The Effect of Ventricular Assist Device-Associated Biomaterials on Human Blood Leukocytes

Gemma Radley[1,2], Ina Laura Pieper[1,2], and Catherine A. Thornton[1]

[1]Institute of Life Science, Swansea University Medical School, Swansea, Wales, UK;

[2]Calon Cardio-Technology Ltd, Institute of Life Science, Swansea, Wales, UK

Running Headline: Assessment of biomaterials for leukocyte damage

Corresponding author:

Professor Cathy Thornton,

Institute of Life Science, Swansea University Medical School, Swansea, Wales, UK

Phone: +44 (0) 1792 602122

Email: c.a.thornton@swansea.ac.uk

Abstract

Background: Ventricular assist devices (VADs) are an effective bridging or destination therapy for patients with advanced stage heart failure. These devices remain susceptible to adverse events including infection, bleeding, and thrombus; events linked to the foreign body response. Therefore, the biocompatibility of all biomaterials used is crucial to the success of medical devices.

Methods: Biomaterials common in VADs - DLC: diamond-like carbon coated stainless steel; Sap: single-crystal sapphire; SiN: silicon nitride; Ti: titanium alloy; and ZTA: zirconia-toughened alumina - were tested for their biocompatibility through incubation with whole human blood for 2 hours with mild agitation. Blood was then removed and used for: complete cell counts; leukocyte activation and death, and the production of key inflammatory cytokines. All were compared to time 0 and an un-exposed 2 hour sample.

Results: Monocyte numbers were lower after exposure to DLC, SiN and ZTA and monocytes showed evidence of activation with DLC, Sap, and SiN. Neutrophils and lymphocytes were unaffected.

Conclusions: This approach allows comprehensive analysis of the potential blood damaging effects of biomaterials. Monocyte activation by DLC, Sap, ZTA and SiN warrants further investigation linking effects on this cell type to unfavourable inflammatory/thrombogenic responses to VADs and other blood handling devices.

Key Words

Ventricular Assist Devices; Biomaterials; Human; Blood; Flow Cytometry

1. Introduction

Ventricular assist devices (VADs) are a successful long-term therapy for end-stage heart failure [1, 2]. The use of biocompatible materials to construct these devices is essential. Blood contact with VADs is brief but frequent and the foreign body response towards these materials combined with heavy anti-coagulation likely contributes to VAD-associated clinical complications such as thrombosis, bleeding, and infection [3]. Surface properties of biomaterials drive the foreign body response beginning with protein adsorption, a complex process driven by different protein-surface forces such as surface energy, morphology, and ionic/electrostatic interactions [4]. The composition of the protein matrix adsorbed onto the material is highly variable but largely consists of the most abundant proteins in the blood: fibrinogen, albumin, and globulins. Platelet activation occurs through interaction with these proteins with fibrinogen in particular leading to platelet-platelet aggregation [5] These immobilised platelets are then able to recruit and activate blood borne leukocytes, mainly neutrophils and monocytes, which contribute to the building of thrombus through adhesion whilst also releasing cytokines for further recruitment to the site [6]. Interactions of T cells with this biological surface build-up can cause activation-induced cell death resulting in defective cellular immunity and susceptibility to severe infections [7].

Adherence and activation of leukocytes at the biomaterial's surface are well described for titanium, low density polyethylene (LDPE), Pellethane®, and polytetrafluoroethylene (PTFE) using *in vitro* models. This evidence is provided by changes in the expression of adhesion molecules such as decreased L-selectin (CD62L) and increased CD11b expression, an increase in inflammatory cytokine concentration (e.g. TNFα, MCP-1, IL-8), and the release of reactive oxygen species [8-12]. A similar activation profile is also demonstrable in vivo after implantation of VADs, e.g. platelet-leukocyte aggregates and increased monocyte tissue factor expression [13-15]. Excessive

leukocyte activation could underpin a diminished immune response upon infection [16, 17]; and thrombotic leukocyte-derived microparticles [18, 19] might lead to stroke, embolism, or device replacement due to blockages.

Titanium is the biomaterial most commonly used for the VAD body [20]; its resistance against fatigue and corrosion makes it durable for long-term implantation [21]. However, titanium can activate the coagulation system *in vitro* and thus be a thrombogenic material that activates leukocytes [22]. Other VAD components have been made from ruby alumina [23], sapphire [24], and zirconium [25] but the biocompatibility testing of these ceramic materials is limited to their applications as dental or joint implants [26, 27]. Silicon nitride could be a good candidate for use as a bearing due to its high strength and oxidation resistance [28] but its biocompatibility has been tested with osteoblasts [29] rather than blood cells. Coatings have been suggested as a solution for materials with desirable mechanical properties but poor biocompatibility. Diamond-like carbon (DLC) is a chemically inert nanocomposite coating with properties of natural diamond such as high hardness, low friction, and high corrosion resistance [30, 31]. DLC has been deemed biocompatible through prolonging clotting time, and suppressing platelet and complement activation [32].

This study aimed to evaluate the leukocyte response to biomaterials and test a methodological approach implementable within most device development laboratories that would enable rapid evaluation of multiple biomaterials. Biomaterials that are of interest to the development of cardiovascular devices such as VADs formed the focus of this investigation: diamond-like carbon coated stainless steel (DLC), single-crystal sapphire alumina (Sap), silicon nitride (SiN), titanium alloy Ti6Al4V (Ti), and zirconia-toughened alumina (ZTA). Biomaterials were exposed to whole blood rather than isolated cells or plasma to better mimic the clinical scenario [33].

