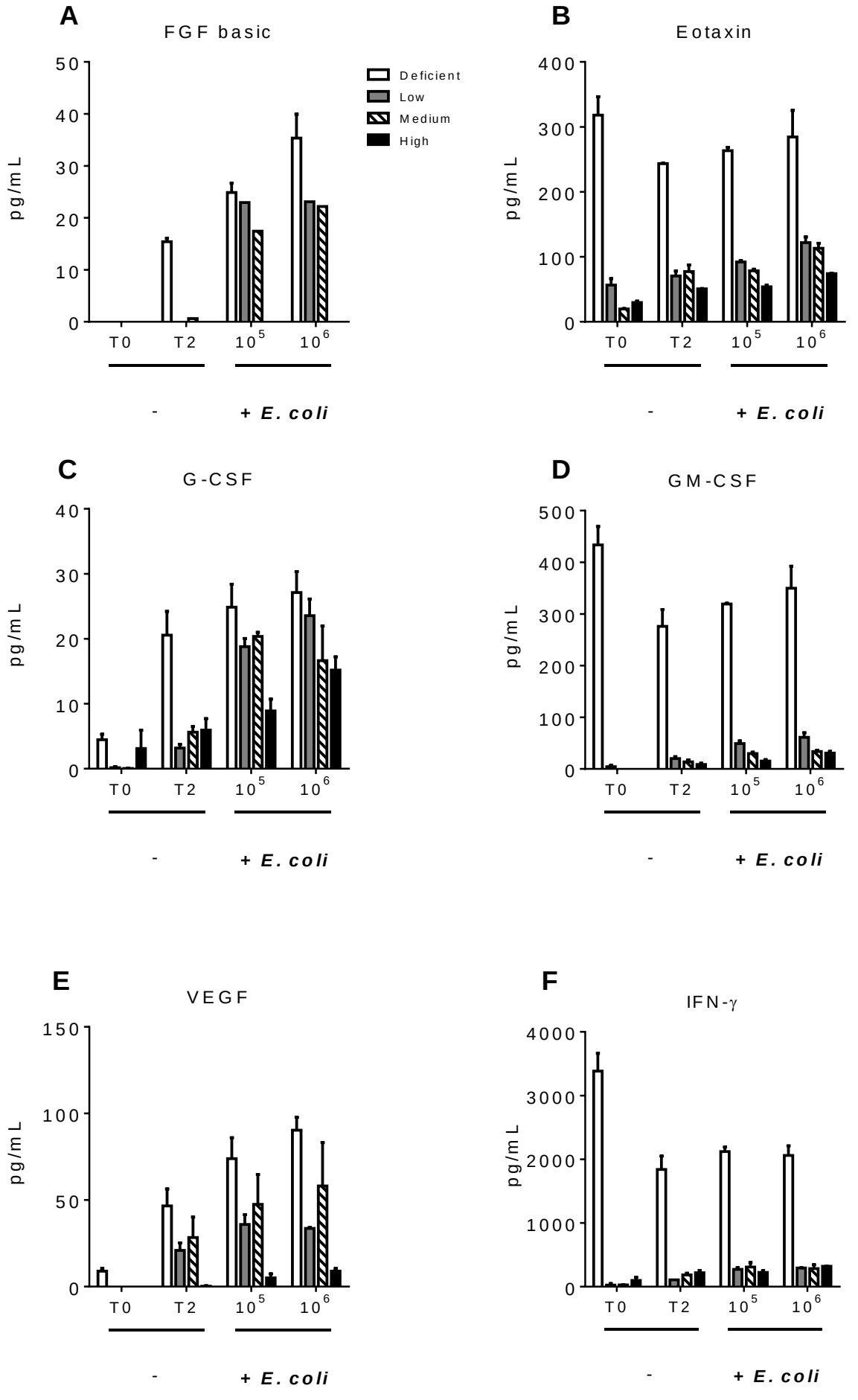
Fig. 8


A
E. coli on erythrocytes
Bacteria (10^8/mL)
1.00
0.75
0.50
0.25
0.00
*
*
T0 + PBS
PBS
PBS
anti-CR1
Ctr. mAb
-
+ E. coli
B
Free E. coli in plasma
Bacteria (10^8/mL)
1.00
0.75
0.50
0.25
0.00
*
T0 + PBS
PBS
PBS
anti-CR1
Ctr. mAb
-
+ E. coli
C
Monocytes
Phagocytosis (MFI)
80000
60000
40000
20000
0
*
T0 + PBS
PBS
PBS
anti-CR1
Ctr. mAb
-
+ E. coli
D
Monocytes
Oxidative burst (MFI% of control)
600
400
200
0
*
T0 + PBS
PBS
PBS
anti-CR1
Ctr. mAb
-
+ E. coli

