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Artificial shear stress effects on leukocytes at a biomaterial surface

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Abstract

Medical devices, such as ventricular assist devices (VADs), introduce both foreign materials and artificial shear stress to the circulatory system. The effects these have on leukocytes and the immune response are not well understood. Understanding how these two elements combine to affect leukocytes may reveal why some patients are susceptible to recurrent device-related infections and provide insight into the development of pump thrombosis.

Biomaterials - DLC: diamond-like carbon coated stainless steel; Sap: single-crystal sapphire; and Ti: titanium alloy (Ti6Al4V) were attached to the parallel plates of a rheometer. Whole human blood was left between the two discs for 5 min at +37°C with or without the application of shear stress ($0s^{-1}$ or $1000s^{-1}$). Blood was removed and used for: complete blood cell counts; flow cytometry (leukocyte activation; cell death; microparticle generation; phagocytic ability; and reactive oxygen species (ROS) production); and the production of pro-inflammatory cytokines.

L-selectin expression on monocytes was decreased when blood was exposed to the biomaterials both with and without shear. Applying shear stress to blood on a Sap and Ti surface led to activation of neutrophils shown as decreased L-selectin expression. Sap and Ti blunted the LPS-stimulated macrophage migration inhibitory factor (MIF) production, most notably when sheared on Ti.

The biomaterials used here have been shown to activate leukocytes in a static environment. The introduction of shear appears to exacerbate this activation. Interestingly, a widely accepted biocompatible material (Ti) utilised in many different types of devices, has the capacity for immune cell activation and inhibition of MIF secretion when combined with shear stress. These findings contribute to our understanding of the contribution of biomaterials and shear stress to recurrent infections and vulnerability to sepsis in some VAD patients as well as pump thrombosis.

Key Words

Introduction

The use of ventricular assist devices (VADs) as a therapy for end-stage heart failure patients ineligible for transplant has grown over recent decades (1, 2). There have been many improvements during this time with devices becoming smaller, their use more broadly acceptable, and with fewer device-related complications (3). Although the most recent device in clinical trials, HeartMate 3 (HM3, Abbott Laboratories, IL, USA), has successfully tackled pump thrombosis, other complications such as infection, ischaemic stroke, and gastrointestinal bleeding remain (4). Infection rates have been deemed lowered in second generation pumps with approximately 7% of HeartMate II (HMII; Abbott Laboratories) and HVAD (HeartWare Inc., Framingham, MA, USA) patients being affected (5, 6). However, recent data from HeartMate 3 patients has shown an increased rate of 22-36% (3, 7).

VAD-related complications are thought to be the result of non-physiological shear stress patterns causing activation and damage of blood cells and proteins (8). Red blood cells (RBCs) can tolerate relatively high shear stress in comparison to other blood cell types with haemolysis occurring due to deformation and fragmentation around $200 - 300$ Pa (9, 10). Flow conditions within VADs disrupt natural haemodynamics through briefly exerting high shear stresses (10-800 Pa) on platelets (11, 12). Platelets exposed briefly to high shear stresses become sensitised to lower shear stress causing rapid activation of platelets and predisposition to thrombus formation (13).

In comparison to RBCs and platelets, the effects of shear stress and VADs on leukocytes are largely overlooked but are equally important being linked to both infection and thrombus formation. *In vitro* studies using a concentric cylinder viscometer on whole human blood found a significant effect on white blood cell viability, count, and enzyme release at shear stresses of $10 - 60$ Pa, much lower value than is critical for red blood cell

damage (14, 15). Leukocyte morphology and function also changes under shear stress. Isolated neutrophils seeded on cardiovascular device materials then exposed to shear stresses above 0.6 Pa become compact, irregularly shaped, and incapable of interacting with bacteria (16). Another study using micropipettes to apply fluid stress identified an immediate retraction of neutrophil pseudopodia upon the application of a shear stress of ~ 0.04 Pa and neutrophil granule velocity was enhanced significantly by shear stress (0.1 Pa) (17). These observations suggest that shear stress has multiple effects on leukocytes including mediating water entry into the cell and the breakdown of F-actin (17) to cause cell swelling/lysis (14). Such damaging effects serve to reduce the normal function of leukocytes to protect the body from invading pathogens through impaired phagocytic ability and decreased cell proliferation.

Clinically, VAD implantation has been associated with a decline in peripheral blood leukocyte counts and an increase in activation marker macrophage antigen-1 (MAC-1) expression on granulocytes (18). Higher levels of apoptosis in $CD4^+$ and $CD8^+$ T cells and a defective proliferative response to pathogens have also been noted in VAD recipients (19-22) which could contribute to recurrent infections. A reduction in the expression of human leukocyte antigen-D related (HLA-DR) on monocytes has also been observed in VAD recipients and suggested to be a marker of mortality (23). VADs are associated with increased formation of microparticles from platelets, leukocytes, and endothelial cells (24). MPs are potentially related to VAD-related thrombosis and infection through their pro-thrombotic and pro-inflammatory characteristics (25). *In vitro* testing of the CentriMag centrifugal pump using bovine blood showed a significant increase in the number of leukocyte-derived microparticles (LMPs) with minimal impact on the levels of plasma free haemoglobin (26) which are generated by high shear stress (24, 27). Subtypes of LMPs that emerge in the CentriMag with ovine blood differ with

levels of shear applied to the blood (28) – evidence that the mechanism of shear-induced damage may not be identical in all leukocyte types.

Another factor in VAD-complications are the choice of biomaterial. The biomaterial surface of such devices adsorb proteins onto the surface immediately upon contact with blood. Cell activation and adhesion by this matrix is dependent on composition and can lead to formation of a thrombus composed of platelets, fibrin, leukocytes, and red blood cells (29, 30). The biomaterials used here have been investigated previously by us (29) and showed monocyte activation.

The aim here was to examine the combined effects of shear stress and VAD-relevant biomaterials on human blood leukocytes using our previously developed a whole blood model (29). Here, this model is developed further to enable the combined effects of shear stress and biomaterials to be studied using a rheometer.

Materials and Methods

Blood collection

Peripheral blood was collected from healthy adult volunteers into lithium heparin (18 I.U. lithium heparin salt per mL; Vacutainer tubes; Greiner Bio-One, Stonehouse, UK). All blood samples were collected with informed written consent. This study was approved by Wales Research Ethics Committee 6 (13/WA/0190).

Biomaterial discs

Biomaterial discs were prepared for use and surface area and surface roughness were measured as per previous study (29). The order of roughness from highest to lowest was DLC (Ra = 0.774 μm) > Ti (Ra = 0.099 μm) > Sap (Ra = 0.044 μm).

Rheometry

Calibration

An ARG2 rheometer (TA Instruments, New Castle, DE, USA) was used to subject samples to a controlled shear stress. The rheometer was calibrated by measuring the instrument inertia, followed by the inertia and friction of the chosen top geometry, a 60 mm aluminium parallel plate with attached biomaterial disc. Attachment of the biomaterial discs required heating the Peltier to +80°C and applying Crystal Bond Adhesive (Agar Scientific, Stanstead, UK) with a melting temperature of +66°C. One of the two biomaterial discs was laid onto the centre of the pre-heated and glue covered Peltier. The other disc was placed on top of the first, heat conduction permitted the application of glue and the top plate geometry was then lowered onto it. Adjustments were made so that the two discs were parallel with one another and then the Peltier temperature was brought down to +25°C. The top plate was lowered down within 1 cm of the bottom Peltier plate and 'zero gap' was established through automated normal force calibrations. A final calibration for rotational mapping was completed (standard, 2 iterations). The temperature was increased to +37°C for the experiment. Calibration of the plate geometry with attached disc was performed between samples to ensure the rheometer was balanced and inducing the correct level of shear was performed as above.