2. Materials and methods

2.1 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).

2.2 Biomaterials

Highly polished discs of DLC, Sap, SiN, Ti, and ZTA (Table 1; SAK Equipment Ltd, East Sussex, UK) were sonicated in 3% Neutracon® (Decon Laboratories Ltd., East Sussex, UK) at +40°C for 10 min and then sonicated in warm, soapy dH_2O for 3 min followed by a 3 min sonication in fresh dH_2O. Discs were air-dried, sprayed with 70% denatured ethanol and left to dry on lint-free cloths. Polypropylene beads were soaked with 70% denatured ethanol for 30 min then rinsed in sterile PBS before use.

2.3 Experimental model

Twenty polypropylene beads (2-3 mm diameter) were placed in a circle in a 70 mm petri dish (Cole Parmer, London, UK). The biomaterials were then carefully balanced on top before the addition of blood (15 mL) (Supplementary Figure 1). Beads only served as a negative control. Dishes were incubated at +37°C on a shaker plate set to 50 rpm. A time course was performed by sampling at 2, 4, and 8 h; the 2 h samples were deemed most appropriate to continue with for further analysis as longer incubation showed high levels of cell death and platelet activation/aggregation with little difference between biomaterials. As plasma-free haemoglobin (pfHB) can upregulate tissue factor expression on macrophages [34] haemolysis in all the samples

was measured using the Harboe assay [35] to check the condition of the red blood cells before progressing to further experiments.

2.4 Complete cell counts

Total and subset cell counts were measured in triplicate using an automated haematology analyser (CELL-DYN Ruby; Abbott Diagnostics, Berkshire, UK).

2.6 Leukocyte activation, aggregation and death through flow cytometry

Leukocyte activation and death

Changes in leukocyte activation - decreased L-selectin (CD62L), increased CD11b, and increased fMLP receptor - were monitored using flow cytometry. These were measured as mean fluorescence intensity (CD62L) or percentage positive cells (CD11b, fMLP receptor) on $CD15^+$ neutrophils, $CD14^+$ monocytes, and $CD3^+$ T cells. Changes in expression are presented as fold change compared to baseline (time 0 sample). Whole blood (20 µL) was stained for flow cytometry using standard protocols with various antibodies (Table 2). Red blood cells were lysed with EasyLyse (Dako, Ely, UK), and DRAQ7 (BioStatus, Leicester, UK) was used for viability monitoring without need for a wash step [36]. Whole blood stimulated with 10 ng/mL LPS for 4 h at +37°C, 5% CO_2-in-air as below was used as a positive control and unstained samples were used for gating purposes. Blood exposed to 1% Triton-X 100 (Fisher Scientific, Loughborough, UK) was used to set a gate for DRAQ7 positive cells. Single-stained AbC beads (Life Technologies, Paisley, UK) were used for compensation. Samples were acquired immediately (Navios flow cytometer, 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 5,000 total lymphocytes and 1,000 monocytes. Post-acquisition compensation and data analysis were performed using Kaluza 1.3 (Beckman Coulter).

Leukocyte-platelet aggregation

The leukocyte-platelet aggregation assay was performed using CD15 and CD14 as above combined with 12.5 ng/μL CD41 (APC, clone P2, Beckman Coulter); CD41 is a platelet marker, CD15 a neutrophil marker and CD14 a monocyte marker – co-expression on an event identified using flow cytometry is indicative of aggregates containing these cells [37]. Samples were stained and lysed with a wash step included before running. Whole blood stimulated with 4 μM PMA for 20 min was used as a positive control; tubes were run on a logarithmic scale with 10,000 $CD15^+$ events as the stopping gate.

2.7 Whole blood cultures

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).

2.8 L-selectin, cytokines and endotoxin levels

Levels of plasma soluble L-selectin and IL-1β, TNFα, IL-6, and IL-8 in cell culture supernatants from whole blood cultures were measured using specific ELISAs as per the manufacturer's instructions (DuoSet, R&D Systems, Oxford, UK). Presence of endotoxin was measured in unstimulated cell culture supernatants using the limulus amebocyte lysate LAL Chromogenic Endotoxin Quantitation Kit according to manufacturer's instructions (Thermo Fisher Scientific, Loughborough, UK).

2. 9 Statistical analysis

GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analysis. Gaussian distributions were tested using D'Argostino & Pearson omnibus normality or Kolmogorov-Smirnov tests. Comparisons between baseline and biomaterials were analysed using one-way ANOVA or the Kruskal-Wallis non-parametric test. Repeated measures ANOVA was used for time course samples. Tukey or Dunn's tests were applied *post hoc*. A p-value ≤ 0.05 was considered significant.

3. Results

3.1 Condition of blood

Total erythrocyte counts in comparison to the baseline sample at time 0 (n = 16; Figure 1A) were not significantly affected by biomaterials. Concentrations of pfHB were not significantly higher than the baseline time 0 sample (n = 9; Figure 1B).

3.2 Leukocyte cell counts

Total leukocyte, neutrophil, and lymphocyte counts in comparison to the baseline sample at time 0 (n=16; Figure 2A, B and D) were not significantly affected by biomaterials. Monocyte counts decreased significantly upon exposure to DLC (p = 0.018), SiN (p = 0.025), and ZTA (p= 0.009) (Figure 2C).

