Title: Granulocyte and monocyte CD11b expression during plasma separation is dependent on complement factor 5 (C5) - an *ex vivo* study with blood from a C5 deficient individual.

Running head: CD11b expression depends on C5.

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Abstract

Hardersen R, Enebakk T, Christiansen D, Bergseth G, Brekke OL, Mollnes TE, Lappegård KT, Hovland A. Granulocyte and monocyte CD11b expression during plasma separation is dependent on complement factor 5 (C5) - an *ex vivo* study with blood from a C5 deficient individual.

The aim of the study was to investigate the role of complement factor 5 (C5) in reactions elicited by plasma separation using blood from a C5 deficient (C5D) individual, comparing it to C5 deficient blood reconstituted with C5 (C5DR) and blood from healthy donors. Blood was circulated through an *ex vivo* plasma separation model. Leukocyte CD11b expression and leukocyte-platelet conjugates were measured by flow cytometry during a 30-minute period. Other markers were assessed during a 240-minute period. Granulocyte and monocyte CD11b expression did not increase in C5D blood during plasma separation. In C5DR samples granulocytes CD11b expression, measured by mean fluorescence intensity (MFI), increased from 10481±6022 (SD) to 62703±4936, and monocytes CD11b expression changed from 13837±7047 to 40063±713. Granulocyte-platelet conjugates showed a 2.5-fold increase in the C5DR sample compared to the C5D sample. Monocyte-platelet conjugates increased independently of C5. In the C5D samples platelet count decreased from 210 $x10^9$/L (201-219) (median and range) to 51$x10^9$/L (50-51), and C3bc increased from 14 CAU/mL (21-7) to 198 CAU/mL (127-269), whereas TCC formation was blocked during plasma separation. In conclusion, upregulation of granulocyte and monocyte CD11b during plasma separation was C5 dependent. The results also indicate C5 dependency in granulocyte-platelet conjugates formation.

Keywords: C5 deficiency; bioincompatibility; plasma separation; CD11b/CD18; leukocyte-platelet conjugate.

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Introduction

CD11b is part of the integrin heterodimer containing CD11b (integrin α_M) and CD18 (integrin $\beta_{2)}$. CD11b/CD18 (CR3) has approximately 40 reported protein ligands. Biologic functions during the inflammatory response include adhesion of leucocytes, regulation of cytokine secretion, and there are indications of direct adhesion to platelets (1). In commonly accepted biocompatibility models activated leukocytes expressing CD11b/CD18 binds iC3b molecules included in the initial protein layer bound to the artificial surface, which is considered one of the first steps in the bioincompatibility reaction (2, 3).

Studies have shown that hemodialysis upregulates the adhesion molecule CD11b on leukocytes, corresponding to elevated platelet-leukocyte aggregate counts, concluding that expression of CD11b is a reliable marker of leukocyte activation during hemodialysis (4).

When plasma separation is used in treatment of e.g. vasculitis, the plasma fraction is disposed and substituted with either fresh frozen plasma or a Ringer/albumin solution before it is merged with the cellular component and returned to the patient. In plasma separation used in LDL apheresis systems the cell fraction is merged with the plasma fraction before returned to the patient after LDL removal has taken place from the plasma fraction. If the plasma separation process in itself has adverse effects on the cellular components, either directly or by activation of the innate immunesystem, this may prove disadvantageous to the patient.

It has previously been demonstrated that plasma separation induces complement activation during *ex vivo* low-density lipoprotein (LDL) double filtration apheresis (5), and that CD11b upregulation is dependent on the presence of complement factor 5 (C5) in an *ex vivo* model with polyvinylchloride tubing (PVC) (6). Studies have also shown upregulation of CD11b on

monocytes and granulocytes, and an increase in monocyte- platelet and granulocyte- platelet conjugates after circulating blood through PVC tubing. Furthermore, blocking of C5a receptor 1 (C5aR1, CD88) on granulocytes and monocytes largely counteracted the CD11b presentation. Thus, CD11b upregulation by PVC is mediated trough complement activation, mainly by C5a (7), and CD11b upregulation on granulocytes is thus to some extent a proxy for complement activation. Crosstalk between parts of the innate immune systems (complement system, coagulation system and contact activation system) in plasma separation procedures leads to activation of the cellular component of the immune system, and thus enhancement of the immune response. The result is an inflammatory response in the patient undergoing treatment including blood exposure to artificial surfaces, also involving the cellular component of the immune system (2). This treatment-induced systemic response may have unwanted consequences for the patient.

The aim of the present study was to investigate the role of complement in leukocyte activation during plasma separation, as measured by expression of CD11b and formation of leukocyte-platelet conjugates. We developed an *ex vivo* model of plasma separation and compared blood from an individual with C5 deficiency (C5D) with C5 deficient blood reconstituted with C5 (C5DR), and blood from healthy donors as control (CTR). C5 deficiency is extremely rare, with only a few dozen individuals reported worldwide (8), but as these individuals represent nature´s own knock-outs their blood is well suited to study the role of complement in general and the role of C5 in particular in various models of inflammation.

Material and methods

Ethics:

The regional ethics committee approved the study and all blood donors signed an informed consent.

Donors:

Blood from a previously described C5 deficient individual (9), and blood from three healthy donors was used. Blood was drawn four times from the C5 deficient donor, and twice from each healthy donor. The individuals donated 450 mL of blood on each occasion. Time duration between blood donations was approximately six months.