Shearing whole blood between parallel plates and biomaterials

Using the TRIOS software (TA Instruments) a procedure was set up for the use of the 60 mm parallel plate geometry with the environment set to +37°C. The gap between top geometry plate and bottom Peltier plate was set to 400 µm which would allow approximately 1.2 mL of blood to be pipetted onto the surface. The soak time was set to 30 secs, the duration set to 300 secs, and the shear rate used was either 0 s^{-1} or 1000 s^{-1}. The blood was added and then the top plate was brought down to the 'loading gap' ready

for initiation of the procedure. After shearing, as much blood as possible was removed from the surface yielding ~ 500 µL for analysis. Blood was left between the parallel biomaterials without shear to observe the effect of the biomaterial alone and pipetted into a tube after 5 min. After cleaning, another 1.2 mL blood was sheared at 1000 s^{-1} to look at the effect both biomaterial and shear have in combination. This shear rate is representative of the wall shear rates found in regions of centrifugal VADs such as along the outer wall and areas of the secondary flow path (31).

Haematology

Total and subset cell counts were measured using an automated haematology analyser (CELL-DYN Ruby; Abbott Diagnostics, Berkshire, UK). Baseline sample descriptive statistics are available in Supplementary Table 1.

Flow Cytometry

Neutrophil and monocyte activation

Whole blood (20 µL) was stained with 110 ng/µL CD15-VioGreen (clone VIMC6, IgM, Miltenyi Biotec Ltd., Surrey, UK); 500 ng/µL CD14-Pacific Blue (clone M5E2, IgG2a, BioLegend, London, UK); 25 ng/µL CD62L-PE (clone DREG-56, IgG1κ, eBioscience); and 100 ng/µL CD11b-APC (clone CBRMI/5, IgG1κ, eBioscience). Samples were vortexed and incubated on ice in the dark for 30 min. Red blood cells were lysed using 600 µL EasyLyse (Dako). Samples were vortexed and incubated at room temperature in the dark for 15 min. Finally, 1 µL DRAQ7, final concentration of 20 µM (BioStatus, Leicester, UK), was added to detect dead cells (32). Whole blood stimulated with 10 ng/mL LPS for 4 h at +37°C, 5% CO_2-in-air was used as a positive control and unstained samples were used for gating purposes. Blood exposed to 1% Triton-X 100 (Fisher Scientific) was used to determine DRAQ7 positive cell gates. AbC beads (Life

Technologies, Paisley, UK) were stained singly with each antibody and used for compensation. Within 2 hours of staining, samples were acquired on the Navios flow cytometer (Three lasers (violet: 405 nm, blue: 488 nm, red: 638 nm and the standard filter configuration as supplied by the manufacturers, Beckman Coulter) using linear forward scatter (FSC) versus side scatter (SSC) scale, flow rate set to high, and stop gate on 10,000 CD15$^+$ events which would allow for acquisition of approximately 10,000 total lymphocytes and 1,000 monocytes. Compensation and data analysis were performed using Kaluza 1.5a (Beckman Coulter).

Leukocyte microparticles

20 µl blood samples were stained with 0.2 µg CD45-PE, clone 1.11.32, mouse IgG1 (Thermo Fisher Scientific, Gloucester, UK) for 30 min on ice in the dark. The red blood cells were lysed by adding 600 µl EasyLyse (1:20 with dH$_2$O, DAKO, Alere, Cheshire, UK), followed by vortexing and 15 min incubation at room temperature in the dark. Necrotic cells were stained with 0.1 µg 7AAD solution (eBioscience, Hatfield, Ireland, UK) at room temperature in the dark for 15 min before 60 sec acquisition on the Navios flow cytometer (Beckman Coulter, High Wycombe, UK).

Leukocytes were gated and displayed on a contour density dot plot with 7AAD against CD45-PE. The healthy cell gate was set on the CD45$^+$ population with high SSC. Any events to the right of this border were considered 7AAD-positive necrotic leukocytes. Fragmented white blood cells (LMPs) still display CD45 but no longer contain DNA and because of their reduced size and complexity, and therefore lower side scatter, end up below the healthy leukocytes on an SSC axes in the gate designated MP (Supplementary Figure 1).

Phagocytosis

BioParticles *E.Coli* K-12 strain, fluorescein conjugate were reconstituted as per manufacturer's instructions (Thermo Fisher). Whole blood (100 µL) was added to tubes and 10 µL BioParticles (diluted 1:10 with PBS) added. Samples were incubated in a shaking water bath at +37°C for 30 min. Phagocytosis was stopped through the addition of 2.5 µL 200 µM Cytochalasin D (Sigma Aldrich, Dorset, UK). Tubes were then placed on ice and 3 mL ice cold PBS added before being centrifuged (+4°C, 515 xg, 7 min). Supernatant was discarded, and the tubes washed in ice cold PBS again. Blood was stained with CD14-PB and CD15-VG as above. 3 mL FACS lyse (BD Bioscience) was added and samples vortexed vigorously to encourage full lysis of red blood cells. Samples were incubated for 15 min in the dark on ice then centrifuged. Surface fluorescence was quenched by washing in 1 mL ice-cold PBS with 12.5 µL 0.2 µm-filtered trypan blue (33). A final wash with ice-cold PBS was performed before fixing samples in 200 µL FACS fix (BD Bioscience). Acquisition on the Navios occurred within 48 hours.

Reactive Oxygen Species assay

Stock solution of ROS assay stain concentrate (eBioscience) was reconstituted in 40 µL of DMSO and stored in aliquots at -20°C. A 1X working solution was prepared by diluting the stain concentrate with ROS assay buffer (eBioscience). 100 µL of 1X ROS assay stain was added per 1 mL blood and incubated for 60 min at +37°C, 5% CO_2. Blood stimulated for 60 min with PMA at a final concentration of 1 µM was used as a positive control. ROS assay stained blood was then sheared as above and 20 µL of sheared blood sample added to a tube for lysis of red blood cells using EasyLyse as before, and acquisition on the flow cytometer (Navios). The median fluorescence intensity of ROS was measured in FL1 (FITC) channel.

Cytokine production

Whole blood cultures

To model the effect of materials and shear on the function of leukocytes through their response to pathogenic stimuli, whole blood cultures (50 µL blood/150 µL RPMI 1640 containing 2 mM GlutaMax and 0.1 mM 2-mercaptoethanol; Life Technologies) were left unstimulated or stimulated with 10 ng/mL *E. coli OIII:B4* lipopolysaccharide (Ultrapure LPS, Invivogen, Toulouse, France) in a U-bottomed 96-well plate. After 24 h incubation at +37°C, 5% CO_2-in-air cell-free supernatants were harvested by centrifugation (+4°C, 515 x g, 7 min). Supernatants were used for cytokine analysis.

ELISA

Cytokines of interest were identified on the Human Cytokine Proteome Profiler Array (R&D Systems) performed as per manufacturer's instructions (data not shown). Cytokines chosen for further analysis were macrophage migration inhibitory factor (MIF) and interleukin-1 alpha (IL-1α) (DuoSet; R&D Systems, Minneapolis, MN, USA) which were measured using a specific ELISAs.

Statistical analysis

GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analysis. Absolute values were compared to baseline values for each donor to obtain a fold change. Results were tested for normality using the column statistics function and were non-parametric. A Friedman test was conducted to compare material effects (baseline vs no shear), shear effects (baseline vs shear at 1000 s^{-1}), and the effects of the two combined (material at 0 s^{-1} vs material at 1000 s^{-1}). A p value of < 0.05 was deemed significant.

Results

Haematology

Erythrocyte counts were not affected by biomaterials or shear (Figure 2A). Blood sheared on Sap had a significantly lower leukocyte count than non-sheared (p = 0.0004; Figure 2B) which translated to significantly lower neutrophil (p = 0.018; Figure 2C) and lymphocyte (p = 0.0004; Figure 2E) counts. The monocyte count was significantly lower in comparison to baseline when sheared on DLC, Sap, and Ti (p = 0.002, 0.025, and 0.007. respectively; Figure 2D). The platelet count was lowered on DLC and Sap without shear (p = 0.045, and 0.0005, respectively) whereas the inclusion of shear exacerbated the low platelet counts on DLC, Sap, and Ti (p = 0.0002, 0.001, and 0.0014, respectively; Figure 2F).