3.3 Leukocyte activation and death

Expression of CD62L (n = 9) on neutrophils (Figure 3Ai) and lymphocytes (Supplementary Figure S3) was not affected significantly by the biomaterials whereas expression of CD62L on monocytes (Figure 3Aii) decreased significantly upon exposure to DLC (p = 0.049), Sap (p = 0.009), and SiN

($p = 0.026$). However, this was not detectable as soluble CD62L in the fluid phase (Supplementary Figure S4).

The percentage of $CD11b^{bright}$ cells after exposure to biomaterials did not differ significantly in comparison to baseline for either neutrophils or monocytes (Figure 3Bi, Bii). CD11b is expressed on only 20-30% of lymphocytes so these data are not shown for $CD3^+$ T cells [38].

Incubation alone increased fMLP receptor expression by neutrophils and monocytes after 2 h incubation with no significant effects of the biomaterials (Figure 3Ci, Cii). Expression of fMLP receptor on T cells was not studied.

Cell death was monitored throughout using DRAQ7 [36] and there was no significant effect of biomaterials exposure on cell death within any of the leukocyte subsets of interest (Figure 3Di-ii and Supplementary Figure S3B).

3.4 Leukocyte-platelet aggregation

Aggregation of leukocytes with platelets contributes to the build-up of thrombus on the surface of materials. Neutrophil-platelet and monocyte-platelet aggregates were elevated in comparison to the baseline time 0 sample in all biomaterials but this was not significant (Figure 4, $n = 5$).

3.5 Cytokine production

The effect of exposure to biomaterials on leukocyte functionality was considered by studying the response to the prototypic inflammatory stimuli LPS using whole blood cultures after exposure to biomaterials. LPS induced a significant increase in inflammatory cytokine levels in blood exposed to biomaterials as well as control blood in keeping with the expected effects of LPS [39]. Levels of LPS-stimulated pro-inflammatory cytokines IL-8 (Figure 5A; $n = 7$), IL-1β (Figure 5B; $n = 7$),

IL-6 (Figure 5C; n = 5) and TNFα (Figure 5D; n = 5) were unchanged by exposure to any of the biomaterials.

4. Discussion

Assessing the potential total blood damage effects of multiple biomaterials *in vitro* are desired to minimise adverse events in VAD patients. Our model has focused on how leukocytes respond to the VAD candidate biomaterials to quickly identify which cells are affected. Such a model might prove an effective early stage screening tool for evaluating biomaterials. The simple suspension of a disc in a petri dish can be performed by anyone and allows for full blood coverage over a large, flat, uniform surface – more representative of the implantation of a medical device than thin films, wires, and beads used by others [40-42]. Additionally, our model allows for erythrocytes, platelets, and leukocytes in whole blood to be studied simultaneously in an experimental system which is recommended by the International Standards [43]. A key feature of this model is that the assays can be used to quickly identify leukocyte subsets most susceptible to each biomaterial type and the level of activation, all of which might contribute to thrombus formation within the VAD [44].

The biomaterial discs used in this study were manufactured to have a smooth surface (Table 1) to comply with the internal surface condition of many VAD types (MicroMed DeBakey, Jarvik-2000, HeartWare HVAD) [20] and were cleaned thoroughly to reduce the presence of contaminants; endotoxin levels were below 1 EU/mL (Supplementary Figure 5). The method was designed to allow the effects of the biomaterials to emerge without requiring complicated experimental rigs. Initial time course analysis revealed that incubation times longer than 2 hours introduced time-related artefacts, thus more detailed analysis was performed at the 2 h time-point for the rest of the study. Activation of blood cells can occur very quickly in response to biomaterials with intracellular calcium flux in T cells within 2 minutes [45], and up-regulation of CD11b as early as

30 minutes in some studies [46]. However, a 2 h time point allows early stage evaluation and a comparison of the biomaterials of interest before progressing to more complex studies incorporating experimental conditions, such as shear stress, better representative of the *in vivo* scenario where residence time is transient but repetitive. Importantly, the model used also allows the research question to be refined based on these early stage findings. Reasonable volumes of blood can be used in this model to reveal functional impact on the cells of interest. Scalable experimental approaches and flow cytometry are useful for this purpose.

The titanium alloy Ti6Al4V is the biomaterial of choice for the VAD body [20] and is therefore the largest surface area for blood contact. Titanium can be thrombogenic by promoting the formation of a surface protein matrix composed of fibrinogen, thrombin, complement factors, and coagulation cascade proteins such as kallikrein that encourages cell activation and adhesion [47]. In this study, titanium had no significant effect on the leukocyte count, leukocyte activation/aggregation/death indicating that the surface protein matrix formed was relatively inert in terms of leukocyte activation/adhesion. Titanium has been shown to cause high levels of TNFα (~600 pg/mL) in PBMCs cultured for 24 h [48] and a reduction in TNFα in MNCs stimulated with LPS [49] which was not observed in this study. However, titanium disc implants in rats have shown low levels of TNFα (~25 pg/mL), IL-1β (~15 pg/mL) and IL-1α (~100 pg/mL) in the exudate after 1 h and even 3 h [10] further highlighting the need to test these biomaterials with whole blood rather than isolated cells to mimic the *in vivo* response.