Blood sampling and plasma separation:

Lepirudin (Refludan®, Celgene, Marburg, Germany), 25mg in 50 mL of 0.9% NaCl, was added to a 600 mL filterless Blood Pack Unit (Fenwal, Lake Zürich, USA, made from polyvinylchloride copolymer plasticized with di-2-ethylhexyl phthalate, without other additives, before blood donation, giving a final concentration of 0.05 mg lepirudin/mL blood and a final volume of 500 mL in the blood pack unit. Lepirudin in this concentration gives efficient anticoagulation without affecting complement activation (10).

Fifty mL of blood was then transferred to an empty blood pack to serve as a control for time-dependent, contact-induced activation (no-plasma separation blood reservoir, NPS). The remaining 450 mL served as the reservoir for blood circulating in the plasma separation model (plasma separation blood reservoir, PS) (Fig. 1). Both blood packs were placed in a temperature controlled heater (Binder, Binder GmBH, Tuttlingen, Germany) set at 37°C, with constant movement by means of a modified test tube rotator (Rock ’n Roller, Labinco BV, Breda, The Netherlands). The blood reservoir was attached to the plasma separation system which consisted of an Octo-Nova (MeSys Gmbh, Hannover, Germany) machine with a PlasmaFlo OP-05W column (Asahi Kasei Medical Europe) plasma separation column and

PVC tubing. The flow rates were 100 mL/min for the blood pump and 20 mL/min for the plasma pump. Blood samples for flow cytometry were obtained at 0, 5, 15, and 30 minutes (T0-T30), while the other markers were obtained at 0, 5, 30, 120 and 240 minutes (T0-T240). All blood samples were drawn simultaneously (within a time frame of one minute) for each sample time and location. Blood samples were drawn into polystyrene tubes containing EDTA (to block any further complement activation) to a final concentration of 10 mM and then placed on ice before centrifugation for 15 min at 3220g at 4°C. The plasma was frozen in aliquots at -80°C until analysis in batches. Blood smears were made immediately after blood sampling, at T0 and at T240.

Flow cytometric studies of CD11b:

Flow cytometric studies were performed with an LSRII (Becton Dickinson (BD), San Jose, Ca, USA). At each point of time blood (100 μL) was fixed with 100 μL 0.5% paraformaldehyde for 4 minutes at 37°C, and 25 μL fixed blood was then incubated for 15 minutes at room temperature in the dark with anti-CD11b-PE (BD) or the isotype control IgG2a-PE (BD). For threshold for the nuclear stain LDS-751 (Molecular Probes, Life Technologies) was added. In addition anti-CD14 FITC (BD) was used for gating purpose. One mL PBS was added and samples were acquired after 15 minutes. Granulocytes and monocytes were gated in an SSC/anti-CD14-dotplot, and the mean fluorescent intensity values for CD11b were calculated (Fig. 2). The antibody used in our study (mouse anti human-CD11b-Phycoerythrin, clone D12, Becton Dickinson, San Jose, Ca, USA) is specific for the 165-kilodalton (kd) α subunit of the CD11b/CD18 antigen heterodimer, and is as such unable to disclose if the CD11/CD18 integrin is conformationally changed into its active form. However, the same anti human CD11b antibody is used by our and other groups in studies exploring CD11b upregulation in inflammation, and taken into account for the

conformational change of the heterodimer into its active form and activation of leukocytes (11-14).

Leukocyte-platelet conjugates:

Fixed blood cells were stained with anti-CD14 PE (BD), anti-CD61 FITC (BD), LDS-751 and re-suspended as described above. Granulocytes and monocytes were gated in an SSC/anti-CD14-dotplot, and the mean fluorescent intensity values for CD61 were calculated.

Routine biochemistry:

Hemoglobin, leukocytes and platelets were analyzed using a Siemens ADVIA® 2120 Hematology System (Siemens Healthcare Diagnostics Ltd., Camberly, UK). Total protein, albumin, C4, IgG, IgM and IgA were analyzed in an ADVIA®1800 system (Siemens Medical Solutions Diagnostics, Japan) with reagents from Siemens Healthcare Diagnostics Ltd.

Complement components and functional activity assays:

Purified human complement protein C5 was obtained from Quidel (Quidel Corporation, San Diego, USA). Purified C5 was added to C5 deficient blood to give a final plasma concentration of 80 µg/mL, corresponding to the concentration of C5 in normal individuals (15). The complement activation products C3bc and the terminal complement complex (TCC) were measured using enzyme immunoassays based on capture antibodies reacting with neoepitopes exposed selectively in the activation product and not in the native component as described in detail previously (16).

Correction for dilution:

A small amount of priming solution (isotonic saline) was used to prepare the tubing and columns before the *ex vivo* loop was started. Hematocrit was used to correct the concentration for the plasma parameters, according to a standardized formula (17).

Statistics:

Formation of leukocyte-platelet conjugates and CD11b expression was measured as mean fluorescent intensity ± standard deviation (SD), all other measurements are median ± range. The rarity of the C5-deficiency precluded use of many repeated samples in this study. Due to the few numbers of observations we have presented the data without further tests of statistical significance. All calculations presented were performed with Prism 5.0 for Windows, Graphpad software (San Diego, California, USA).

Results

Expression of CD11b on leukocytes (Fig 3):

Granulocytes (Fig 3A): Blood from control individuals showed an increase in CD11b expression in the plasma separation sample from 2621±498 (mean and SD) at T0 to 30727±9165 at T30 (CTR-PS: Fig.2 A, left panel). In contrast the C5 deficient blood (C5D) showed no increase in granulocyte CD11b expression in the plasma separation sample changing from 5351±919 at T0 to 7935±1648 at T30, (C5D-PS: Fig 3A, left panel). After reconstituting the C5 deficient blood with purified C5 (C5DR), granulocyte CD11b expression in the plasma separation sample increased from 10481±6022 at T0 to 62703±4936 at T30 (C5DR-PS: Fig.2 A, left panel).