Leukocyte activation

Given that total leukocyte and subset counts differed by exposure to shear and biomaterials, the activation status of neutrophils and monocytes was considered. L-selectin expression by neutrophils decreased significantly in blood sheared on Sap and Ti compared to the baseline (p = 0.0007 and 0.0034, respectively; Figure 3A) whereas shearing on the Ti surface decreased expression compared to non-shear (p = 0.0076). Exposure to Sap without shear significantly increased CD11b[Bright] expression on neutrophils (p = 0.039), but not with shear (Figure 3B). Significantly decreased L-selectin expression was a feature of monocytes exposed to all biomaterials both with and without shear (Figure 3D). Results from neutrophils and monocytes without shear were consistent with previous results exposing these biomaterials to blood in a static environment (34). CD11b[Bright] expression on monocytes increased significantly in all samples apart from those in contact with DLC without shear (Figure 3E). Cell death was unchanged for both neutrophils and monocytes (Figure 3C&F).

Leukocyte microparticles

The blood from the rheometers was stained with CD45/7AAD to determine the effect of shear stress and biomaterials on their prevalence of leukocyte microparticles (LMPs). However, this was not the case as neither biomaterial nor shear affected the number of CD45$^+$ putative LMPs (Figure 4).

Phagocytosis

Cell functionality was then considered, again with a focus on neutrophils and monocytes as the rapid response cells of the innate immune system.

Phagocytic function was measured through adding fluorescent *E. Coli* BioParticles to blood that had been in contact with the biomaterials with/without shear. The BioParticles were incubated with the blood for 5 min to observe initial phagocytosis and 30 min to full phagocytosis. Monocytes and neutrophils were identified with CD14 and CD15 antibodies respectively (Supplementary Figure 2).

A significant difference in the phagocytic ability of neutrophils was observed at 5 min in the sheared DLC, Sap, and Pel samples compared to the baseline ($p = 0.009$, 0.045, and 0.023, respectively; Figure 5Ai). There was no difference in neutrophil phagocytosis at 30 min (Figure 5Aii). Monocyte phagocytosis was unaffected by biomaterial or shear at either 5 or 30 min (Figure 5Bi and Bii).

ROS production

ROS production was not evident for neutrophils or monocytes when sheared between the biomaterials although it could be detected in response to the positive control PMA (Figure 6; n = 3).

Cytokine production

There was no significant difference compared to baseline in any of the samples for IL-1α (n = 4; Figure 7Ai). There was a significant increase between the baseline stimulated with LPS and the Sap 0 s^{-1} (S0), Sap 1000 s^{-1} (S1000), and Ti 1000 s^{-1} (T1000) stimulated for MIF concentration (p = 0.011, 0.028, and 0.006, respectively; n = 7; Figure 7Bi). When compared as a fold change from the unstimulated samples, an attenuated response to LPS in the samples sheared on Ti (T1000) was observed for MIF (p = 0.019; Figure 7Bii) but not IL-1α from the same samples.

Discussion

Increasing our understanding of shear-dependent blood trauma is important to the development of safer VADs as high shear stresses within the pumps causes haemolysis (35), platelet activation (13), leukocyte damage (14), and degradation of vWF (36). Heart failure is an inflammatory disorder which greatly affects leukocyte responses and patient outcomes (37, 38), making leukocytes critical to the success of VAD therapy. *In vitro* testing of shear stress on different blood components can aid in the design of VADs especially if consideration of the impact of biomaterials in this setting can be incorporated. The simple model presented here enables testing of shear stress and biomaterials simultaneously on multiple blood cell types, allowing an insight into how these affect leukocytes and how this may contribute to complication rates in VAD patients.

To study the effects of both shearing and VAD candidate biomaterials on leukocytes, a rheometer model was developed. By attaching biomaterial discs to a rheometer, it was possible to shear healthy human blood between relevant biomaterials: DLC, Sap, and Ti. The rheometer model incorporates large gaps and long residence times, however, using a shear rate of 1000 s^{-1} represents some regions of centrifugal VADs, mainly in the

secondary flow path (31). Whilst this level of shear is neither stagnant (< 50 -100 s^{-1}) resulting in thrombosis (39) , nor significantly high enough to cause cell damage ($> \sim 5000$ s^{-1} (40)), it is a mid-range level of shear that causes leukocyte activation which contributes to thrombus formation (41). Also, surface roughness of the discs was monitored (29) with order of roughness from highest to lowest of DLC > Ti > Sap.

Five minutes was chosen as the rheometer application time for two main reasons: 1) it was adequate for the user to add the blood, set the rheometer up, run, and then remove the blood without excessive haste, and 2) it was deemed long enough that changes might occur but not too far removed from the typical VAD residence time.

Cell counts were measured using a haematology analyser to identify changes that could indicate cellular activation and/or damage. A problematic study limitation here was the blood volume pipetted from the surface of the biomaterials was approximately 500 µL which had to be divided between numerous assays. Consequently, the decision was made to prioritise the other assays and use the remaining blood for the haematology analyser. The CELL-DYN Ruby haematology analyser requires a minimum sample volume of 150 µL with anything lower than that potentially affecting the counts. Equally, the analyser uses electrical impedance to count cells and therefore can be affected by cellular aggregation causing a clump of cells to be identified as a single cell. For example, the pseudolymphocytosis observed in Figure 2E is likely a process error due to aggregated cells (platelet-platelet/platelet-leukocyte) being identified as lymphocytes (42). These process errors were taken into account when interpreting the data.

There was no significant effect on erythrocyte counts compared to the baseline sample or biomaterials with or without shear, but leukocyte counts were affected. Total leukocyte counts were decreased when blood was sheared on a Sap surface and this was associated

with a decreased neutrophil and lymphocyte count. Monocyte counts were significantly decreased when sheared on DLC, Sap, and Ti. Platelet counts were significantly reduced on non-sheared Sap, and when sheared on DLC, Sap, and Ti. Decrease in platelet counts under shear has been noted previously as a sign of platelet activation (43, 44). This cell type was not studied further here but is the focus of ongoing work. Decreases in leukocyte counts under shear have been noted in the past in a model wherein whole blood was sheared for 10 min and there was a 25% decrease in leukocyte count at 60 Pa and membrane disruption at 15 Pa (14). This is not only a longer time but a higher shear stress than in our study (~ 10 Pa).

There was also evidence of activation of neutrophils and monocytes measured as decreased L-selectin expression. L-selectin (CD62L) is shed from the surface of leukocytes when they are activated (45). Neutrophil expression of CD62L was decreased in the sheared Pel, Ti, and Sap blood and there was a significant decrease between non-sheared and sheared blood on Ti. L-selectin on neutrophils is cleaved from the surface 'during rolling on a ligand-bearing surface under hydrodynamic shear flow' according to Lee et al., and the mechanical shedding depends on the shear stress applied (46). Only neutrophils in contact with Sap with no shear increased CD11b expression. This may simply reflect the short duration of neutrophil shear exposure or that of the integrin subunit partners of CD11b, namely CD18, might have been a better measure. Fluid shear stress applied to whole blood on a glass surface significantly down-regulated neutrophil CD18 expression in comparison to non-sheared cells (47). Monocytes also showed decreased CD62L in response to all biomaterials, as observed previously (29), and irrespective of the application of shear. Interestingly, CD11b expression was significantly increased in the sheared DLC monocytes likely relating to increased adhesion of monocytes as observed by Linder et al. (48) and in our previous studies with these

biomaterials (29). This also relates to the fact that macrophages adhere preferentially to rough surfaces under flow, as DLC had the highest surface roughness (49). The difference in the activation profile of the two leukocyte subtypes emphasises that they respond to certain conditions differently. Biomaterials with or without shear did not have any effect on neutrophil and monocyte death nor on the generation of leukocyte microparticles (LMP).