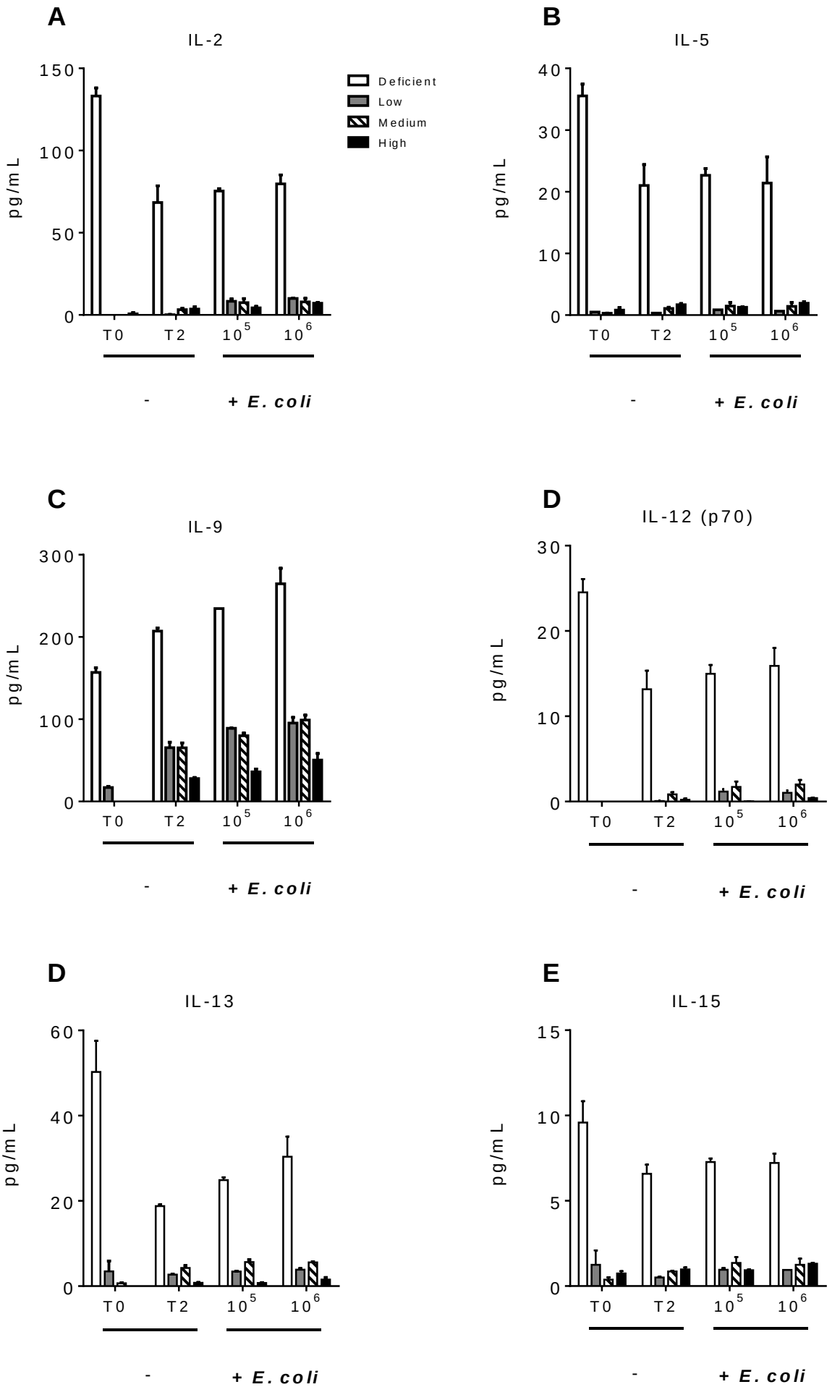
Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Fig. 3



Supplementary Fig. 4