In the time-dependent, spontaneous activation, no-plasma separation sample (NPS) there was a small increase in CD11b expression in blood from the control persons from 2443±725 at T0 to 6419±218 at T30, (CTR-NPS: Fig. 3A, right panel). Similarly there was a small increase in C5 deficient blood from 5351±919 at T0 to 10537± 890 at T30 (C5D-NPS; Fig. 3A right panel). After reconstitution there was an increase in CD11b expression from 10481±6022 at T0 to 47080±17186 at T30, (C5DR-NPS: Fig. 3A, right panel).

Monocytes (Fig. 3B): In blood from control individuals the plasma separation sample showed a marked increase in monocyte CD11b expression from 3894±285 at T0 to 23575±6765 at T30, (CTR-PS; Fig. 3B, left panel). In C5 deficient blood there was no increase in CD11b expression as it changed from 9027±456 at T0 to 11478±1461 at T30, (C5D-PS: Fig 3B, left panel) similar to that seen for the granulocytes. Upon reconstitution with C5 monocyte expression of CD11b in C5 deficient blood also increased, from 13837±7047 at T0 to 40063±713 at T30, (C5DR-PS: Fig. 3B, left panel).

In the no-plasma separation samples there was a small and equal increase in CD11b expression on monocytes for controls and C5D (CTR-NPS and C5D-NPS: Fig. 3B, right panel). Similar to the granulocytes, there was an increase in CD11b expression in the monocyte C5DR-NPS samples from 13837±7047 at T0 to 40532±543 at T30, (Fig. 3B, right panel).

Formation of leukocyte-platelet conjugates (Fig.4):

Granulocyte-platelet conjugates (Fig. 4A)*:* In blood from control individuals the plasma separation sample showed an increase in granulocyte-platelet conjugate formation from 1325±250 at T0 to 5633±3199 at T30, (CTR-PS: Fig. 4A, left panel). In C5D blood granulocyte-platelet conjugate formation during plasma separation increased from 1931±337 at T0 to 3247±1066 at T30, (C5D-PS: Fig 4A, left panel). The reconstituted C5 deficient blood increased from 1851±805 at T0 to 4743±485 at T30, (C5DR-PS: Fig. 4A, left panel). In the no-plasma separation samples (Fig. 4A, right panel), there were a small and equal increase during the observation time in the three groups.

Monocyte-platelet conjugates (Fig. 4B): There were increases in monocyte-platelet conjugate formation for all groups during plasma separation from T0 to T30 (Fig. 4B left panel). The increases seen in the no-plasma separation samples during the 30 minute observation time

were less than in plasma separation samples and also similar in all groups (Fig. 4B right panel).

Platelet and leukocyte counts (Fig.5):

Platelet count was reduced in all groups during plasma separation (T0-T240); CTR: 235 10^9/L (218-246) (median and range) to 67 10^9/L (26-68), C5D: 210 10^9/L (201-219) to 51 10^9/L (50-51), C5DR: 191 10^9/L (158-224) to 31 10^9/L (26-36), (Fig. 5A, left panel). Blood smears obtained from the plasma separation samples at the end of the apheresis session showed platelet agglutination (data not shown). None of the groups changed in platelet count in the no-plasma separation samples (Fig. 5A right panel). Furthermore there were no significant changes in the leucocyte counts during plasma separation or in the no-plasma separation samples in the different groups (Fig. 5B).

Total protein and complement factor 4 (C4) (Fig.6):

In the plasma separation control sample there was a reduction in total protein from T0 53 g/L (45-54) (median and range) to 25 g/L (18-31) at T5 with no further reduction at T240 (CTR-PS: fig 6A, left panel). There were similar reductions in total protein for all groups during plasma separation (C5D-PS and C5DR-PS: fig. 6A, left panel). No such reduction was seen in the no-plasma separation samples (fig. 6A, right panel). The same pattern was seen for albumin, IgG, IgM and IgA (data not shown).

Resembling the pattern for total protein, there were reductions in C4 for all groups during plasma separation starting at T5, with no further reduction at T240 (Fig. 6B, left panel), whereas the levels in the no-plasma separation samples remained unchanged during time (Fig 6B, right panel).

Complement activation (Fig.7):

In all plasma separation samples there were increases in the complement activation product C3bc, starting at 5 min with further increase after 30 min (Fig. 7A, left panel). In the no-

plasma separation samples the increase occurred later, starting at 30 min and continuing up to 240 min, however not reaching the same maximum as in the plasma separation samples (Fig. 7A, right panel).

The terminal C5b-9 complement complex (TCC) was measured to assess endpoint complement activation. In the controls and in the C5 reconstituted samples there was a fifteen-fold increase in TCC during plasma separation (CTR-PS, C5DR-PS: Fig. 7B, left panel), and as expected there was no TCC formation in the C5D sample consistent with the lack of C5 (C5D-PS: Fig. 7B, left panel). Similarly, in the no-plasma separation samples, there was a ten-fold TCC increase in the CTR sample and a twenty-fold increase in the C5DR sample, while there was no TCC formation in the C5D sample (Fig. 6B, right panel).

