Platelet number and function alterations in preclinical models of sterile inflammation and sepsis patients: implications in the pathophysiology and treatment of inflammation

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Platelet alterations in sepsis patients

Keywords: platelet transfusion, sepsis patient, TRALI, flow cytometry, platelet aggregation

ABSTRACT

Platelets are the blood cells in charge of maintaining the body haemostasis, recognising the damaged vessel wall, and providing the appropriate cellular surface for the coagulation cascade to act locally. Additionally, platelets are active immunomodulators. At the crossroads of haemostasis and inflammation, platelets may exert either beneficial actions or participate in pathological manifestations and have been associated to the prothrombotic nature of multi-organ failure in systemic inflammation. Platelet number alterations have been reported in sepsis, and platelet transfusions are given to thrombocytopenic patients. However, the risk to develop transfusion related acute lung injury (TRALI) is higher in sepsis patients. In this manuscript we show that platelets produced during inflammation in preclinical mouse models of sterile inflammation display lower aggregation capacity when stimulating certain receptors, while responses through other receptors remain intact, and we name them “inflammation-conditioned” platelets. In a cohort of sepsis patients, we observed, as previously reported, alterations in the number of platelets and platelet hyperreactivity. Furthermore, we identified a receptor-wise platelet aggregation response disbalance in these patients, although not similar to platelets from preclinical models of sterile inflammation. Interestingly, we generated evidence supporting the notion that platelet aggregation capacity disbalance was partially triggered by plasma components from sepsis patients. Our findings have implications in the indication of platelet transfusions in sepsis patients: Are fully functional platelets suitable for transfusion in sepsis patients? Current Clinical Trials (RESCUE) will answer whether platelet production stimulation with thrombopoietin receptor agonists (TPO-RAs) could be a substitute of platelet transfusions.

INTRODUCTION

Platelets are known as the blood cells responsible for maintaining the body haemostasis; they form blood clots (thrombi) at the site of endothelial damage, preventing bleeding.[1] They have a life-span of 5-9 days and around 10^{11} platelets are produced every day by an average healthy adult. In mice, the life-span is 3-5 days, and production is around 10^{10} platelets/day. Haemostatic platelet function is highly dependent on the synergistic action of a number of receptors and can be summarised in three different steps: recognition of the damaged vessel wall, platelet activation (including granule release and shape conformation changes) and thrombus formation.[2] When the endothelial layer is injured, collagen and vWF, amongst other molecules, are exposed. Collagen and vWF are recognised by platelet $\alpha 2\beta 1$/GPVI and vWF receptor respectively, and this leads to a cascade of synergistic events inducing platelet granule release and shape conformational changes, and the activation of $\alpha IIb\beta 3$ integrin (fibrinogen/fibrin receptor) on platelets, which is absolutely necessary for thrombus formation.[2] These events assure the formation of the blood plug and endothelium repair, through the interaction with the endothelium and other cells recruited such as leukocytes, fibroblasts and platelets themselves.[3]

However, there is emerging acceptance that platelets play other roles than mere haemostasis.[4] Platelets have inflammatory functions and influence both innate and adaptive immune responses and it has been shown that they play essential opposite roles in cancer.[5-8] Furthermore, recent identification of the podoplanin receptor in platelets (Clec2) has led to the discovery of the crucial role of platelets in various physiological processes through this receptor.[9, 10]

Platelets are formed from precursor megakaryocytes derived from hematopoietic stem cells, whose commitment to the megakaryocytic lineage is stimulated by rising plasma levels of the hormone thrombopoietin (TPO).[1, 11] However, the exact mechanism how this occurs has been a matter of debate, and platelet production has not yet been successfully mimicked *in vitro*.[12, 13] In fact, how other humoral factors affect megakaryopoiesis is not yet completely understood, since cMpl (TPO receptor) or TPO null mice still produce low levels of platelets, so do patients with deleterious mutations in cMpl.[14-16]

Platelet apheresis products are used for transfusion to thrombocytopenic patients who are at risk of bleeding or that have developed bleeding episodes. One of the major populations receiving platelet transfusions are sepsis patients, in which as a consequence of their condition and/or treatment the platelet counts drop severely. A serious concern considering sepsis patients is the prevalent high risk of transfusion related acute lung injury (TRALI, 1 in 5000), which in 50% of the cases becomes fatal, making it the leading cause of death after transfusion therapy.[17, 18] There

is emerging evidence that platelets and neutrophils are major contributors to TRALI.[19, 20] In a mouse model of TRALI that is neutrophil- and platelet-dependent, targeting platelet activation with either aspirin or αIIbβ3 integrin inhibitor decreased lung injury, proving the direct role of platelets in the development of TRALI.[20] While it seems that targeting platelet function or their interaction with neutrophils sounds as a promising solution against TRALI,[19] αIIbβ3 integrin inhibition would also compromise the haemostatic function of platelets and would therefore not be suitable as a treatment, when sepsis patients suffer from bleeding.

In an effort to gain knowledge on platelet function in the context of subjacent inflammation, we characterised the platelet aggregation capacity upon stimulation with a battery of agonists in murine preclinical models of acute or chronic inflammation and in a cohort of sepsis patients. Our findings have implications in the indication of platelet transfusions in sepsis patients, which we discuss below.

MATERIAL AND METHODS

Mice

Mice were maintained at the Netherlands Cancer Institute (NKI) or the Academic Medical Center (AMC) in Amsterdam under specific pathogen-free conditions. Animal experiments were approved by the NKI or AMC Animal Ethics Committee, respectively. The acute sterile inflammation model was induced by injecting intravenously 5 µg of LPS (Sigma-Aldrich) in 100 µL of sterile PBS in wild-type C57BL/6J mice. Control C57BL/6J mice were injected with the same volume of sterile PBS. Blood samples were obtained 24 hr and 5 days after injection. The chronic sterile inflammation model, *i.e.* CD70Tg mice, were generated on a C57BL/6J background, and maintained heterozygously as previously described.[21, 22] These mice bear a murine *CD70* transgene under the *CD19* promoter; wild-type non-transgenic littermates were used as controls. Blood samples were obtained from adult mice (approximately 12-weeks old).

Mouse blood processing

Blood was drawn by heart or cheek puncture and collected in heparin-coated vials. Blood parameters were determined on a scil Vet abc Plus+ instrument (scil animal care company GmbH).

Mouse flow cytometry based platelet aggregation assay (FCA)

FCA was performed as described.[23] In short, CD9-APC and CD9-PE (Abcam antibodies) labelled platelets were mixed 1:1 in the presence of 2% wildtype mouse plasma. As agonists, we used 100 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich); 30 nM aggretin A, 10µg/mL collagen (Horm; Nycomed Arzneimittel GmbH) and 10µg/mL Botrocetin (Sigma-Aldrich). Time-series samples fixed in 0.5% formaldehyde/PBS were measured on an LSRII + HTS flow cytometer and analysed for double-coloured events by FACSDiva Version 6.1 software (BD Biosciences).