Given there was some effect on neutrophil and monocyte activation we then undertook a more detailed investigation of cell function by studying phagocytosis, ROS production and cytokine outputs in response to an inflammatory stimulus. Functional assays have been cited as 'a more appropriate way to evaluate the effect of shear stress on leukocytes rather than simple cell counts' (10).

The host response to pathogens largely depends on the first defence provided by neutrophils which engulf pathogens via phagocytosis and the generation of ROS (50). Interestingly, neutrophils exposed to DLC, Pel, and Sap demonstrated a significantly reduced immediate phagocytic ability in comparison to baseline (5 min incubation). This appeared resolved when incubated for longer (30 min) with the *E. Coli* Bioparticles apart from in sheared Sap neutrophils which had lower, although again not significant, levels of phagocytosis. These possible alterations in phagocytic ability were not evident in the monocyte population. Diminished phagocytic ability has been noted in neutrophils adhered to cardiovascular device material subjected to physiological shear stresses for 60 min (16). The effect of shear on ROS production focus largely on endothelial cells rather than leukocytes (51) and despite a robust ROS response to the positive control PMA, we could not detect ROS production in response to shear stress for any of the leukocyte subsets studied. Finally, cytokine response to a commonly used prototypic inflammatory stimuli – LPS - was considered (52). Instead of simply measuring the same cytokines as

previously (29) or used by other investigators (53-55), an array approach was used first to better identify candidates for further analysis. IL-1α is an IL-1 family member that interacts with the IL-1 receptor to produce a pro-inflammatory response (56). Biomaterials and shear did not alter the levels of IL-1α produced into the supernatant or the fold change of LPS-stimulated compared to baseline levels in the absence of stimulation. The difference in surface roughness of the materials did not appear to influence the production of IL-1α. Surface roughness has been shown to positively correlate with cytokine production (57), however this has been observed in surfaces above 4 μm, far higher than that in this study. Macrophage migration inhibitory factor (MIF) is involved in cell-mediated immunity and is considered an inflammatory cytokine that regulates macrophage function (58). Macrophages release MIF upon stimulation with LPS (59) with high levels of MIF in circulation known to exacerbate sepsis (60). Here, the most notable effect on MIF is the complete abrogation of the LPS-stimulated MIF response after shearing on Ti. Why there should be such a dramatic effect on MIF when the IL-1α response is unaffected remains to be determined. Lack of MIF might contribute to an immunosuppression environment. Many pathogens, trauma, burns, and major surgery can cause acute immunosuppression leading to a diminished capability of responding to infection (61). The combination of major surgery and implantation of a VAD that is largely composed of titanium alloy could potentially increase patient susceptibility to infection.

It is important to note that the trajectory of an individual leukocyte is variable throughout the pump: therefore residence time and exposure to shear stress will be variable. Biomaterial contact is limited in the primary flow path, but a small number of leukocytes will travel through the secondary flow path which is prone to smaller gaps, higher shear, and low volumetric flow rates (62). Activation and adhesion in this area would be more

apparent and influenced by a variety of factors including surface finish, device design, circulatory stasis, inflammatory profile, and anticoagulation. Leukocytes are the most complex blood cell types and understanding their response to shear stress will prove useful during VAD development.

Conclusion

Our previous work showed that Sap causes higher levels of leukocyte activation than the other biomaterials studied (29) and the rheometer model demonstrates that these are likely exacerbated by shearing. This could pose a problem for VAD developers as Sap is considered for VAD bearings, an area of very high shear and prone to thrombus generation. The finding here that titanium, which is used often for medical devices and implants, could cause a reduction in the normal cytokine response towards pathogenic stimuli could open new avenues of research into why infections occur in both VAD patients and those with titanium implants in general. The rheometer model has its limitations, but it does allow the combined effects of shear and biomaterials to be evaluated on a small volume of blood making the assay possibilities vast. The small blood volume recovered (~ 500 µL) allows for complete blood cell counts, flow cytometry, and other whole blood approaches to be considered. This also lends use of the model to clinical scenarios such as adult and paediatric patients with heart failure or congenital anomalies who will most likely have different baseline leukocyte responses towards VADs than healthy adults.

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Conflict of interest

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Author contributions

Removed for peer-review

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Figure legends

Figure 1: Rheometer model. A & B) Biomaterial discs (Ti here) were attached in parallel to the geometry (bottom plate) and Peltier (top plate) using crystal bond adhesive. Blood was sandwiched between the biomaterial discs for 5 min at +37°C with/without shear (0 s^{-1} /1000 s^{-1}). Sample was pipetted from the surface after elapsed time (C).

Figure 2: Complete cell counts before and after shear on biomaterials. Human whole blood was placed between biomaterial discs attached onto a rheometer. Shear (1000 s^{-1}) or no shear (0 s^{-1}) was applied for 5 min. Samples were then measured on the clinical haematology analyser (mean ± SD; n = 7; significant decrease compared with baseline indicated with '*'; significance of applying shear to materials indicated with '$').

Figure 3: Leukocyte activation and death before and after shear on different biomaterials. Human whole blood was placed between biomaterial discs - diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), and titanium alloy (Ti) - attached onto a rheometer. Shear (1000 s-1) or no shear (0 s-1) was applied for 5 min. Leukocyte activation was investigated by: A) Change in median fluorescent intensity (MFI) of CD62L on A) $CD15^+$ neutrophils and D) $CD14^+$ monocytes; Change in percentage of $CD11b^{Bright}$ cells on B) $CD15^+$ neutrophils and E) $CD14^+$ monocytes. Percentage of C) $CD15^+$ neutrophils and F) $CD14^+$ monocytes cells are dead as determined by DRAQ7. All were compared to the time 0 baseline as a fold change (dashed line). The LPS positive control is indicated with a dotted line (mean ± SD, n = 9; significance compared with baseline indicated with '*'; significance of applying shear to materials indicated with '$').

Figure 4: Leukocyte microparticle generation before and after shear on different biomaterials. Human whole blood was placed between biomaterial discs- diamond-like

carbon coated stainless steel (DLC), single-crystal sapphire (Sap), and titanium alloy (Ti) - attached onto a rheometer. Shear (1000 s^{-1}) or no shear (0 s^{-1}) was applied for 5 min (mean ± SD; n = 3). The blood was stained with CD45 and 7AAD before acquiring on the flow cytometer. The number of CD45$^+$ putative LMPs was compared as a fold change to the baseline sample.

Figure 5: Phagocytic ability of neutrophils and monocytes before and after shear on different biomaterials. Human whole blood was placed between biomaterial discs - diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), and titanium alloy (Ti) - attached onto a rheometer. Shear (1000 s^{-1}) or no shear (0 s^{-1}) was applied for 5 min. Samples were added to tubes and incubated in a +37°C shaking water-bath with fluorescent *E.coli* bioparticles for 5 min and 30 min. Phagocytosis was compared to the baseline sample and measured as neutrophils (CD15$^+$) or monocytes (CD14$^+$) that were in the *E. coli*^{Bright} region at 5 minutes (Ai and Bi, respectively) and 30 minutes and compared to the baseline sample (Aii and Bii, respectively) (mean ± SD, n = 6; significance compared with baseline indicated with '*')

Figure 6: ROS production before and after shear on different biomaterials. Blood was incubated at +37°C for 1 h with 1X ROS assay stain before being placed between biomaterial discs- diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), and titanium alloy (Ti) - attached onto a rheometer. Shear (1000 s^{-1}) or no shear (0 s^{-1}) was applied for 5 min (n = 3). Baseline blood stimulated with 1 µM PMA was used as a positive control. A) Overlay dot-plot for controls with and without ROS stain, B) Overlay histogram for controls with and without ROS stain. ROS production in C) granulocytes, D) monocytes, and E) lymphocytes from blood sheared between biomaterials.