Discussion

We have previously shown that individuals deficient of C5, nature's own knock-outs, can be used as a robust model for exploring the role of C5 in different experimental settings (14). In the present study, using C5D blood compared to controls in an *ex vivo* model of plasma separation, we demonstrate that CD11b expression was C5 dependent both for granulocytes and monocytes supporting previous published observations. Formation of granulocyte-platelet conjugates was to some extent C5 dependent. Monocyte-platelet conjugates, however, were C5 independent. These findings underscore the importance of the complement system in leukocyte activation during extra-corporeal treatments involving surface activation.

Expression of CD11b on leukocytes:

Our finding that CD11b expression on granulocytes is C5 dependent in our model of *ex vivo* plasma separation is supported by previous studies. Rinder et al showed that C5aR1 blockade significantly decreased CD11b upregulation on granulocytes and that anti-human C5 antibody blocks CD11b upregulation on granulocytes in an *ex vivo* model with simulated

extracorporeal circulation (18, 19), and it has also been demonstrated that blocking the C5aR1 in a model with PVC tubing counteracted the CD11b expression on granulocytes (7). Our group has previously demonstrated that CD11b expression on granulocytes in a *Neisseria meningitides* model using blood from a C5 deficient donor only occurred after reconstitution of C5, also indicating the importance of C5 in CD11b expression on granulocytes (14) and Bergseth et al showed that C5 deficiency decreased CD11b expression on granulocytes in a model with C5 deficient and C5 reconstituted blood in PVC tubes (6).

Our findings also indicate that CD11b expression on monocytes in the plasma separation samples is C5 dependent. This is also partly in accordance with former studies. Rinder et al found that anti C5a only trended towards blocking CD11b expression on monocytes (19). We have previously shown that CD11b expression on monocytes to some extent was dependent on C5 in a model using C5aR1 antagonist for blocking C5 effect on monocytes in a PVC tubing model (7), and Bergseth et al found that lack of C5 decreased monocyte CD11b expression in a PVC tubing model, using C5D deficient blood reconstituted with C5 (6). Rinder et al also demonstrated that CD11b expression on monocytes can be reduced by down regulating both classical and alternative C3/C5 convertases using the complement activation blocker (CAB-2; CD46-CD55 conjugate). Thus, blocking formation of C3 cleavage products points to C3a and probably other C3 fragments as additional candidates possibly able to facilitate upregulation of CD11b expression on monocytes (20). This indicates that biocompatibility between different materials cannot be readily compared, and that every material and model has to be evaluated separately. The main difference between our study and others is the presence of the plasma separation column and the blood and plasma flow rates. Since plasma separation involves shear stress and shear force, this could also affect CD11b expression.

Formation of leukocyte-platelet conjugates:

Formation of leukocyte-platelet conjugates is recognized as a component of inflammation in many circumstances, and conjugate formation can be induced by artificial surfaces and thus seen as a marker of bioincompatibility (21, 22). In an in vitro model of artificial surface induced inflammation using monoclonal antibodies and small peptides as complement inhibitors, we have previously shown that conjugate formation is mediated by activation of complement and the formation of C5a, which also upregulates CD11b on leukocytes (7).

In the present study, formation of granulocyte-platelet conjugates increased fairly equal in the C5DR-PS and C5D-PS samples until T15. From T15 to T30 there was a 2.5-fold increase in granulocyte-platelet conjugate formation in the C5DR-PS sample compared to the C5D-PS sample. In the CTR-PS sample there was a 4-fold increase in granulocyte-platelet conjugate formation. Other studies have shown increase in formation of granulocyte-platelet conjugates in models including PVC tubing and a membrane oxygenation device and at the same time also shown that granulocyte-platelet conjugate formation can be reduced either by blocking C5aR1 or by blocking cleavage of C5 (7, 19).

We found an increase in monocyte-platelet conjugate formation in the plasma separation samples in our study, and this increase appeared to be C5 independent. Previous studies have indicated C5 dependence to a certain degree in the formation of monocyte-platelet conjugate formation (6, 7, 19).

The difference in result from other studies regarding formation of leukocyte-platelet conjugates indicate that the plasma separation column and the blood bag or the model as a whole can mediate conjugate formation also trough mechanisms other than complement C5a generation and CD11b expression on leukocytes. Previous studies have pointed out the ability of shear stress and shear force in blood circulating circuits both *ex vivo* and *in vivo* to activate cellular components of the blood (23-25). Gutensohn et al described, in a model of

platelet apheresis, interaction between platelets and monocytes simultaneously as upregulation of P-selectin and CD63 was observed on platelets (26). At the same time activated platelets binding CD41a+ leukocytes forming conjugates was observed. Importantly, they noticed that most of the binding between the platelets and leukocytes happened during the first 5 min of circulation. This was also the case in our study with regard to the monocyte platelet conjugate formation in the plasma separation samples.

The plasma separation column used in the current study has been described to mediate complement activation, but not to activate cellular components of the blood (27). By measuring C3, C3a and C5a in models with different types of lipoprotein apheresis and in lone plasmapheresis models, other studies have also concluded that the plasmapheresis column activates complement (5, 28). Taken together, these findings indicate that shear stress and shear force also mediate conjugate formation trough mechanisms other than C5 activation.