Human Patients and Healthy Donor cohorts

Peripheral blood samples were collected from hospitalized sepsis patients and healthy donors after informed consent according to the guidelines of the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of the Hospital Clínico San Carlos (Madrid, Spain). All patients included in the study were managed at the Dept. of Emergencies of the Hospital Clínico San Carlos. A total of 35 sepsis patients were recruited (18 men and 17 women; age 86.71 ±

0.90 years [average ± standard error of the mean]). As controls, 27 healthy donors were recruited for the study. Of these, 11 were age-matched controls (2 men and 9 women; age 86.18 ± 1.70 years [average ± standard error of the mean]), and 8 were AB0 group-matched controls. The study was done in the period 2015-2017.

Blood Sample Processing

Blood samples were collected in EDTA or citrate tubes. On the day of collection, the complete blood counts (CBC) were measured on a Beckman Coulter haemato-counter. After that, platelet rich plasma (PRP) was obtained by differential centrifugation and platelets were counted again in the haemato-counter. The PRP was subject to differential centrifugation, in order to separate platelets from plasma. In order to discard remaining cells/debri from the plasma fraction, it was centrifuged an extra round at 10 krpm 1 min, transferred to a clean tube, and kept frozen at -80ºC until further use. The platelets were resuspended in HEPES buffer (132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM HEPES, pH 7.4) previously set to RT on the concentration required for flow cytometry-based platelet aggregation (FCA) as described.[23]

Platelet Surface Marker Expression Analysis by Flow Cytometry

The following antibodies were used for flow cytometry analysis: CD9-FITC, CD61-FITC, CLEC2-APC, GPVI-PE, CD42B-PerCP, CD49B-FITC or CD49B-PE. All antibodies and reagents were from BD, and were used in a 1:200 dilution, except CD49B (1:100) and CLEC2 (1:10).

Antibody cocktails were prepared in 1% human serum albumin (HSA) in PBS. 100 μl of antibody cocktail was used per staining (using a million platelets per panel/incubation). Incubations were done at RT for 10 min, after which 100 μl of 1% formaldehyde (FA) was added to fix the stained platelets prior measurement on a FACSCanto-II flow cytometer. Data was analysed with FlowJo Version 10.8.1 software (Tree Star, Inc).

Human flow cytometry based platelet aggregation assay (FCA)

The flow cytometry based platelet aggregation assay (FCA) was performed as previously described.[21] In brief, platelets previously incubated with CD31-APC or CD31-APCCy7 (BD) were mixed in a 1:1 proportion in the presence of 20% plasma. When studying the aggregation capacity of sepsis platelets, reactions were performed in the presence of AB+ plasma. When studying the effect of plasma from sepsis patients on platelet function, AB0-matched platelets from healthy donors were mixed with 20% of matching sepsis patient plasma. Agonists used were 100 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich); 300 nM aggretin A, 30μg/mL collagen

type-I (Horm; Nycomed Arzneimittel GmbH), 0.5 ng/ml convulxin (Enzo) and 0.5 mg/ml Ristocetin (Sigma-Aldrich). Analysis was performed on a FACSCanto-II (BD Biosciences), and data analysed using FlowJo version 10.8.1.

Statistical Analysis

All data was analysed using Excel, and 2-tailed Student´s t-tests were applied in order to identify significant differences. Data was considered significant when $p < 0.05$, as indicated.

RESULTS

Platelet specific receptor shut down responses in murine preclinical models of sterile inflammation

We examined platelet aggregation capacity in murine preclinical mouse models of sterile inflammation. On one hand, we induced an acute sterile inflammation response on wildtype mice by injecting intravenously LPS, as described. Following LPS injection, the platelet count drops and, by day 5, the platelet counts are restored (Figure 1A). This suggests that by day 5, these mice have in circulation platelets that have been produced under systemic inflammatory signals, which we hypothesize would have qualitative differences compared to platelets that are produced in the steady-state.

We performed flow cytometry based aggregation (FCA) assays on platelets from day 5 LPS- and mock-treated mice, with a battery of agonists, as described. FCA allows the study of platelet aggregation capacity, in a receptor-wise manner, as it measures the first steps of (micro) aggregation. In short, PMA triggers αIIbβ3-mediated aggregation; aggretin A triggers CLEC2-mediated aggregation (also dependent on a functional αIIbβ3 integrin); collagen triggers α2β1/GPVI-mediated aggregation and botrocetin triggers vWF receptor-mediated agglutination. As shown in Figure 1B, the platelet aggregation responses upon stimulation with aggretin A and collagen, were severely impaired in LPS-treated mice as compared to mock-treated or wildtype mice, while responses to botrocetin or PMA were intact.

Next, we set out to study whether this distinct aggregation capacity profile was present in platelets from CD70Tg mice, which represent a preclinical model of chronic sterile inflammation.[22] CD70Tg mice express constitutively CD70 antigen on B cells, resulting on T-cell activation and increased interferon gamma (Ifn-γ). These mice have been extensively analysed and display persistent severe thrombocytopenia (Figure 1A).[21] Our FCA assays show that platelets from CD70Tg mice display a more acute platelet aggregation response disbalance, where PMA-induced aggregation is affected in addition to aggretin A and collagen-mediated responses.

Overall, these results suggest that, in preclinical models of sterile inflammation, the platelet Clec2 and collagen receptors are specifically “shut-down”, while the vWF receptor function remains largely intact when aggregation is induced with specific agonists. Only in chronic sterile inflammation, the function of the fibrinogen receptor (αIIbβ3 integrin) is also compromised. We could not identify significant changes in the expression of any of the receptors of study (data not shown). This suggests that platelets are differently tuned on situations of acute or chronic inflammation, and we name them tentatively as “inflammation-conditioned” platelets.

Sepsis Patients: complete blood counts and stratification based on platelet counts

We next set out to explore platelets in a cohort of sepsis patients. In Figure 2, selected variables from complete blood counts are shown. As previously reviewed,[24] the platelet counts in sepsis patients are variable, and in our cohort, approximately 10% of patients displayed thrombocytopenia (counts < 100 x 10^9/L), and 20% of patients displayed elevated platelet counts (> 250 x 10^9/L). We have to consider that our cohort of sepsis patients encompasses aged patients, and values from age-matched controls are shown on each graph. Interestingly, the white blood cell counts (specially the neutrophils), followed the same dynamics as the platelet counts.

Surface marker expression in sepsis patients

When looking at the expression of relevant surface molecules expressed on platelets in the total sepsis patient cohort, we could not identify significant alterations, although there was a tendency for reduced expression of CD9, GPVI and CD42B (subunit of the vWF receptor) (Figure 3). Interestingly, when plotting the mean expression stratifying the sepsis patient cohort based on platelet counts, we observed different patterns. Surface proteins that reduced their expression level in a negative correlation manner with platelet counts, *i.e.* CD9, CD61 and CD49B; surface proteins that reduced their expression in a positive correlation manner with platelet counts, *i.e.* GPVI, CD42B, and CLEC2, although this receptor displays reduced expression also when the platelet counts are elevated.