Ceramic materials such as Sap, SiN and ZTA used in this study are desirable bearing materials due to their potential to resist wear damage, their hardness, and wettability [50, 51]. Biocompatibility testing of these materials has so far been limited to bone growth [29], tissue implantation [26], or protein adsorption [52, 53]. Our study is the first to analyse the effects of these materials on

leukocytes using whole blood. We found monocytes were the most affected cell type with a significant decrease in number for SiN and ZTA and a decrease in monocyte expression of CD62L on Sap and ZTA. Monocytes have considerable quantities of tissue factor on the surface and actively aggregate with platelets when stimulated [54]. Our time course studies of tissue factor showed that it wasn't up-regulated on monocytes until 8hrs (data not shown) which was not compatible with the optimised time for these experiments. This delayed tissue factor expression might also explain the absence of significant monocyte-platelet aggregates despite the apparent effects of the biomaterials on the monocytes in particular. Use of these monocyte-activating materials as VAD bearings, could lead to the build-up of thrombus such as that seen in the single-crystal alumina (ruby) bearing of the HeartMate 2 [55, 56] which this model identifies *in vitro*.

To overcome biocompatibility issues in mechanically desirable materials, coatings with excellent biocompatibility have been introduced. DLC is one such coating which rapidly adsorbs albumin and inhibits fibrinogen adsorption [30] therefore strongly reduces platelet adhesion and activation, prolongs clotting time, and suppresses complement convertase activation [32]. In this study, DLC showed a significant decrease in monocyte count with a decreased monocyte CD62L expression – a response like that of the ceramics. DLC has been shown to slightly increase monocyte adhesion [57], with DLC coated rather than uncoated Ti6Al4V favouring cell adhesion [58].

Due to the findings that monocytes are the key leukocyte target type, ongoing work involves optimising methods for visualising cells adhered to the biomaterial surface. A limitation of this study is the continued contact of the blood cells with the biomaterials when residence time would normally be measured in milliseconds rather than hours, and the absence of shear stress. Parallel work is considering the additive effects of biomaterials shear stress on leukocyte activation. This

is being done using a rheometer and this approach has driven the size of the biomaterials discs used and the model optimised for study.

5. Conclusion

This simple model can be used by anyone in the blood-contacting devices field to quickly identify the cells most affected by materials of choice for better targeted research. Such research could include elucidating the mechanism of activation or careful consideration of the cells of interest during further *in vitro* and *in vivo* testing of the material. The methods used here go beyond the tests recommended by the International Standards to include a more thorough investigation of the leukocyte response, such as cytokine release. This is important when designing a device for patients with a disease characterised by haematological effects on multiple cells types. Our study has largely focused on leukocytes and shown that monocytes become activated by ceramic materials considered for bearings and DLC, the classically termed 'inert' material, but not with titanium. Such monocyte activation could contribute to the thrombus build-up which has already been observed on ceramic material bearings in current VAD designs so warrants further investigation. Overall the candidate materials studied caused little activation of healthy, human blood leukocytes. However, the target patient group of people with chronic heart failure have pre-existing inflammation meaning leukocytes, particularly monocytes, are already activated [59, 60] which might render the biomaterials no longer 'inert'. This possibility has been described: platelet adherence on low and medium thrombogenic polymer-based biomaterials was increased for patients with coronary artery disease compared to healthy controls [61]. Therefore, this model also offers a simple approach to determining the effects of biomaterials leukocytes and other blood cells and proteins in blood from paediatric and adult heart failure patients to add real value to the field.

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Conflict of interest

Author Ina Laura Pieper was an employee of Calon Cardio-Technology Ltd (Calon). Gemma Radley is 50% funded by Calon through a PhD studentship jointly awarded to Swansea University Medical School and Calon. Gemma Radley has presented parts of this work at ESC-HF 2015, ESAO 2015, and ISRBP 2015.

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Figures and Tables

Table 1. Biomaterial specifications. Surface roughness was measured using a SurfTest SJ-210 profileometer (Mitutoyo UK) Ltd., Hampshire, UK).

Biomaterial	**Abbreviation used**	**Ø (mm)**	**h (mm)**	**Surface area (mm^2)**	**Surface roughness (Ra, µm)**
Diamond-like carbon coated stainless steel	DLC	60	2	6032	0.774
Single-crystal sapphire	Sap	60	4	6409	0.044
Silicon nitride	SiN	60	4	6409	0.029
Titanium alloy (TiAl6V4)	Ti	60	2	6032	0.099
Zirconia toughened alumina	ZTA	46	3	4072	0.047

Table 2. Flow cytometry antibodies. Whole blood (20 µL) was incubated on ice in the dark for 30 min with antibodies for leukocyte activation (CD15, CD14, CD3, CD62L, CD11b, fMLPr), red blood cells were lysed with EasyLyse and then DRAQ7 added before acquisition (lyse-no-wash protocol).

Cell type/ activation marker	**Antigen**	**Colour**	**Clone**	**Isotype**	**Concentration (ng/µL)**	**Supplier**
Neutrophil	CD15	Krome Orange	80H3	IgM	12.5	Beckman Coulter, High Wycombe, UK
Monocyte	CD14	Pacific Blue	M5E2	IgG2a	500	BioLegend, London, UK
T-Lymphocyte	CD3	APC-AF750	UCHT1	IgG1κ	100	Beckman Coulter, High Wycombe, UK
L-selectin	CD62L	PE	DREG-56	IgG1κ	25	eBioscience, Hatfield, UK
Macrophage-1 antigen	CD11b	APC	CBRMI/5	IgG1κ	100	eBioscience, Hatfield, UK
Formyl peptide receptor 1	fMLPr	FITC	REA169	IgG1	220	Miltenyi Biotec, Woking, UK