Platelets and leukocytes:

Activation, adherence and clotting of platelets are recognized as bioincompatibility in artificial devices. Primary activation of platelets and secondary activation as a result of activation of the coagulation and immune systems have been discussed as possible mechanisms (2, 3). In our study there was a marked reduction in platelets in the plasma separation samples compared to the no-plasma separation samples, but the reduction was not C5 dependent. The observation of C5 independence is not in alignment with earlier studies stating that inhibiting C5 did preserve the platelet count in a cardiopulmonary bypass model (18). On the other hand it is clearly demonstrated that platelets bind vigorously to PVC used in the tubing in our model (29, 30). Shear stress and shear forces can activate platelets enhancing adhesion of platelets to biomaterials (31, 32). Platelet agglutination was also clearly shown in blood smear taken from the plasma separation samples at T240. This is

probably part of the explanation for the observed platelet reduction in the plasma separation samples in our study, despite the use of lepirudin as an anticoagulant. The plasma separation column used in our study is not yet known to interact with the platelets (27). The leukocyte count remained stable and unchanged in all samples in our study despite formation of conjugates and the change in platelet count in the plasma separation samples, indicating that leukocytes did not adhere to the plasma filtration column or the tubing.

Plasma proteins:

We observed a reduction in circulating plasma proteins (albumin, immunoglobulins, and C4) in the plasma separation samples independent of C5. When blood interacts with foreign material the first step in the bio-incompatibility cascade is that a layer of plasma proteins binds to the surface (33). These proteins, bound to the material, undergo conformational changes making the proteins able to activate inflammatory cascade and network systems (34). In the no-plasma separation samples there were no or only marginal changes in protein concentration, consistent with the different biomaterials in the blood bag and in the plasma separation system and the different mode of contact between blood and plasma circulating in the plasma separation column causing shear stress with increased binding of protein to the biomaterial. Thus testing of bio-incompatibility for any material should take place under the same conditions as the biomaterials are supposed to be used in clinical practice.

Complement activation:

Generation of C3bc is formed by C3 cleavage irrespective of which initial pathway(s) that are activated (35). A model for activation of complement on artificial surfaces has been suggested by several authors (2, 36, 37). Put together it is possible for both classical, lectin and alternative pathway to activate the amplification loop of complement and secondly lead to the generation of the terminal complement complex. In accordance with this, we observed an increase in C3bc in our study whereas the lack of C5 as expected prevented the generation of

TCC in the C5 deficient samples. When C5D blood was reconstituted with purified C5, we observed an enhanced activation reflected by increased TCC formation compared to the controls also in the no-plasma separation control sample. The most likely explanation for this is the fact that purified proteins may undergo changes in configuration enhancing their biological effects, in addition to the inherent risk of contamination. We have experienced this with the purified C5 in our laboratory (non-published observations) and it has been described that different forms of C5b have different potency in generating TCC (38). Our data indicate that the purified C5 might have increased capacity to generate TCC.

Conclusion

In an *ex vivo* model of plasma separation the upregulation of leukocyte CD11b was C5 dependent both on granulocytes and monocytes. The results also indicate a possible C5 dependency of granulocyte-platelet conjugate formation. Platelet count was reduced during plasma separation whereas the leukocyte count was unchanged. Further improving biocompatibility and reducing complement activation by materials used in routine plasma separation could prove to be of clinical benefit.

We acknowledge that the findings are not new as such and our results are based on a small sample size and conclusions should be drawn with care, however, previous studies in the field have used inhibitors of the complement system, such as purified or monoclonal antibodies or smaller peptides. Such experimental approaches always carry the risk of contamination and cross-reaction. To our knowledge, our study is the first using blood from a C5 deficient individual to investigate complement activation in a model with plasma separation, and in our opinion this model adds important information even if confirming previous findings. Furthermore, although C5 deficiency is extremely rare, the increasing clinical use of

inhibitors of C5 (e.g. eculizumab) emphasises the importance of studying mechanisms related to complement activation in more depth.