Platelet aggregation capacity in sepsis patients: in the presence of healthy donor plasma

We next set out to study the function of platelets in sepsis patients by using FCA, as described. We preferred to study the responses of isolated platelets first, in the absence of own plasma, as previous studies had been performed on platelets within whole blood or platelet rich plasma fraction.[25, 26] All reactions were set in the presence of AB^+ plasma from healthy donors, as a natural source of fibrinogen. In human, we included convulxin as a specific GPVI ligand, and ristocetin, that triggers vWF receptor activation. Results show no significant alteration of platelet responses (per agonist) in the global sepsis cohort (Figure 4). A tendency to increased aggregation responses upon collagen stimulation was observed. When plotting the mean FCA values stratifying the sepsis patient cohort based on platelet counts, we observed different patterns. Responses upon PMA stimulation were largely intact, while a reduced response in at least one of the stratification groups was observed with aggretin A, convulxin and ristocetin. We

observed a hyperactivation status in sepsis platelets (spontaneous aggregation in unstimulated platelets), which was significantly elevated in thrombocytopenic patients.

Effect of plasma from sepsis patients on the aggregation capacity of platelets from healthy donors

We next performed FCA with plasma from sepsis patients and platelets from AB0-group matched healthy donors. As shown in Figure 5, there was a tendency to reduced responses upon stimulation with PMA and ristocetin. Furthermore, the ristocetin triggered responses appeared to follow a negative correlation pattern with platelet counts. Interestingly, the same dynamics were observed to be induced by sepsis plasma on the spontaneous aggregation of healthy donor platelets without stimulus.

DISCUSSION

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection.[27] The host response to infections conjugates two important arms: the immune and the haemostatic systems. Platelets, as members of the haemostatic system, are active immunomodulators, and their role in sepsis has been largely acknowledged.[25, 28-30] However, as a double edge sword, they may exert beneficial or deleterious actions in the pathophysiology of sepsis.[31-33] As an extra level, alterations in platelet counts are often observed in sepsis patients, and when thrombocytopenia is observed, prophylactic platelet transfusions may be indicated to prevent bleeding. However, the risk of transfusion related adverse events is higher in sepsis patients.[17]

In this manuscript we provide proof that platelets display a tuned functional capacity under inflammatory conditions, in mouse and human. Results showed a more constant reproducible pattern on platelets from mouse models of sterile inflammation (acute or chronic), suggesting a specific “shut-down” of the so-called non-haemostatic or immune receptors CLEC2 and GPVI.[34] These results led us to hypothesize that “inflammation-conditioned” platelets are produced with a specific receptor function profile to avoid an exacerbated inflammation response that could represent danger to the body’s well-being. How megakaryocytes sense inflammation to produce “inflammation-conditioned” platelets is unknown, but there are a number of studies which support the notion.[35-38] In line with this assumption, we think that the high risk of TRALI in sepsis patients is due to the fact that transfused platelets are derived from healthy donors, and therefore fully functional, representing a danger to the septic patient. Interestingly, podoplanin, the Clec2 natural ligand, has been reported as a marker for lung injury,[39] and “inflammation-conditioned” platelets have “shut-down” this receptor.

From our studies in a cohort of sepsis patients, a disbalance of the platelet aggregation capacity was evidenced, although the variability of patterns was higher than the ones found previously on the murine preclinical models of sterile inflammation. Platelet dysfunction in sepsis patients was previously documented by others.[25, 26, 28, 30] One consideration is the limited size of our cohort, and on the other hand, inbred mice are far more homogeneous than randomly recruited patients, which are also prescribed with different medication plans or are managed with different treatment regimes. Interestingly, we provide results that support the notion that, in addition to the different platelet production under inflammation, platelets within a sepsis/infectious milieu are also “primed” in the circulation. When performing aggregation assays of AB0-group matched platelets

from healthy donors with the plasma from the matched sepsis patient, platelet hyperactivation was induced (there was spontaneous aggregation without stimulus), and a tendency to reduced responses towards the agonists used was observed.

Understanding the molecular mechanisms governing megakaryocyte differentiation during inflammation and dissecting better the functional differences of the “inflammation-conditioned” platelets, and how the inflammation milieu affects them in human opens a new path for improvement of platelet transfusion management, but also of platelet function modulation. This is relevant specifically for septic patients at risk of fatal TRALI, but also for other target populations of platelet transfusions that are at risk of thrombosis (*i.e.* chemotherapy patients), or even to ease the prothrombotic nature of viral infections, as has been put forward with the current COVID-19 pandemic.[40, 41]

Platelet transfusion, in septic patients is still a matter of debate. However, some relevant Clinical Trials have been withdrawn as COVID-19 trials gained priority. Such is the case of INFUSE and PlaTiSep, aimed at defining whether the platelet transfusions are advantageous or to find whether the platelet count transfusion threshold (high or low) associates with lower mortality rate or shorter hospitalizations in the sepsis thrombocytopenic patient. However, our findings and those from other groups might add another variable into the management of platelet transfusion in the septic patient – “Does a septic patient require or admit a transfusion of fully functional platelets? Interestingly, other Clinical Trials are currently progressing in elucidating whether platelet transfusions could be replaced by TPO-RA treatment (RESCUE Trial). Clinical and translational research will advance on the knowledge and will certainly improve the management of sepsis patients and other ICU hospitalized patients requiring platelet transfusions. Our studies would logically continue to cover other situations where platelet transfusion success is a concern, or where platelets could be used therapeutically (*i.e.* immunotherapy against cancer or autoimmune [42] diseases).

Acknowledgements

This study was partially supported by an RYC fellowship (RYC-2013-12587, Ministerio de Economía y Competitividad, Spain) and I+D Excelencia 2017 and 2020 project grants (SAF2017-85489-P and PID2020-117265GB-I00, Ministerio de Ciencia e Innovación -Spain- and Fondos Feder) to L.G and a Severo Ochoa Grant (PA-20-PF-BP19-014, Consejería de Ciencia, Innovación y Universidades del Principado de Asturias) to PMB. JAE was financially supported by the Interdisciplinary Center for Clinical Research of the University of Münster (IZKF grant: Ebl4/009/21). We thank Paloma Cerezo, Almudena Payero, María de la Poveda Colomo, Nuria Urquijo, María del Carmen Ovejero, Ángela Hernández, María Isabel Granados and Ana María Fernández for technical support. We are especially thankful to Dr. Jerard Seghatchian, who over the years has collaborated with us, feeding us with crucial questions in the field and, hence, inspiring research projects.

Author Contributions

LG conceived and designed the study. MVF and MM performed experiments and analysed results. MCYP, JGC and FJMS managed and followed the patients. AAH and PMB analysed data and revised the manuscript. MN provided the CD70Tg mice and revised the manuscript. CBC gave flow cytometry assistance. JAE provided reagents and revised the manuscript.

Declaration of interest

The authors declare they have no conflict of interest.

References

[1] Machlus KR, Italiano JE, Jr. The incredible journey: From megakaryocyte development to platelet formation. J Cell Biol. 2013;201:785-96.

[2] Jackson SP, Nesbitt WS, Kulkarni S. Signaling events underlying thrombus formation. J Thromb Haemost. 2003;1:1602-12.