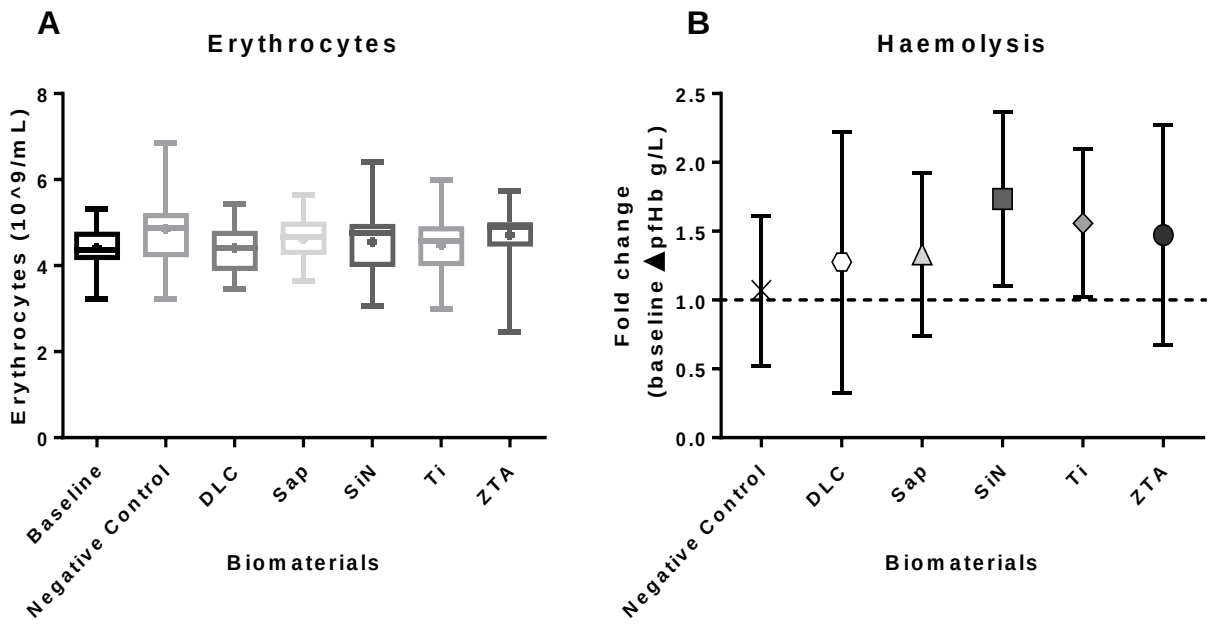

Figure 1. The effect of various biomaterials on erythrocytes. Whole heparinised blood was exposed to each of the biomaterials - diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), silicon nitride (SiN), titanium alloy (Ti), and zirconia toughened alumina (ZTA) - for 2 hours and A) complete cell counts were measured on an automated haematology analyser (n = 16) and B) plasma-free haemoglobin (pfHB) measured in plasma from whole heparinised blood exposed to each of the biomaterials– using the Harboe assay and B) compared to time 0 baseline and shown as a fold change (n = 9).