Figure 7: IL-1α and MIF expression before and after shear on different biomaterials. Human whole blood was placed between biomaterial discs - diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), and titanium alloy (Ti) - attached onto a rheometer. Shear (1000 s-1) or no shear (0 s-1) was applied for 5 min. The samples were cultured with (stimulated – diagonal pattern) or without (unstimulated – chequered) 10 ng/mL LPS for 24 h at +37°C, 5% CO2. Supernatants were harvested and processed for Ai) IL-1α and Aii) as a fold change from the unstimulated sample (n = 4) and Bi) MIF and Bii) as a fold change from the unstimulated sample (mean ± SD, n = 7; * $p \leq 0.05$, dashed line represents unstimulated sample) ELISA.

Supplementary Figure 1: Gating strategy for leukocyte microparticles. Human blood stained with CD45-PE and 7AAD then acquired for 60 secs on the Navios flow cytometer. A) Forward-versus side-scatter plot (FSC/SSC), B) detection of CD45$^+$ events on a CD45-FITC versus side-scatter plot (CD45-FITC/SSC), C) CD45$^+$ events were displayed on SSC vs 7AAD plot to display healthy, necrotic and leukocyte microparticles (CD45$^+$ MPs).

Supplementary Figure 2: Gating strategy for phagocytosis. Baseline blood was incubated in a +37°C shaking water-bath with fluorescent *E. coli* bioparticles for 5 min and 30 min. Phagocytosis was stopped through the addition of cytochalasin D and two washes with ice-cold PBS. Samples were stained with CD14 and CD15 antibodies, red blood cells were lysed, then samples were washed with ice cold PBS containing trypan blue to quench the bioparticles on the cell surface. *E.coli* bioparticles in PBS were used to gate the 'Bright' population indicating full phagocytosis. A) Forward-versus side-scatter plot (FSC/SSC), B) detection of monocytes (CD14-PB/SSC), C) detection of neutrophils (CD15-VG/SSC), D) E. Coli BioParticles in PBS for gating purposes, E)

baseline CD15$^+$ events phagocytosis after 5 min, E) baseline CD15$^+$ events phagocytosis after 30 min.

Supplementary Table 1: Baseline haematology descriptive statistics. Peripheral blood collected from healthy adult volunteers into lithium heparin anticoagulant was measured for complete cell count on the clinical haematology analyser. The minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation, and standard error of the mean were calculated.
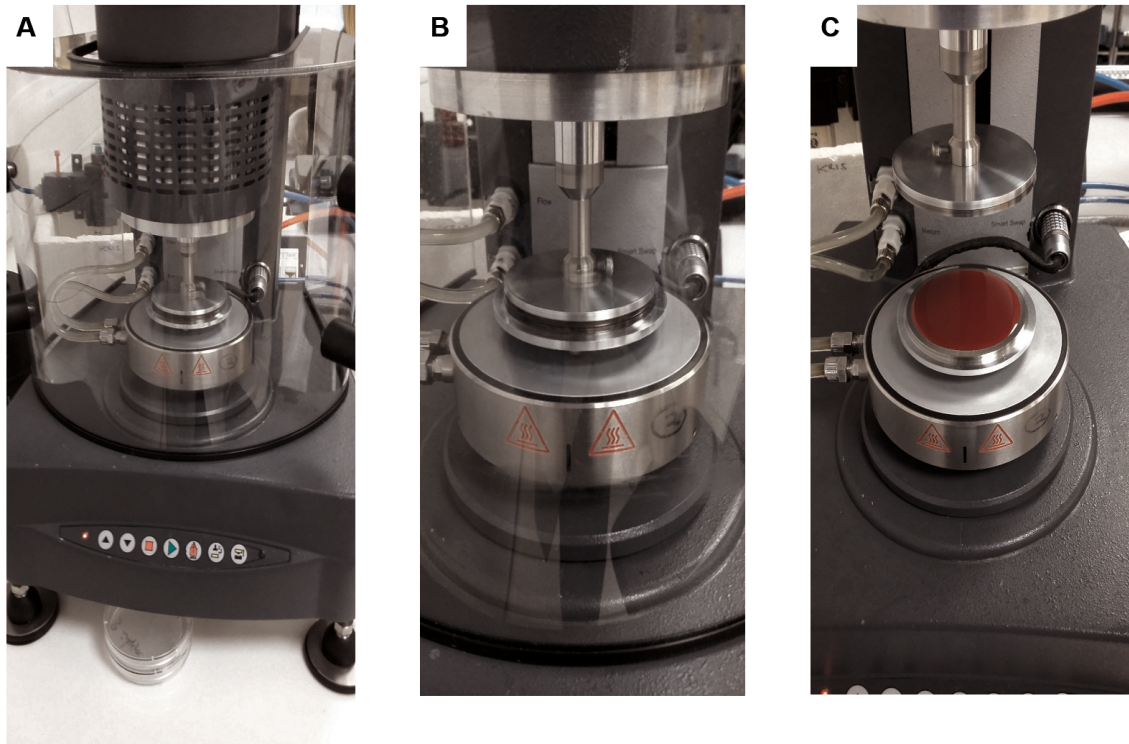
Figure 1
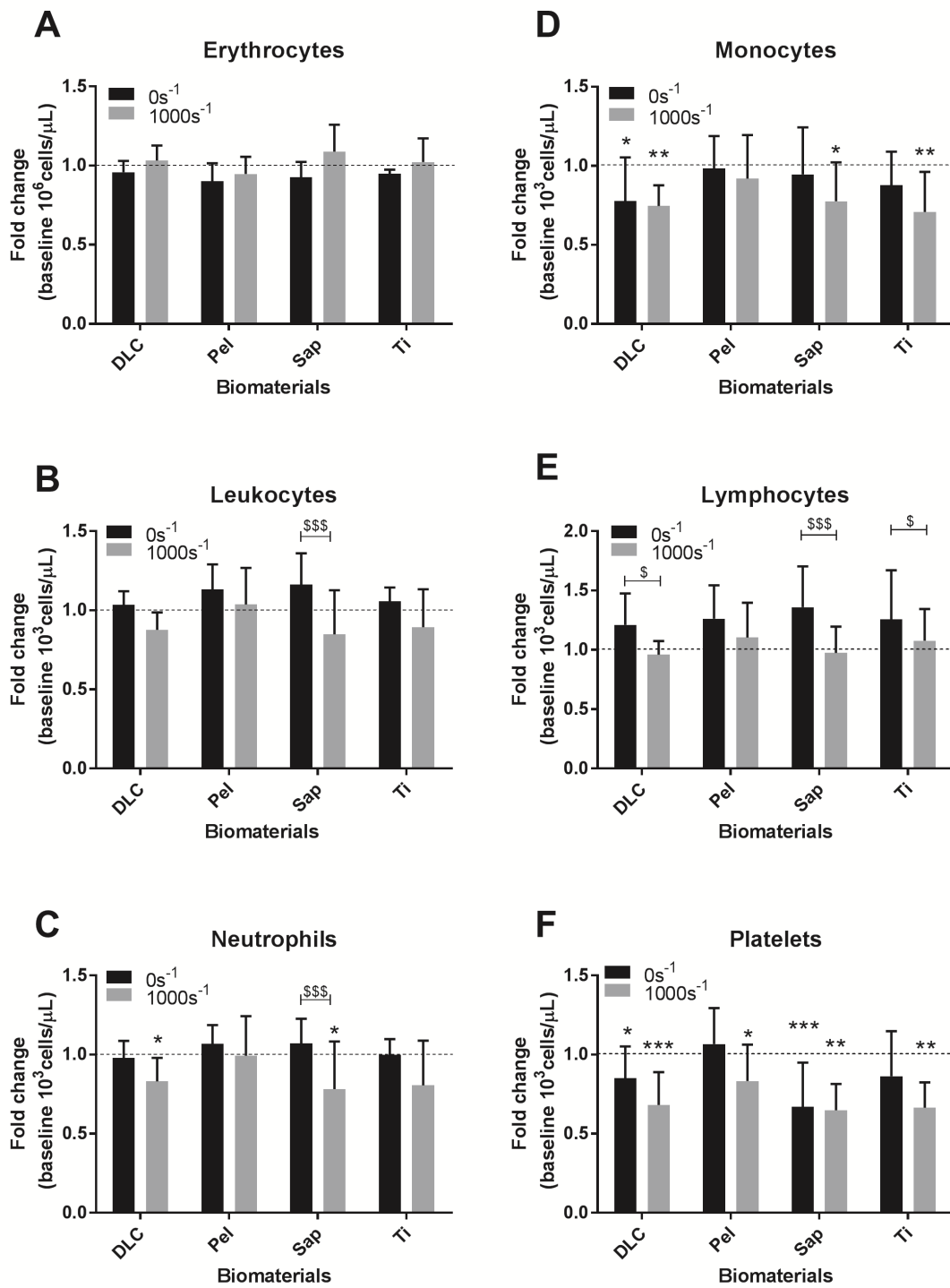
Figure 2