[3] Laurens N, Koolwijk P, de Maat MP. Fibrin structure and wound healing. J Thromb Haemost. 2006;4:932-9.

[4] Leslie M. Cell biology. Beyond clotting: the powers of platelets. Science. 2010;328:562-4.

[5] Feng W, Madajka M, Kerr BA, Mahabeleshwar GH, Whiteheart SW, Byzova TV. A novel role for platelet secretion in angiogenesis: mediating bone marrow-derived cell mobilization and homing. Blood. 2011;117:3893-902.

[6] Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. Nat Rev Cancer. 2011;11:123-34.

[7] Jenne CN, Urrutia R, Kubes P. Platelets: bridging hemostasis, inflammation, and immunity. Int J Lab Hematol. 2013;35:254-61.

[8] Semple JW, Italiano JE, Jr., Freedman J. Platelets and the immune continuum. Nat Rev Immunol. 2011;11:264-74.

[9] Navarro-Nunez L, Langan SA, Nash GB, Watson SP. The physiological and pathophysiological roles of platelet CLEC-2. Thromb Haemost. 2013;109:991-8.

[10] Uhrin P, Zaujec J, Breuss JM, Olcaydu D, Chrenek P, Stockinger H, et al. Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation. Blood. 2010;115:3997-4005.

[11] Miyazaki H. Physiologic role of TPO in thrombopoiesis. Stem Cells. 1996;14 Suppl 1:133-8.

[12] Martinez-Botia P, Acebes-Huerta A, Seghatchian J, Gutierrez L. On the Quest for In Vitro Platelet Production by Re-Tailoring the Concepts of Megakaryocyte Differentiation. Medicina (Kaunas). 2020;56.

[13] Martinez-Botia P, Acebes-Huerta A, Seghatchian J, Gutierrez L. In vitro platelet production for transfusion purposes: Where are we now? Transfus Apher Sci. 2020;59:102864.

[14] de Sauvage FJ, Carver-Moore K, Luoh SM, Ryan A, Dowd M, Eaton DL, et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. J Exp Med. 1996;183:651-6.

[15] Geddis AE. Congenital amegakaryocytic thrombocytopenia. Pediatr Blood Cancer. 2011;57:199-203.

[16] Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient mice. Science. 1994;265:1445-7.

[17] Shaz BH, Stowell SR, Hillyer CD. Transfusion-related acute lung injury: from bedside to bench and back. Blood. 2011;117:1463-71.

[18] Vlaar AP, Straat M, Juffermans NP. The relation between aged blood products and onset of transfusion-related acute lung injury. A review of pre-clinical data. Clin Lab. 2011;57:267-72.

[19] Caudrillier A, Looney MR. Platelet-neutrophil interactions as a target for prevention and treatment of transfusion-related acute lung injury. Curr Pharm Des. 2012;18:3260-6.

[20] Looney MR, Nguyen JX, Hu Y, Van Ziffle JA, Lowell CA, Matthay MA. Platelet depletion and aspirin treatment protect mice in a two-event model of transfusion-related acute lung injury. J Clin Invest. 2009;119:3450-61.

[21] Libregts SF, Gutierrez L, de Bruin AM, Wensveen FM, Papadopoulos P, van Ijcken W, et al. Chronic IFN-gamma production in mice induces anemia by reducing erythrocyte life span and inhibiting erythropoiesis through an IRF-1/PU.1 axis. Blood. 2011;118:2578-88.

[22] Arens R, Tesselaar K, Baars PA, van Schijndel GM, Hendriks J, Pals ST, et al. Constitutive CD27/CD70 interaction induces expansion of effector-type T cells and results in IFNgamma-mediated B cell depletion. Immunity. 2001;15:801-12.

[23] De Cuyper IM, Meinders M, van de Vijver E, de Korte D, Porcelijn L, de Haas M, et al. A novel flow cytometry-based platelet aggregation assay. Blood. 2013;121:e70-80.

[24] Assinger A, Schrottmaier WC, Salzmann M, Rayes J. Platelets in Sepsis: An Update on Experimental Models and Clinical Data. Front Immunol. 2019;10:1687.

[25] Yaguchi A, Lobo FL, Vincent JL, Pradier O. Platelet function in sepsis. J Thromb Haemost. 2004;2:2096-102.

[26] Woth G, Varga A, Ghosh S, Krupp M, Kiss T, Bogar L, et al. Platelet aggregation in severe sepsis. J Thromb Thrombolysis. 2011;31:6-12.

[27] Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016;315:801-10.

[28] Levi M. Platelets in sepsis. Hematology. 2005;10 Suppl 1:129-31.

[29] Sjovall F, Hansson MJ, Elmer E. Platelet mitochondrial function in sepsis. Crit Care Med. 2012;40:357; author reply -8.

[30] Vincent JL, Yagushi A, Pradier O. Platelet function in sepsis. Crit Care Med. 2002;30:S313-7.

[31] Greco E, Lupia E, Bosco O, Vizio B, Montrucchio G. Platelets and Multi-Organ Failure in Sepsis. Int J Mol Sci. 2017;18.

[32] Guo L, Shen S, Rowley JW, Tolley ND, Jia W, Manne BK, et al. Platelet MHC class I mediates CD8+ T-cell suppression during sepsis. Blood. 2021;138:401-16.

[33] Lundahl TH, Petersson J, Fagerberg IH, Berg S, Lindahl TL. Impaired platelet function correlates with multi-organ dysfunction. A study of patients with sepsis. Platelets. 1998;9:223-5.

[34] Rayes J, Watson SP, Nieswandt B. Functional significance of the platelet immune receptors GPVI and CLEC-2. J Clin Invest. 2019;129:12-23.

[35] Couldwell G, Machlus KR. Modulation of megakaryopoiesis and platelet production during inflammation. Thromb Res. 2019;179:114-20.

[36] Freishtat RJ, Natale J, Benton AS, Cohen J, Sharron M, Wiles AA, et al. Sepsis alters the megakaryocyte-platelet transcriptional axis resulting in granzyme B-mediated lymphotoxicity. Am J Respir Crit Care Med. 2009;179:467-73.

[37] French SL, Butov KR, Allaeys I, Canas J, Morad G, Davenport P, et al. Platelet-derived extracellular vesicles infiltrate and modify the bone marrow during inflammation. Blood Adv. 2020;4:3011-23.

[38] Schwertz H, Rowley JW, Portier I, Middleton EA, Tolley ND, Campbell RA, et al. Human platelets display dysregulated sepsis-associated autophagy, induced by altered LC3 protein-protein interaction of the Vici-protein EPG5. Autophagy. 2021:1-17.

[39] McElroy MC, Pittet JF, Hashimoto S, Allen L, Wiener-Kronish JP, Dobbs LG. A type I cell-specific protein is a biochemical marker of epithelial injury in a rat model of pneumonia. Am J Physiol. 1995;268:L181-6.

[40] Martinez-Botia P, Bernardo A, Acebes-Huerta A, Caro A, Leoz B, Martinez-Carballeira D, et al. Clinical Management of Hypertension, Inflammation and Thrombosis in Hospitalized COVID-19 Patients: Impact on Survival and Concerns. J Clin Med. 2021;10.