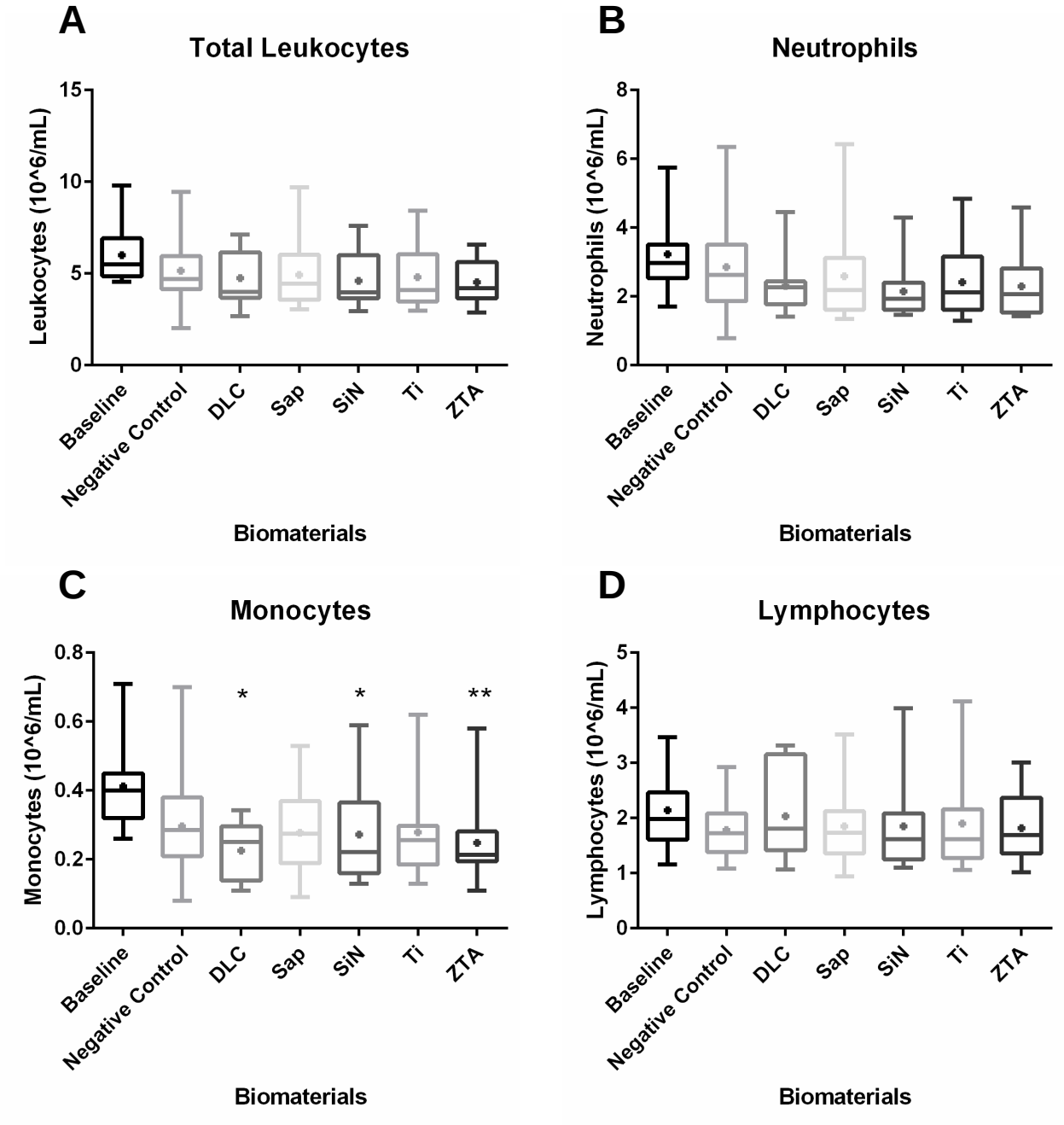

Figure 2. The effects of various biomaterials on total leukocyte and subset counts. Whole heparinised blood was exposed to each of the biomaterials - diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), silicon nitride (SiN), titanium alloy (Ti), and zirconia toughened alumina (ZTA) - for 2 hours and complete cell counts were measured on an automated

haematology analyser. A) Total leukocytes, B) neutrophils, C) monocytes, and D) lymphocyte (n = 16); * $p \leq 0.05$, ** $p \leq 0.01$ on statistical comparison to time 0 baseline.

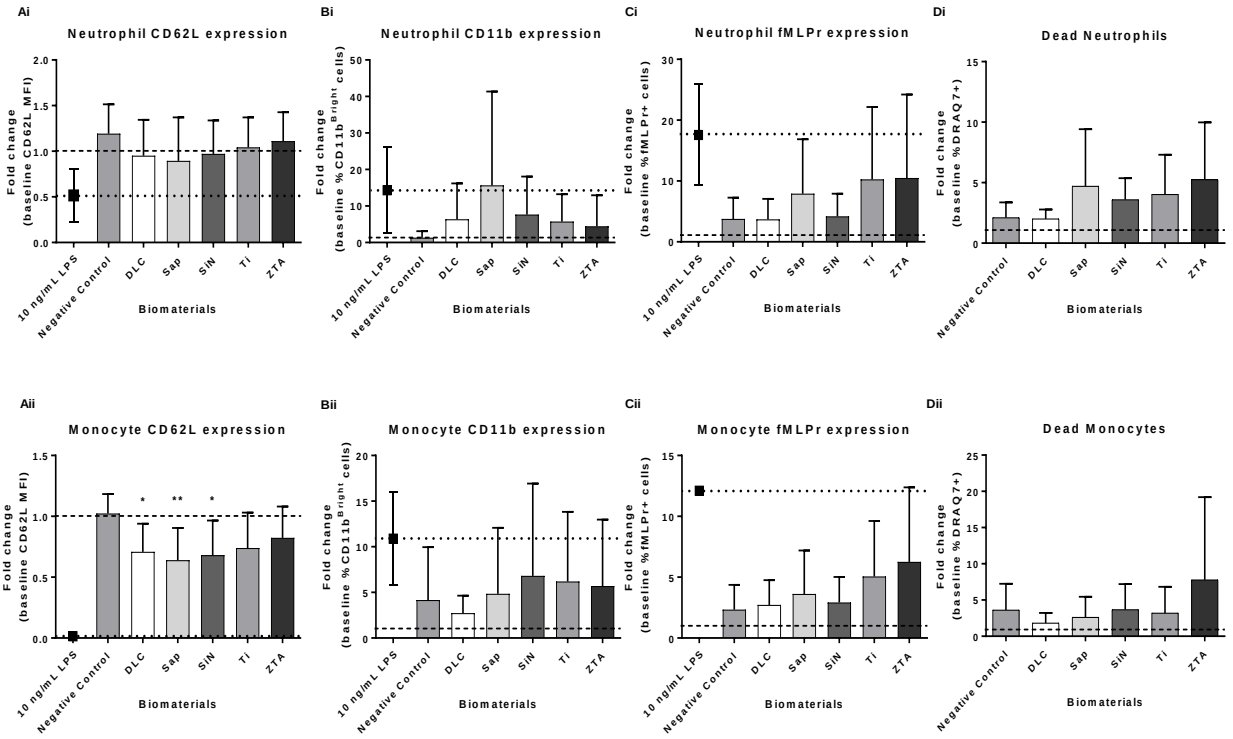

Figure 3. The effect of various biomaterials on neutrophil and monocyte activation and death. Whole heparinised blood was exposed to each of the biomaterials - diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), silicon nitride (SiN), titanium alloy (Ti), and zirconia toughened alumina (ZTA) - for 2 hours. Samples were then stained with CD62L, CD11b, fMLP receptor (fMLPr), CD15, CD14, and DRAQ7. Leukocyte activation was investigated by: A) Change in median fluorescent intensity (MFI) of CD62L on i) $CD15^+$ neutrophils (n = 9) and ii) $CD14^+$ monocytes (n = 9); B) Change in percentage of $CD11b^{Bright}$ cells on i) $CD15^+$ neutrophils and ii) $CD14^+$ monocytes (n = 6); C) Change in percentage of fMLPr on i) $CD15^+$ neutrophils and ii) $CD14^+$ monocytes (n = 5); D) Percentage of i) $CD15^+$ neutrophils and ii) $CD14^+$ monocytes cells are dead as determined by DRAQ7 (n = 5). All were compared to the

time 0 baseline as a fold change (dashed line). The LPS positive control is indicated with a dotted line (* $p \leq 0.05$, ** $p \leq 0.01$).