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Fig. 1

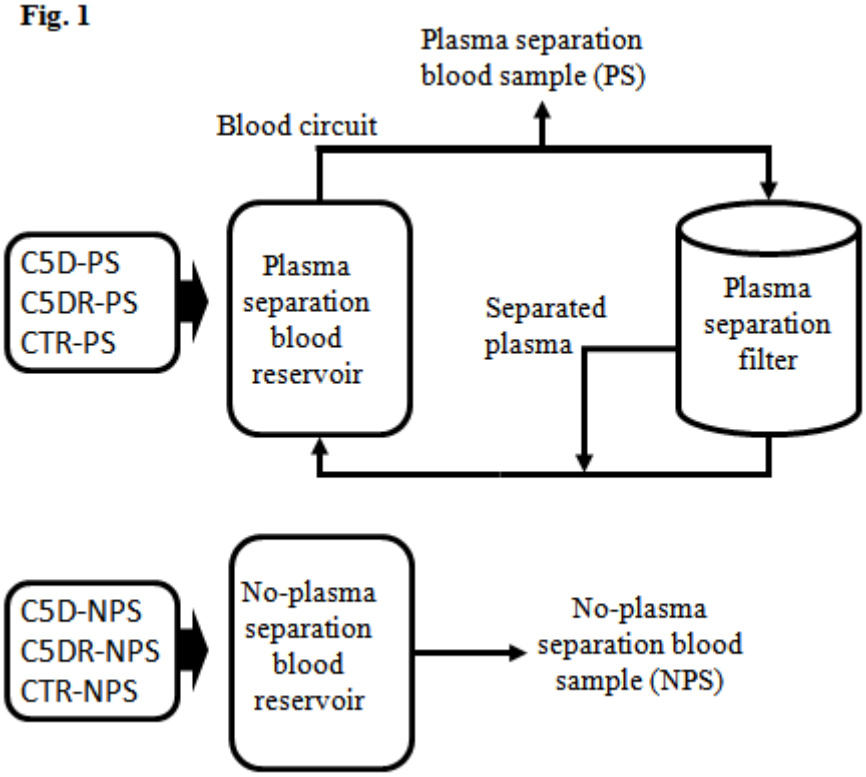

Fig. 1 Schematic drawing of the *ex vivo* model. The plasma separation blood bag served as the reservoir for the plasma separation circuit. Plasma separation blood samples were obtained from the tubing blood sample outlet after the plasma separation blood reservoir. The arrows show the direction of blood flow and plasma flow in the system. The no-plasma separation blood reservoir was kept at 37°C on the test tube rotator next to the plasma separation blood reservoir. No-plasma separation blood samples were drawn directly from the no-plasma separation blood reservoir.

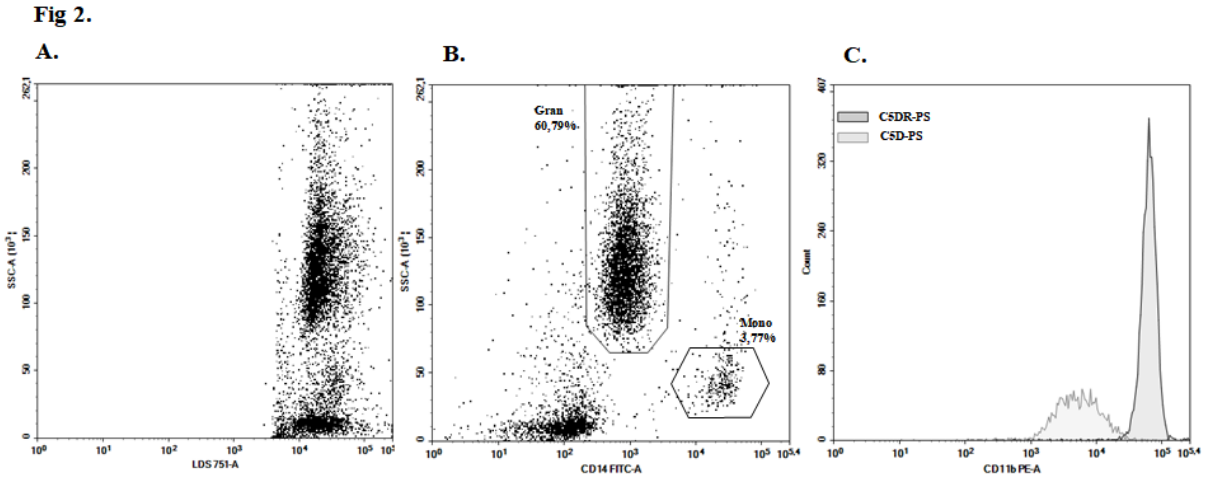

Fig 2 Flowcytometric readouts at 30 min of granulocyte and monocyte CD11b. During acquisition threshold was set on the nuclear stain LDS 751 (A). Granulocytes and monocytes were gated for in a CD14 FITC/SSC dotplot (B). Granulocyte CD11b expression in samples C5DR-PS and C5D-PS shown in a histogram (C).

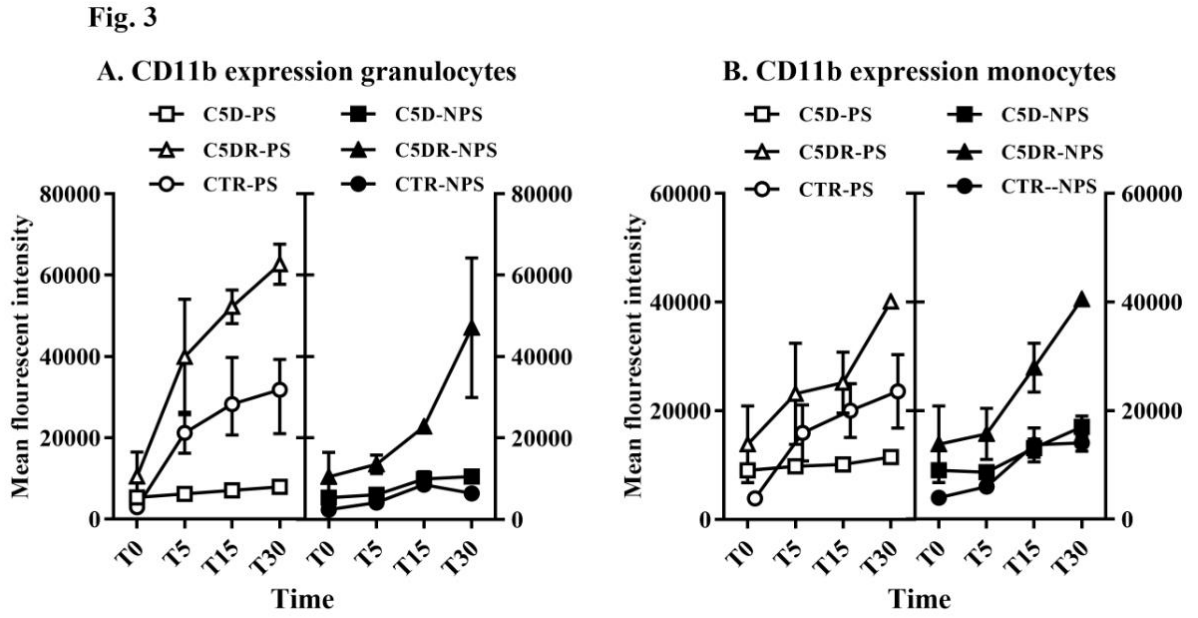

Fig. 3 CD11b expression. Expression of CD11b on granulocytes (A) and monocytes (B). CD11b expression from baseline (T0) through 30 minutes (T30) expressed as mean fluorescent intensity and standard deviation in the C5D, C5DR and CTR samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).