Figure 3

Figure 4

Ai Neutrophil (5 min incubation)

Bi Monocyte (5 min incubation)
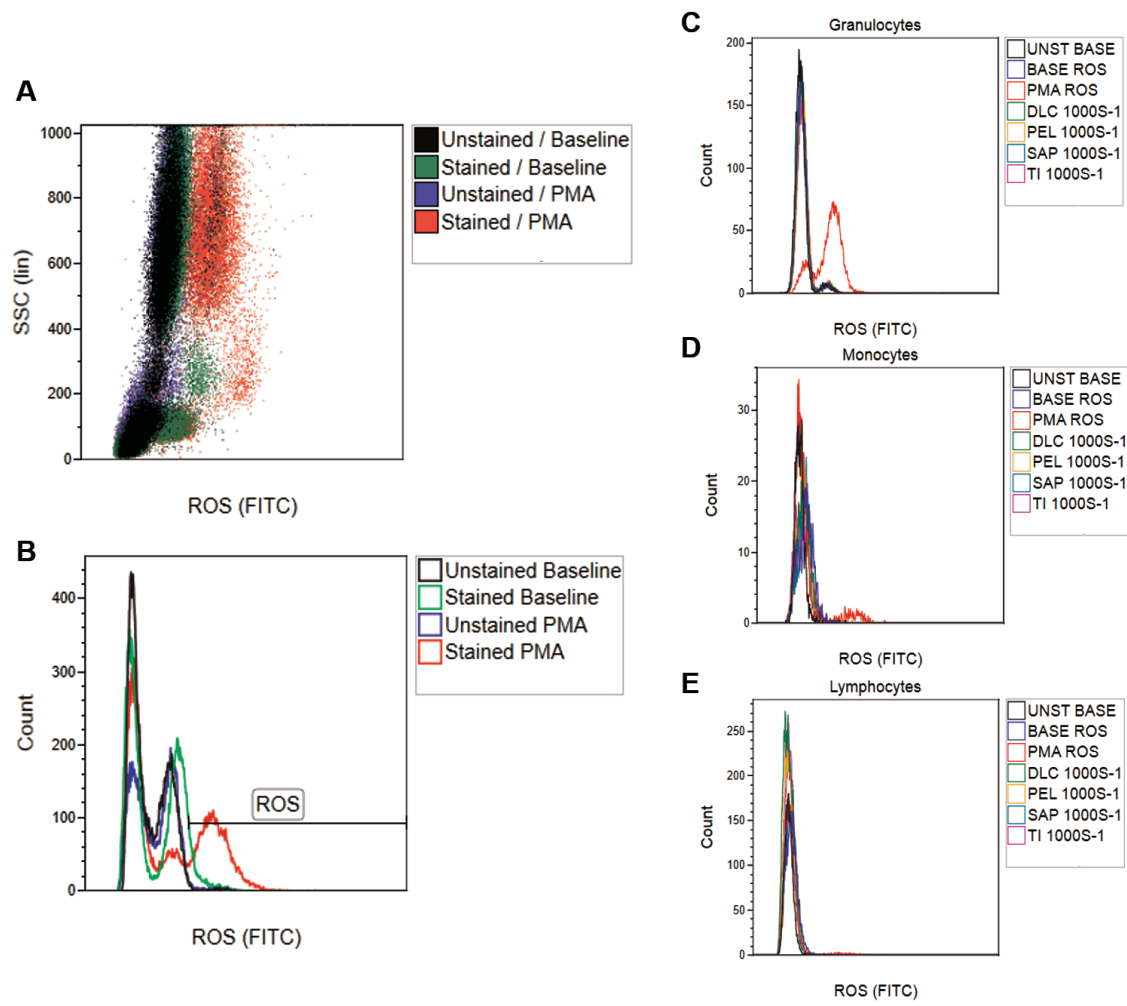
Aii Neutrophil (30 min incubation)