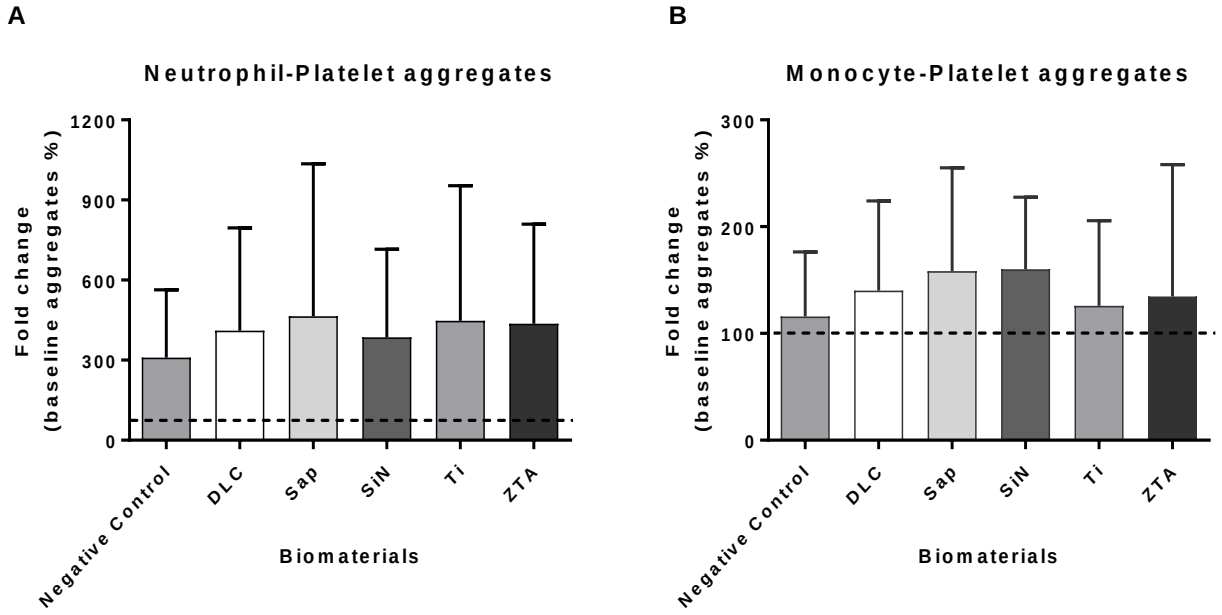

Figure 4. The effects of various biomaterials on leukocyte-platelet aggregation. Whole heparinised blood was exposed to each of the biomaterials - diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), silicon nitride (SiN), titanium alloy (Ti), and zirconia toughened alumina (ZTA) - for 2 hours. Samples were then stained with CD14, CD15, and CD41. Percentage of dual-positive cells ($CD14^+/CD41^+$ or $CD15^+/CD41^+$) were compared to the time 0 baseline as fold change (n = 5).