Fig. 4

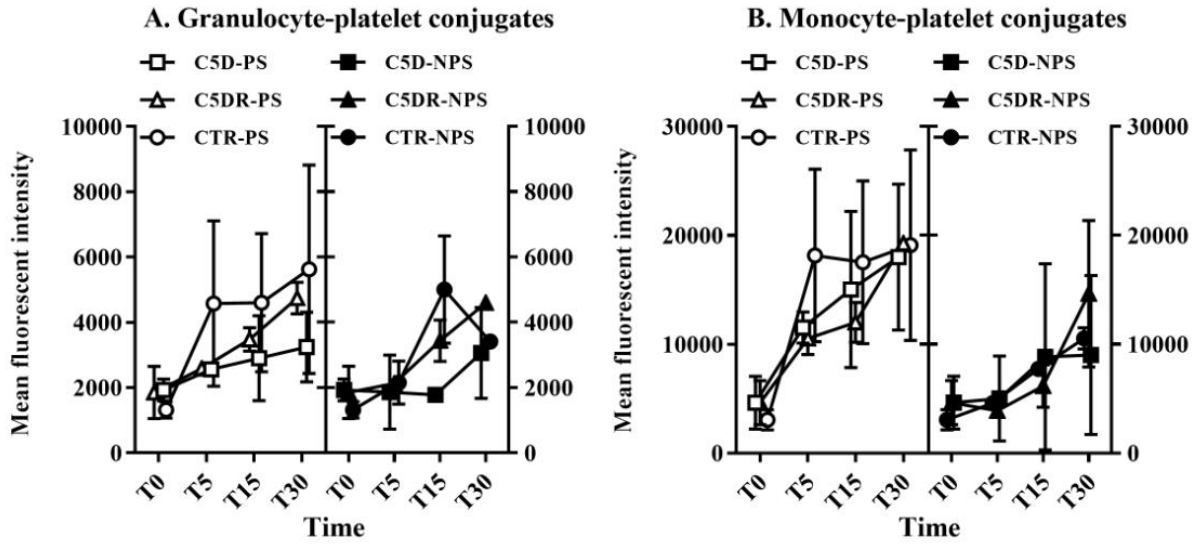

Fig. 4 Leukocyte-platelet conjugates. Granulocyte-platelet (A) and monocyte-platelet (B) conjugate formation from baseline (T0) trough 30 minutes (T30) expressed as mean fluorescent intensity and standard deviation in the C5D, C5DR and CTR samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).

Fig. 5

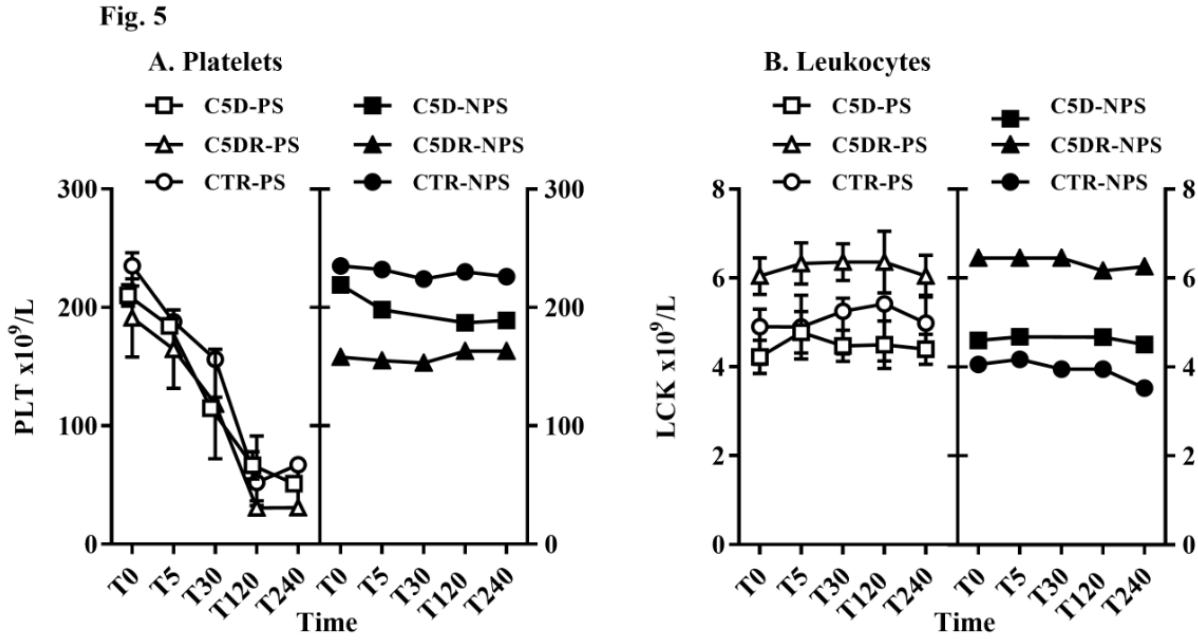

Fig. 5 Platelet and leukocyte counts. Platelet (A) and leukocyte (B) from baseline (T0) trough 240 minutes (T240) expressed as median and range in the C5D, C5DR and CTR

samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).