[41] Campbell RA, Hisada Y, Denorme F, Grover SP, Bouck EG, Middleton EA, et al. Comparison of the coagulopathies associated with COVID-19 and sepsis. Res Pract Thromb Haemost. 2021;5:e12525.

[42] Habets KL, Huizinga TW, Toes RE. Platelets and autoimmunity. Eur J Clin Invest. 2013;43:746-57.

Figure Legends:

Figure 1: Thrombocytopenia and platelet dysfunction in mouse models of sterile inflammation

A) Platelet (PLT) counts in the mouse model of acute sterile inflammation 24 hr and 5 days after LPS or PBS injection (left), and CD70Tg mice (right), as model of chronic sterile inflammation (wildtype -WT- sibling mice were used as controls). The thrombocytopenia induced upon LPS injection disappears by day 5 in the acute sterile inflammation mouse model, while CD70Tg mice display severe and persistent thrombocytopenia. **B)** Flow cytometry based platelet aggregation assays (FCA) were done with platelets from mouse models of sterile inflammation. The platelet aggregation responses upon stimulation with PMA, aggretin A (AggA), collagen and botrocetin were measured. The area under the curve of each time-series was calculated, setting respective WT responses to 100. A specific inability to respond to AggA and collagen stimulation was observed in platelets from acute sterile inflammation mice (day 5, LPS), while CD70Tg platelets also display a defect upon PMA stimulation. Botrocetin responses were not affected in platelets from either mouse model. Mean and standard error of the mean (SEM) are represented. Statistical significance is indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. N ≥ 3 in each group, except when individual values are depicted (CD70g Botrocetin FCA).

Figure 2: Complete blood count variables in the cohort of sepsis patients

The platelet (PLT), white blood cells (WBC), neutrophil and lymphocyte counts are represented. Mean and standard error of the mean (SEM) are represented, in the following groups: day controls, age-matched controls and sepsis patients. Sepsis patients were further stratified based on PLT counts: low < 100 x 10^9/L; normal 100-250 x 10^9/L; high > 250 x 10^9/L. The WBC counts (specially the neutrophil counts) follow the same trend as the PLT counts. Statistical significance is indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Bar Graph represents the distribution (percentage) of sepsis patients based on PLT counts. N-sepsis = 35; N-day controls = 8; N-aged-matched controls = 9.

Figure 3: Platelet immunophenotyping in the cohort of sepsis patients

The expression of typical platelet receptors was analysed by flow cytometry. The Mean Fluorescence Intensity (MFI) is represented, after setting the mean MFI of each surface marker to 100 in the group of Day controls. Alterations in surface marker expression were observed,

some of them associating with the peripheral PLT count. The graph on the bottom represents the mean MFIs of some of the studied surface markers comparing Day and Aged-matched controls. Mean and standard error of the mean (SEM) are represented. Statistical significance is indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. N-sepsis ≥ 20; N-day controls ≥ 4; N-aged-matched controls = 8.

Figure 4: Flow cytometry based platelet aggregation assays (FCA) of platelets from sepsis patients in the presence of healthy donor plasma

Flow cytometry based platelet aggregation assays (FCA) were done with platelets from sepsis patients in the presence of AB^+ plasma from healthy donors. The platelet aggregation responses upon stimulation with PMA, aggretin A (AggA), collagen, convulxin and ristocetin were measured. The area under the curve of each time-series was calculated. Generally, no significant differences were observed in the platelet aggregation capacities, except for a disbalance response tendency upon collagen (hyper) and ristocetin (hypo). Stratification of sepsis patients based on PLT counts, added more complexity to the functional platelet disbalance in sepsis. PMA responses were not affected in platelets from sepsis patients, in either PLT-count based group. The percentage of aggregation in the absence of stimuli is also shown. Mean and standard error of the mean (SEM) are represented. Statistical significance is indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. N-sepsis ≥ 20; N-day controls ≥ 4; N-aged-matched controls = 8.

Figure 5: Flow cytometry based platelet aggregation assays (FCA) of healthy donor platelets in the presence of plasma from sepsis patients

Flow cytometry based platelet aggregation assays (FCA) were done with platelets from platelets from AB0-matched healthy donors in the presence of plasma from sepsis patients. The platelet aggregation responses upon stimulation with PMA and ristocetin were measured. The area under the curve of each time-series was calculated. Generally, a tendency to inhibited responses was observed, which did not reach significance. The percentage of aggregation in the absence of stimuli is also shown, which mimics what was observed on platelets from sepsis patients. Mean and standard error of the mean (SEM) are represented. Statistical significance is indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. N-sepsis = 32; N-AB^+ controls = 8.