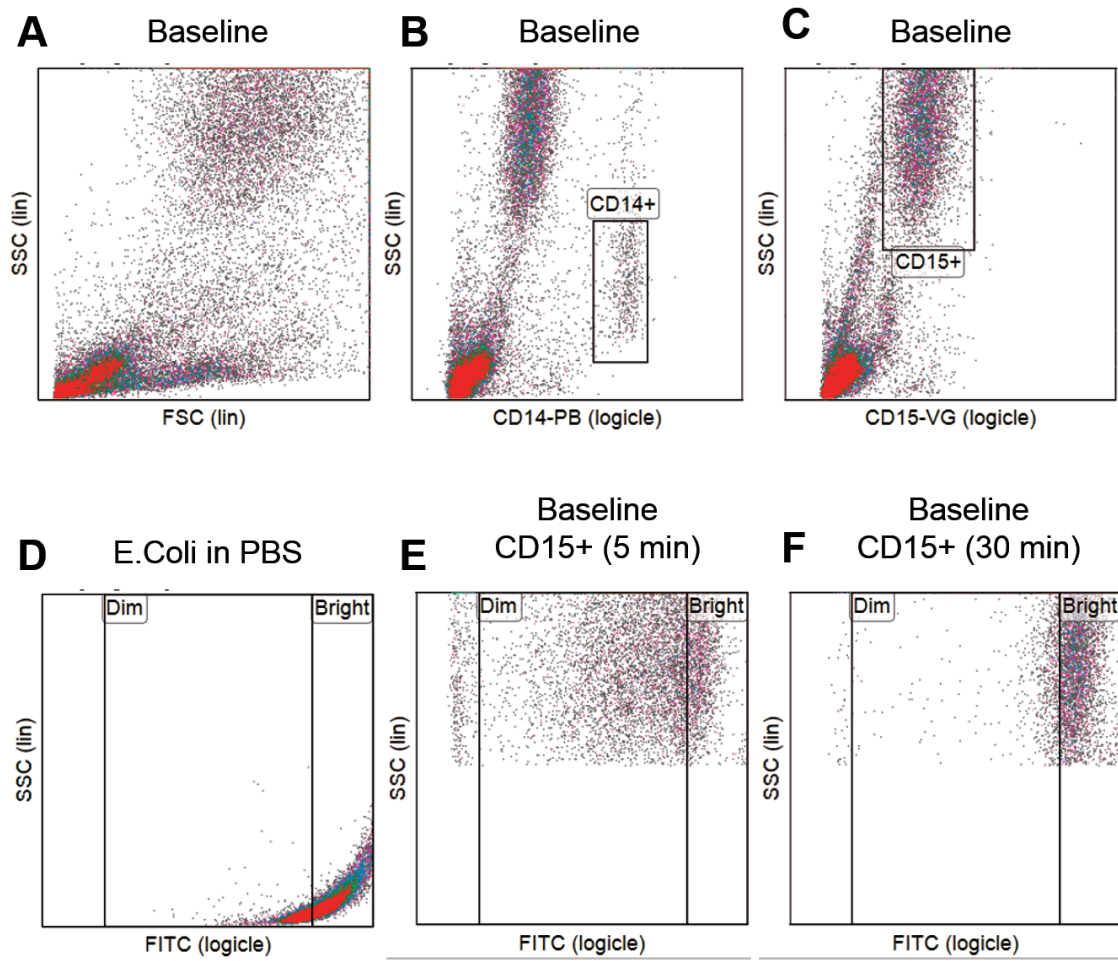**Bii** Monocyte (30 min incubation)
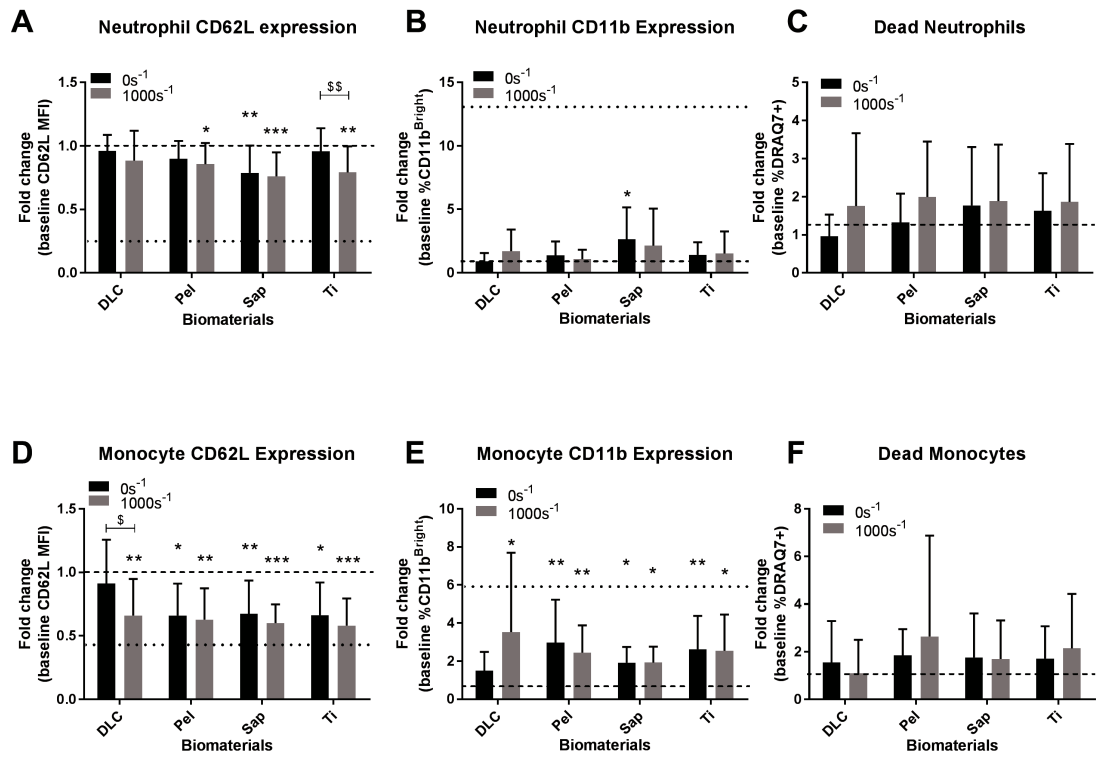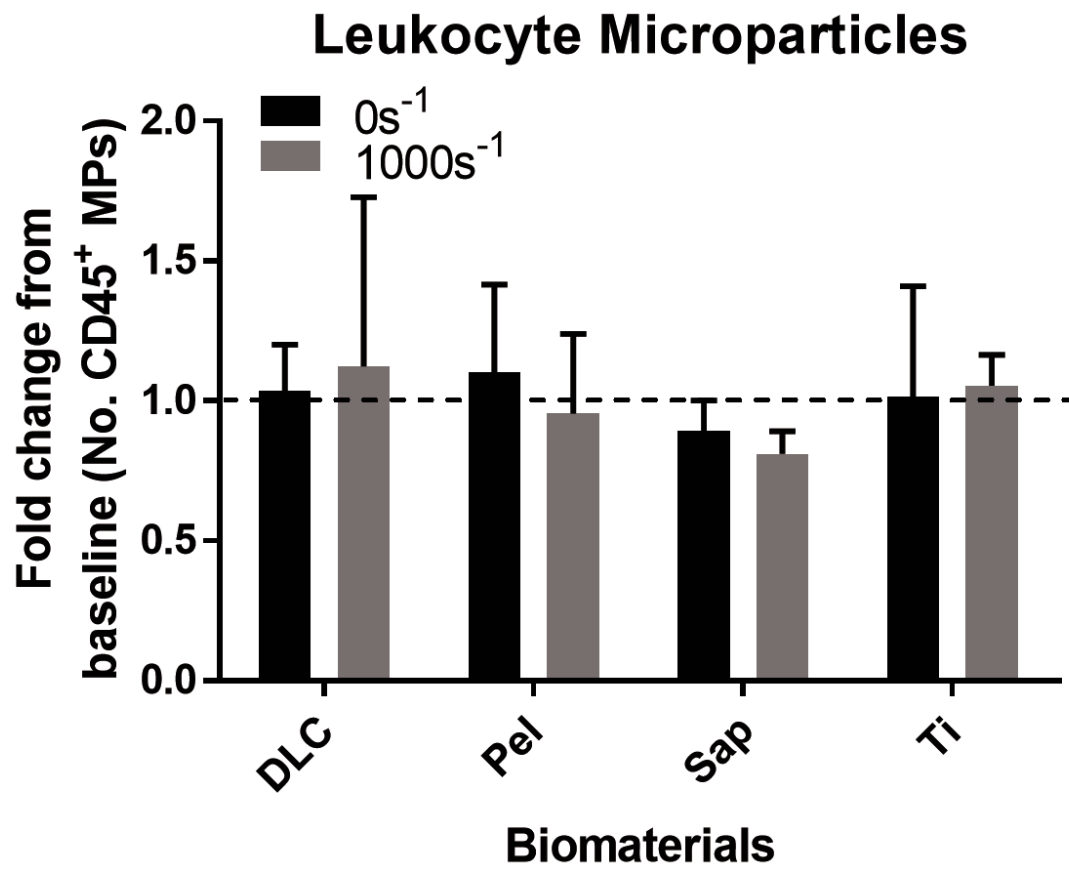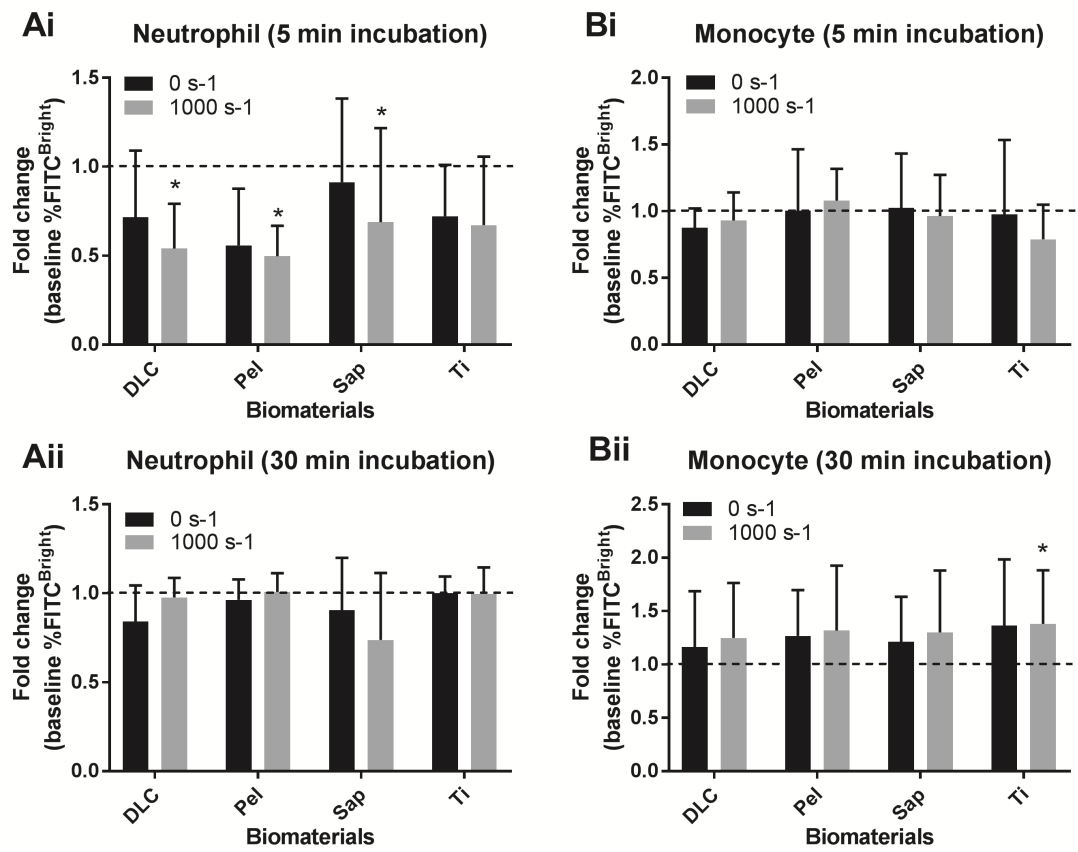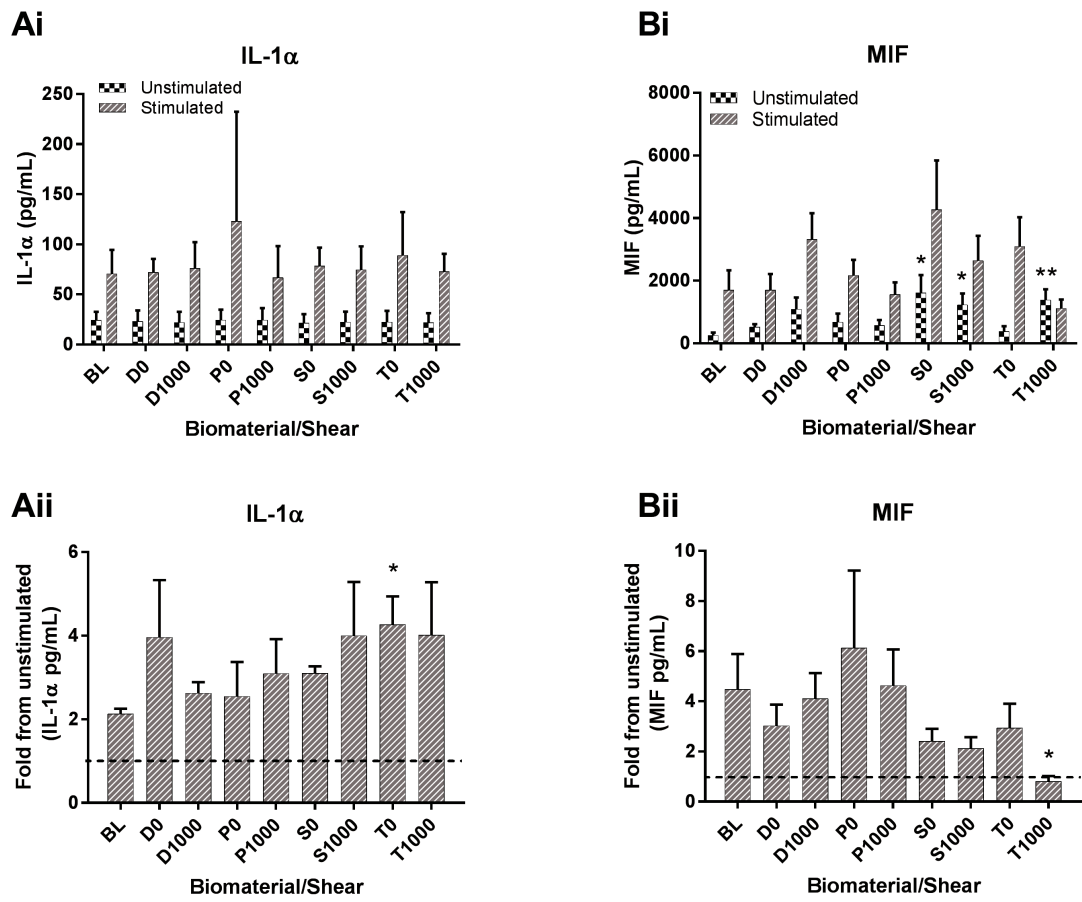
Figure 5