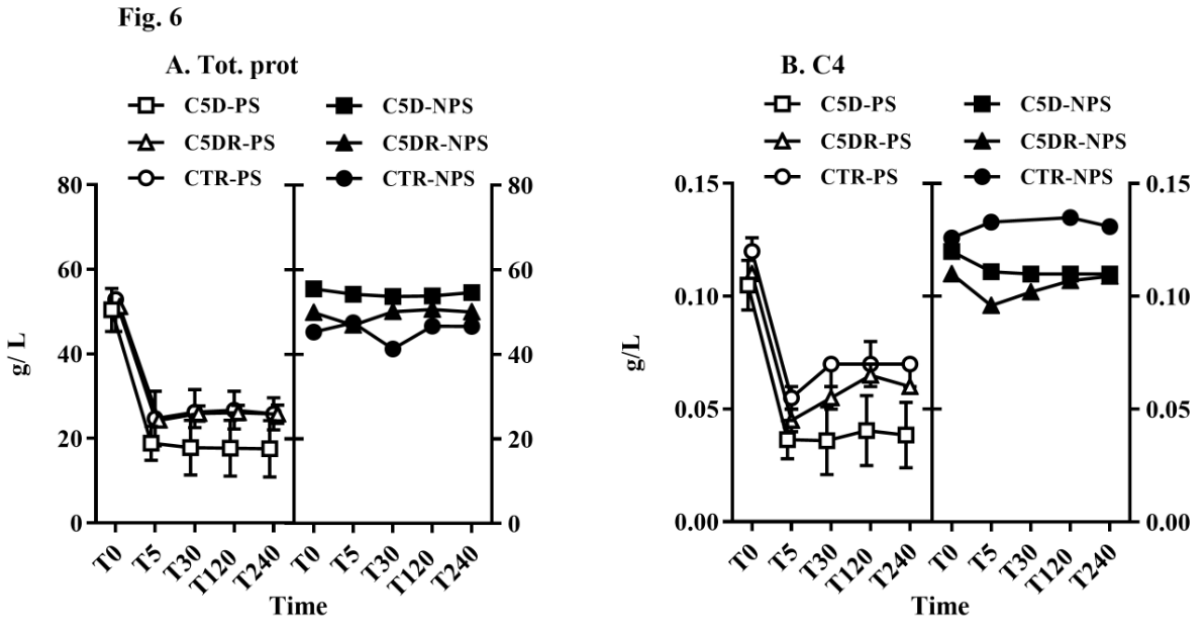

Fig. 6 Plasma protein concentrations. Total protein (A) and complement factor 4 (B) from baseline (T0) trough 240 minutes (T240) expressed as median and range in the C5D, C5DR and CTR samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).

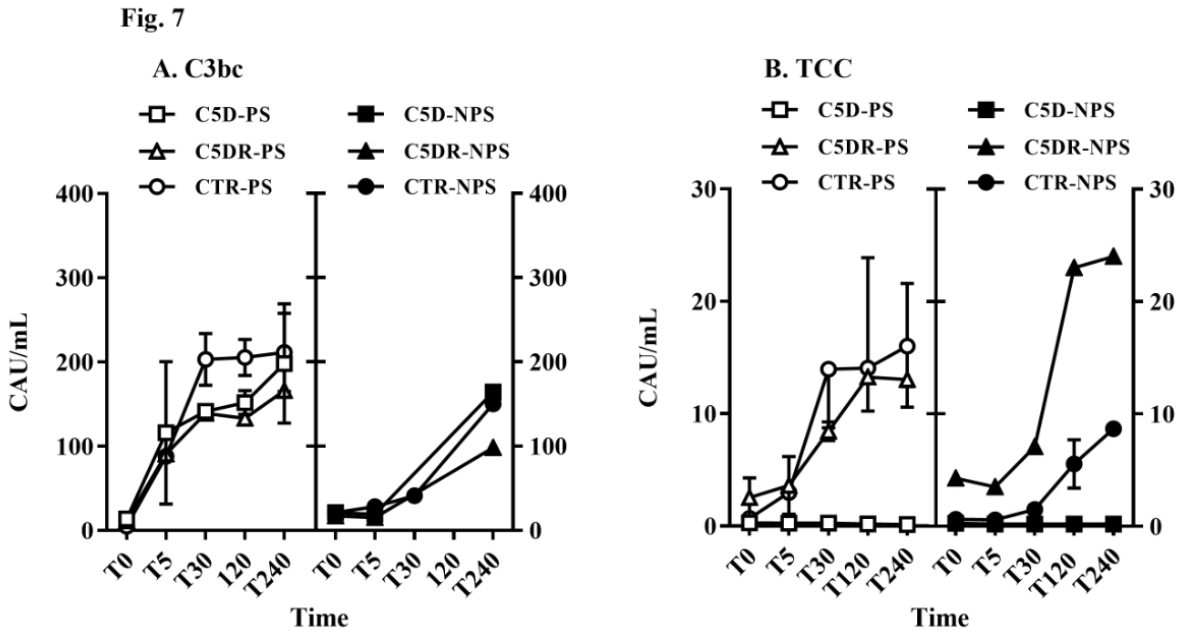

Fig. 7 Complement activation. Complement activation products C3bc (A) and TCC (B) from baseline (T0) trough 240 minutes (T240) expressed as median and range in the C5D, C5DR and CTR samples in the plasma separation blood samples (PS) and in the no-plasma separation blood samples (NPS).