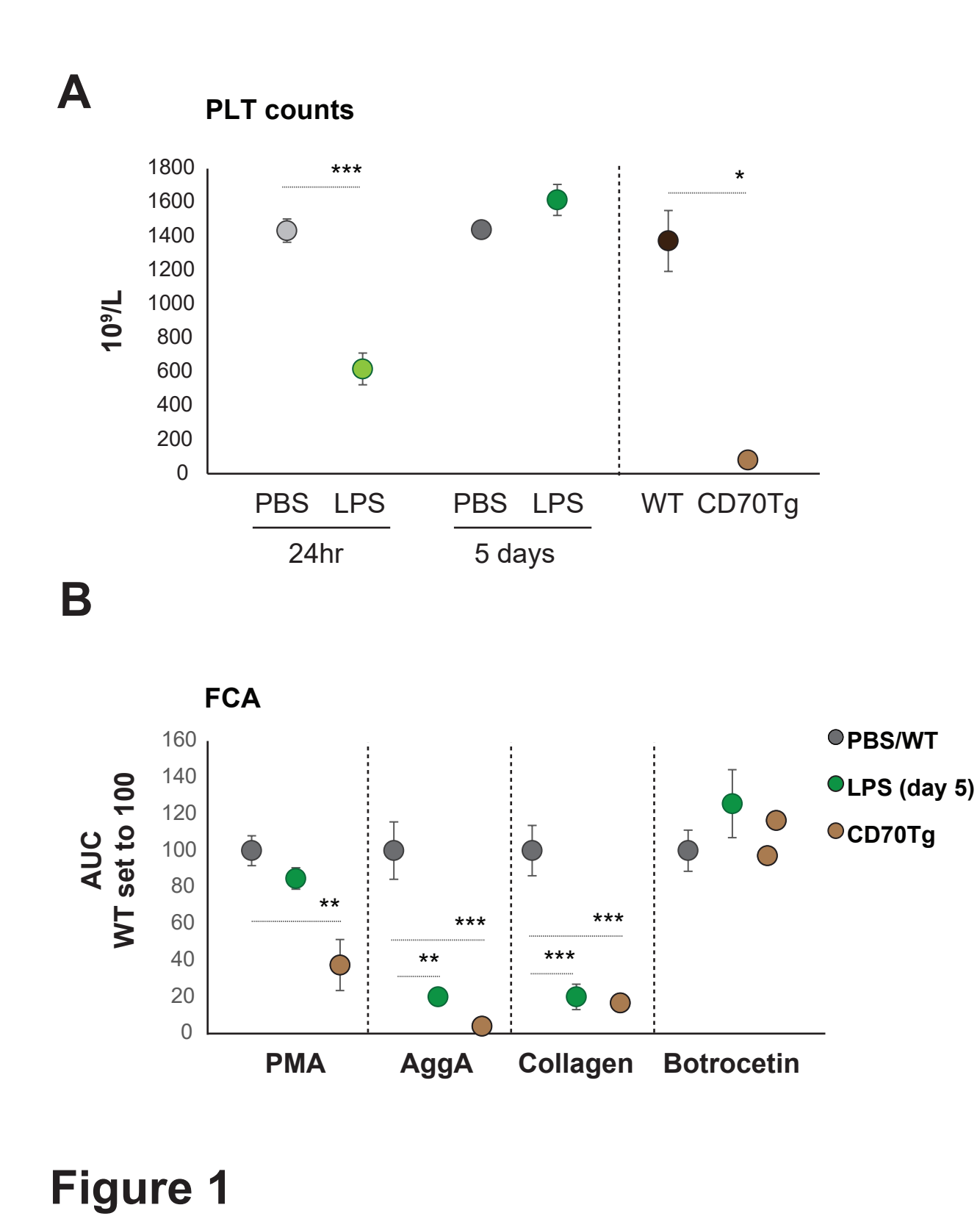
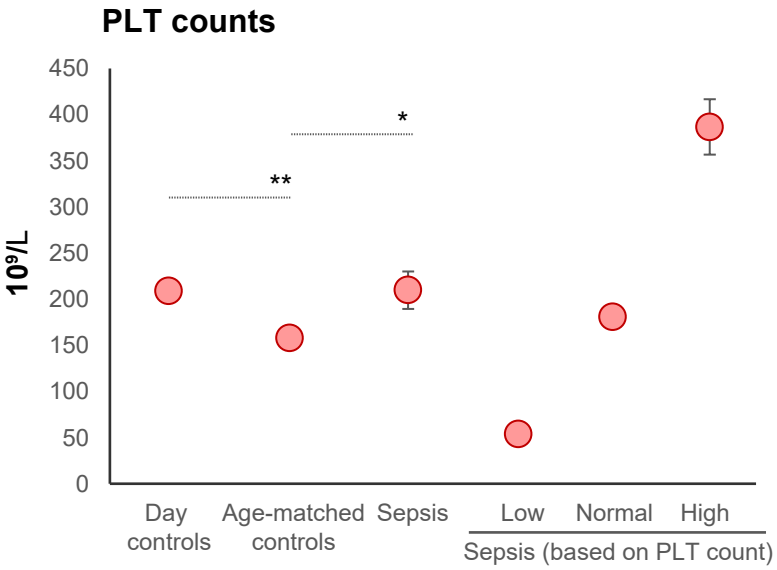
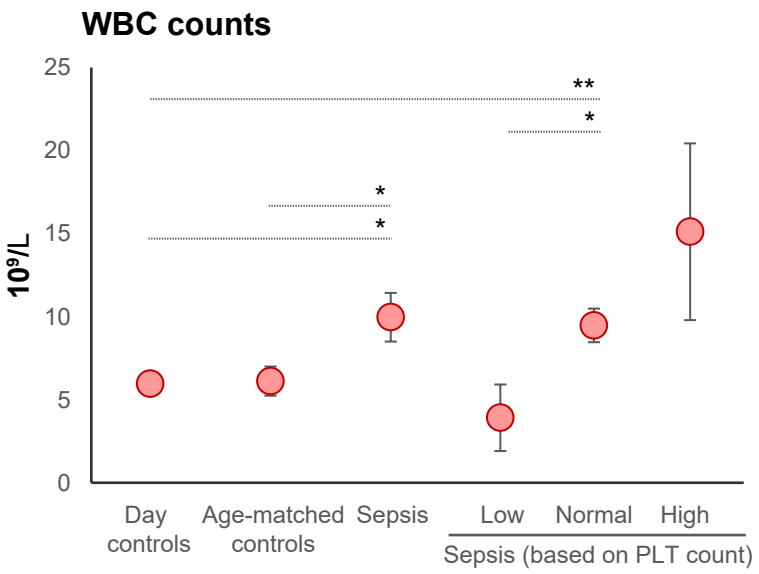
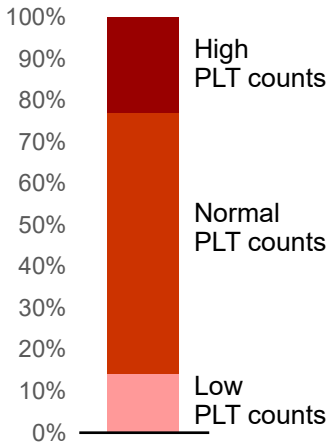
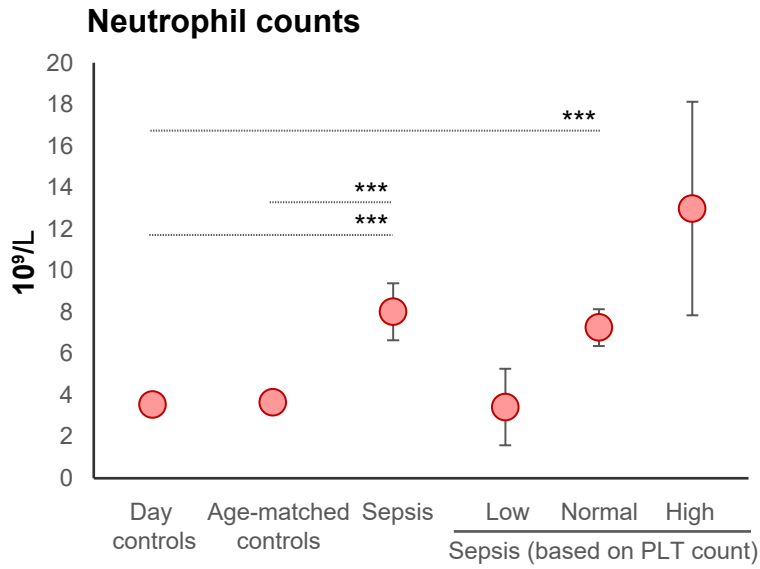

A
PLT counts
1800
1600
1400
1200
1000
800
600
400
200
0
10⁹/L
***
*
PBS
LPS
PBS
LPS
WT
CD70Tg
24hr
5 days
B
FCA
160
140
120
100
80
60
40
20
0
AUC
WT set to 100
**
***
**
***
***
PBS/WT
LPS (day 5)
CD70Tg
PMA
AggA
Collagen
Botrocetin

Figure 1


PLT counts
10⁹/L
450
400
350
300
250
200
150
100
50
0
**
*
Day controls
Age-matched controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)


WBC counts
10⁹/L
25
20
15
10
5
0
**
*
*
*
Day controls
Age-matched controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
High PLT counts
Normal PLT counts
Low PLT counts