Figure 6

Figure 7

Supplementary Table 1

	WBC	NEU	LYM	MON	RBC	PLT
Minimum	4.49	1.96	1.40	0.27	3.64	93
25th percentile	5.51	2.35	1.99	0.34	4.00	144
Median	5.92	2.67	2.12	0.44	4.46	153
75th percentile	6.69	3.76	2.39	0.49	4.74	162
Maximum	9.44	7.20	3.13	0.61	5.51	192
Mean	6.35	3.48	2.20	0.43	4.46	149
STD	1.71	1.97	0.57	0.12	0.67	36
SEM	0.70	0.80	0.23	0.05	0.27	13

Counts in $10^6/\mu L$ for RBC and $10^3/\mu L$ for all others. LYM: Lymphocytes; MON: Monocytes; NEU: Neutrophils; PLT: Platelet; RBC: Red Blood Cell; SEM: Standard Error of the Mean; STD: Standard Deviation; WBC: White Blood Cell.
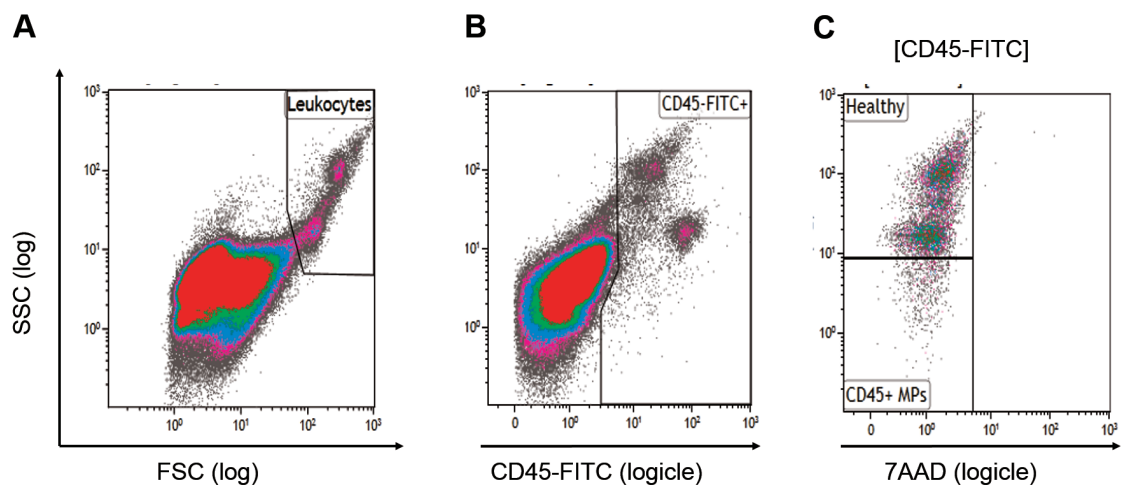
Supplementary Figure 1

Supplementary Figure 2