Neutrophil counts
10⁹/L
20
18
16
14
12
10
8
6
4
2
0
***
***
***
Day controls
Age-matched controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)

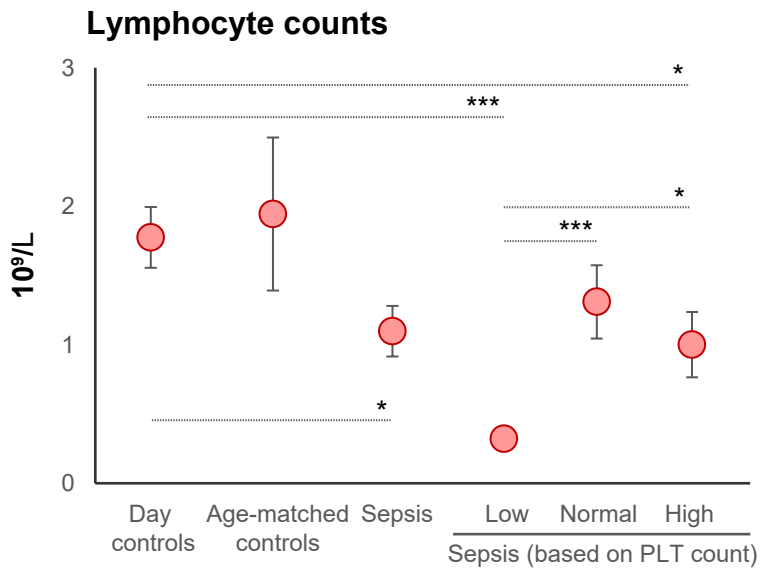

Lymphocyte counts
10⁹/L
3
2
1
0
*
***
*
***
*
Day controls
Age-matched controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)

Figure 2

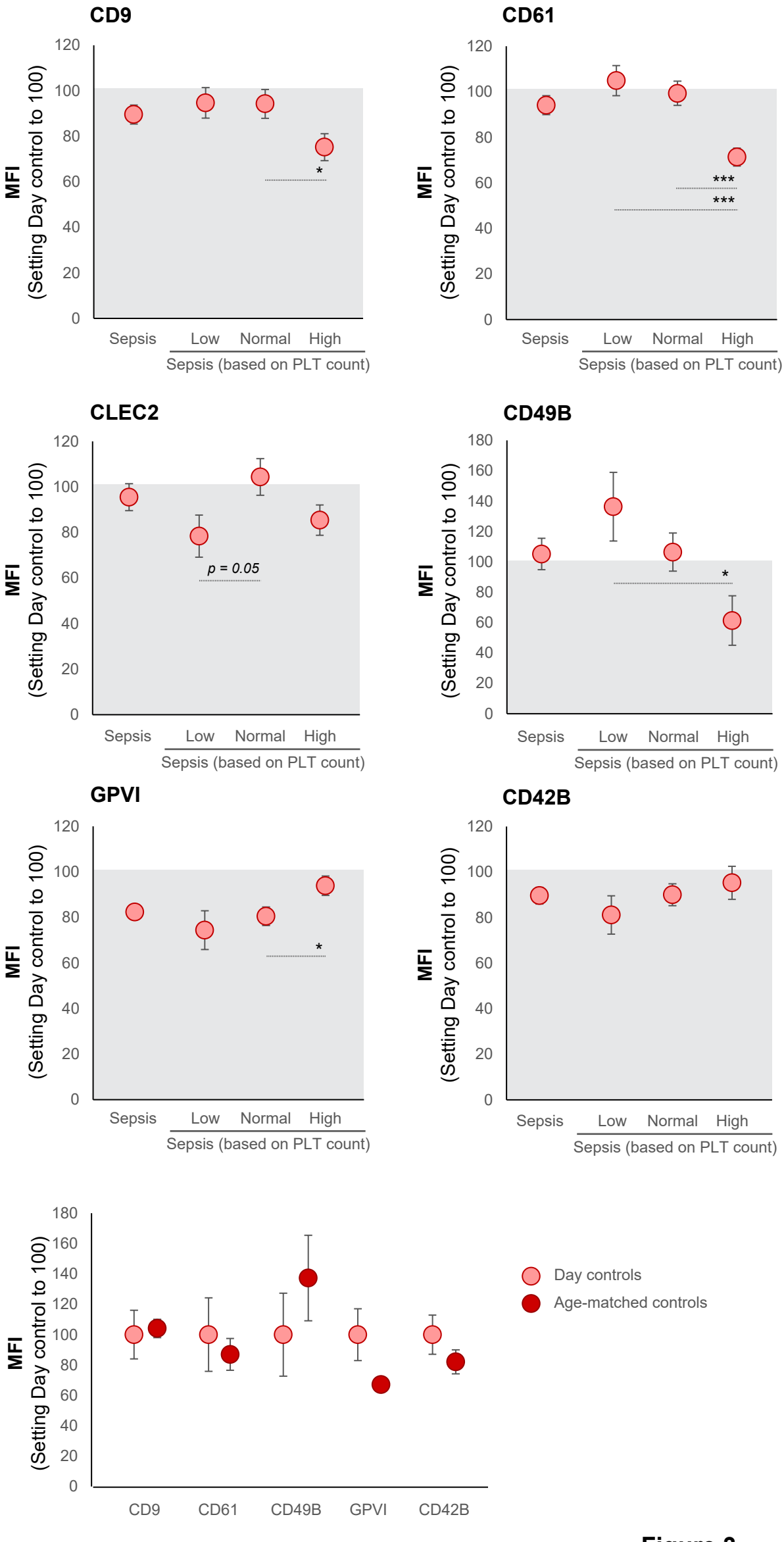

CD9
CD61
CLEC2
CD49B
GPVI
CD42B
MFI
(Setting Day control to 100)
Sepsis
Low
Normal
High
Sepsis (based on PLT count)
*
***
p = 0.05
CD9
CD61
CD49B
GPVI
CD42B
Day controls
Age-matched controls

Figure 3

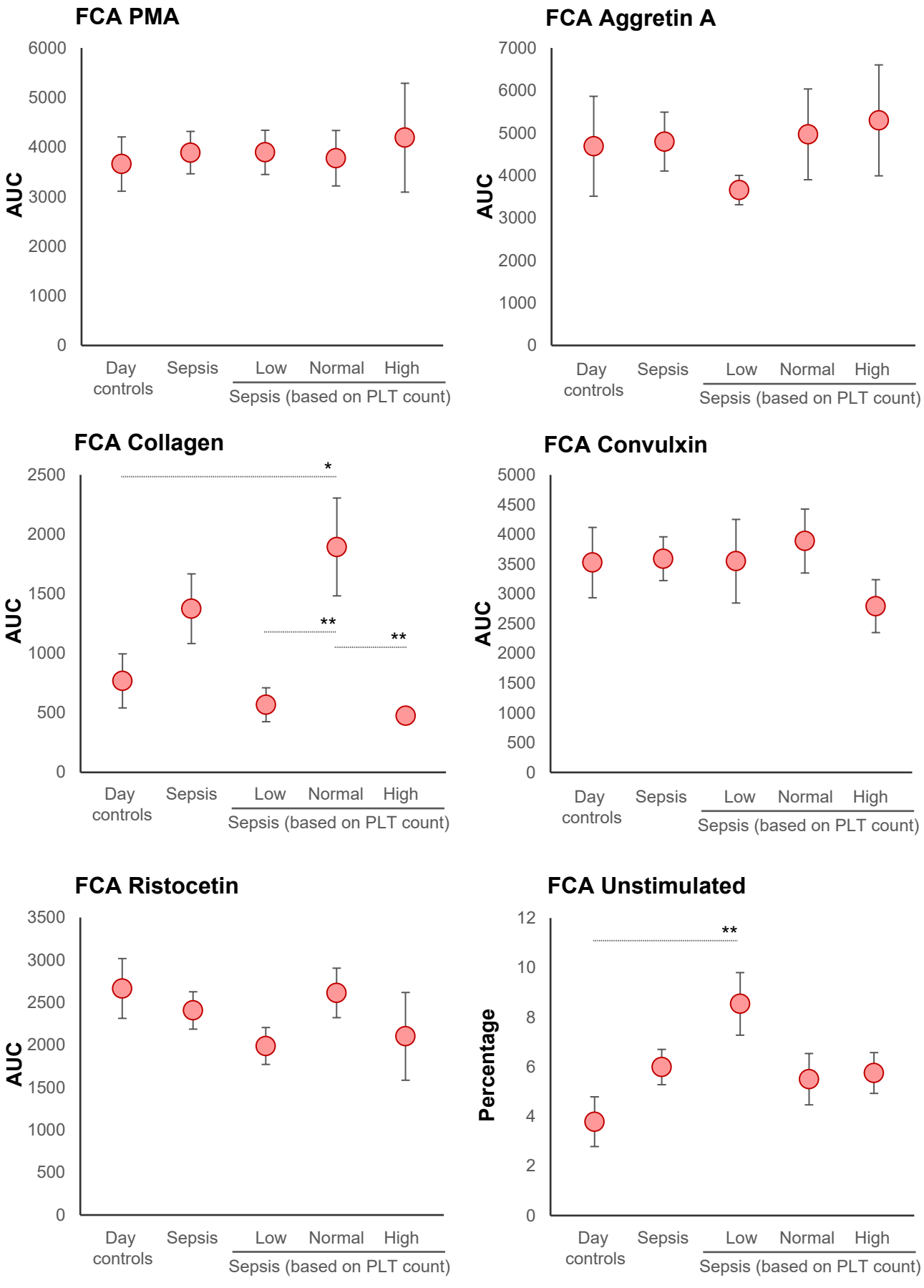
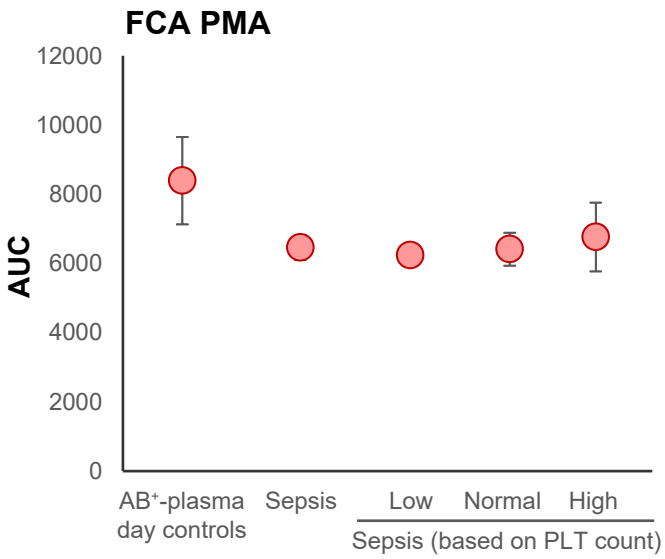
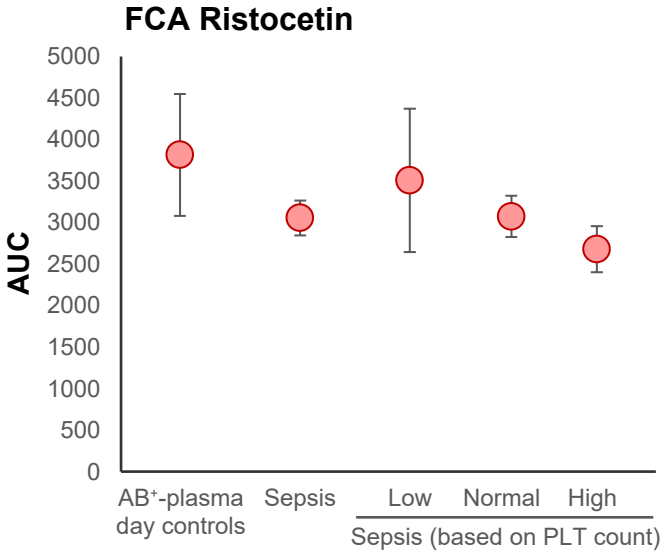

FCA PMA
AUC
6000
5000
4000
3000
2000
1000
0
Day controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)
FCA Aggretin A
AUC
7000
6000
5000
4000
3000
2000
1000
0
Day controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)
FCA Collagen
AUC
2500
2000
1500
1000
500
0
*
**
**
Day controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)
FCA Convulxin
AUC
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Day controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)
FCA Ristocetin
AUC
3500
3000
2500
2000
1500
1000
500
0
Day controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)
FCA Unstimulated
Percentage
12
10
8
6
4
2
0
**
Day controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)

Figure 4


FCA PMA
AUC
12000
10000
8000
6000
4000
2000
0
AB+-plasma day controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)


FCA Ristocetin
AUC
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
AB+-plasma day controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)

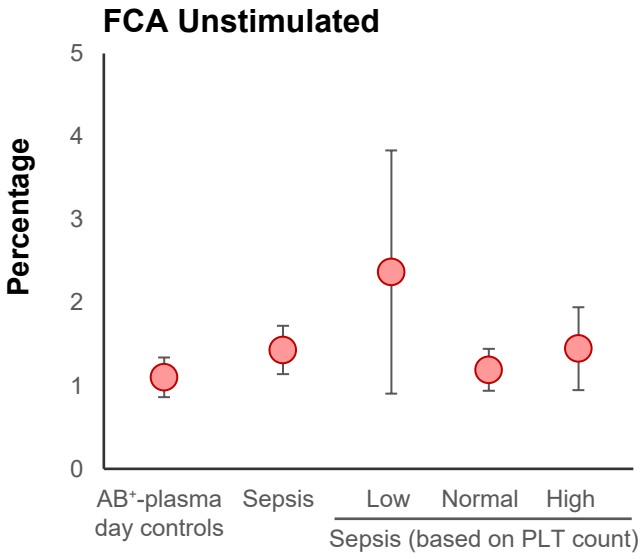

FCA Unstimulated
Percentage
5
4
3
2
1
0
AB+-plasma day controls
Sepsis
Low
Normal
High
Sepsis (based on PLT count)

Figure 5