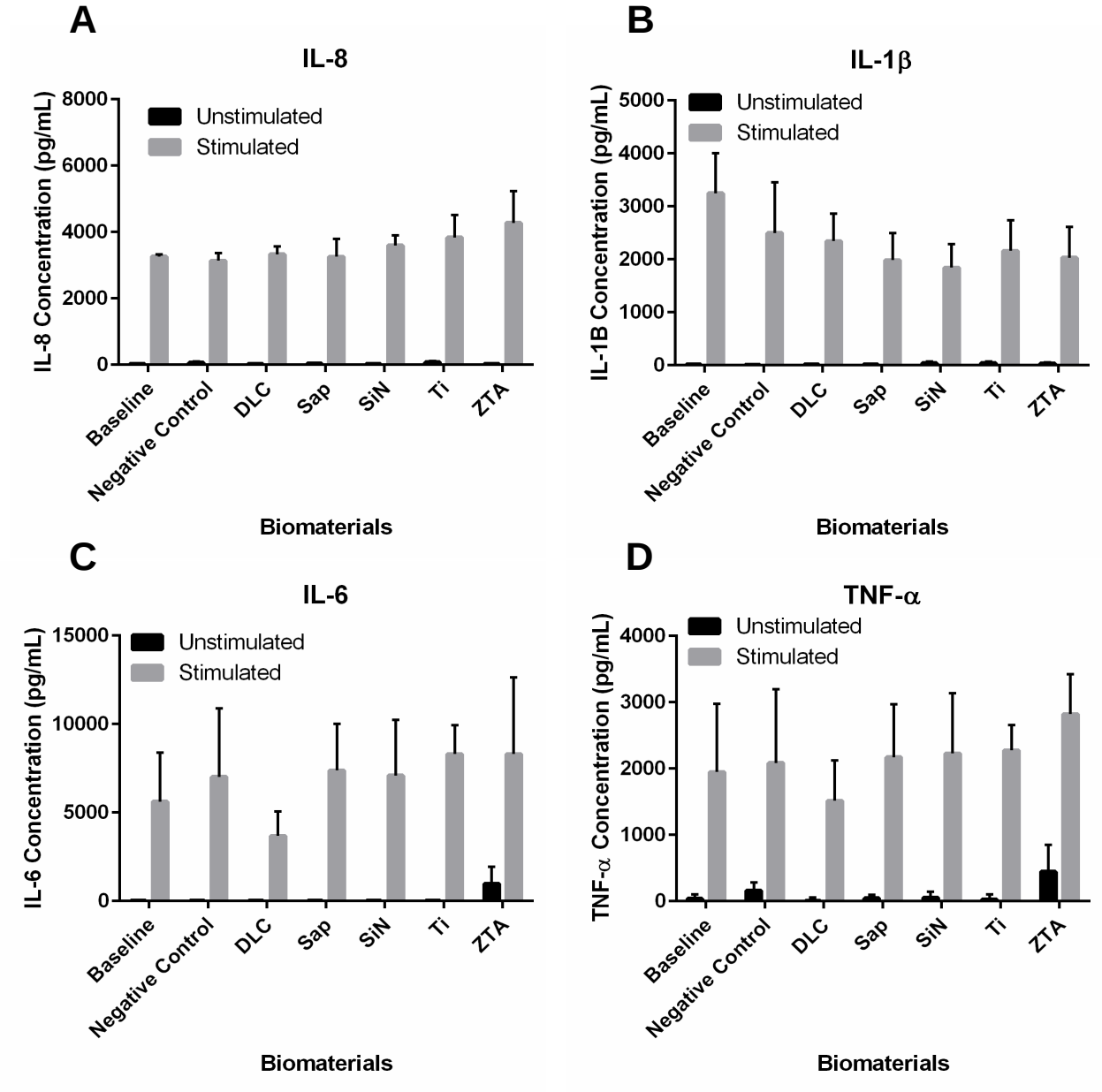

Figure 5. Lipopolysaccharide-stimulated release of cytokines in blood exposed to different biomaterials. Whole heparinised blood incubated with various biomaterials - diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), silicon nitride (SiN), titanium alloy (Ti), and zirconia toughened alumina (ZTA) - for 2 h was placed into culture either in media alone (unstimulated) or with media containing 10 ng/mL LPS (stimulated) for a further 24 h in 5% CO_2, 37°C**.** Pro-inflammatory cytokines A) IL-8 (n = 7), B) IL-1β (n = 7), C) IL-6 (n = 5), and D) TNFα

(n = 5) were measured with specific ELISA. No significant difference in comparison to time 0 baseline although ZTA shows elevated levels of IL-6 and TNF-α in unstimulated samples.

Supplementary Figure 1. Model set-up. 20 polypropylene beads of 2-3 mm diameter were added to a 70 mm petri dish (A&B) in a circle in order to support the biomaterial to allow maximum surface area coverage. The biomaterial was carefully laid on top of the beads (C&D) and blood (15 mL) added. The dishes were then placed on a shaker plate and incubated at 37°C for 2 hours.

Supplementary Figure 2. Flow cytometry gating strategy for leukocyte activation, death and aggregation. Baseline (time 0 sample) and baseline blood stimulated with 10 ng/mL LPS for 4 hours (positive control for leukocyte activation) were stained with CD3, CD14, CD15, CD62L, CD11b, fMLP receptor (fMLPr), and DRAQ7. Samples were used to set up gating strategy for activation profiles of different leukocyte subsets with neutrophils shown in figure. A) Neutrophils ($CD15^+$) were identified by plotting SSC/CD15-Krome Orange. CD15+ events were then plotted as SSC versus CD62L-PE, CD11b-APC, or fMLPr-FITC. Gates were set according to baseline samples having high expression of CD62L, dim expression of CD11b and fMLPr and vice versa for LPS samples. B) 1% Triton-X 100 was added to lysed baseline blood, vigorously vortexed, and DRAQ7 added before running sample as a dead cell control. DRAQ7 positive events ($DRAQ7^+$) identified dead cells and a gate was on these. Baseline was used as a dead cell negative control. C) Baseline and baseline blood stimulated for 20 min with 4 μM PMA stained with CD14, CD15 and CD41. Platelets were identified using CD41 with neutrophil-platelet aggregates also positive for CD15 and monocyte-platelet aggregates also positive for CD14.

Supplementary Figure 3. The effect of various biomaterials on lymphocyte activation and death. Whole heparinised blood was exposed to each of the biomaterials - diamond-like carbon

coated stainless steel (DLC), single-crystal sapphire (Sap), silicon nitride (SiN), titanium alloy (Ti), and zirconia toughened alumina (ZTA) - for 2 hours. Samples were then stained with CD62L, CD3 and DRAQ7. Change in A) median fluorescent intensity (MFI) of CD62L; and B) percentage of $DRAQ7^+$ dead cells. There were no significant differences in comparison to baseline (all n = 3).

Supplementary Figure 4. The effects of various biomaterials on soluble CD62L. Whole heparinised blood was exposed to each of the biomaterials - diamond-like carbon coated stainless steel (DLC), single-crystal sapphire (Sap), silicon nitride (SiN), titanium alloy (Ti), and zirconia toughened alumina (ZTA) - for 2 hours. Samples were centrifuged and the plasma analysed for soluble L-selectin using specific ELISA. There were no significant differences in comparison to baseline (n = 8).

Supplementary Figure 5: Endotoxin levels. Unstimulated, biomaterial-exposed-cell culture supernatants were analysed for endotoxin described in the materials and methods (n = 7). 1 EU/mL is approximately 0.1 ng endotoxin/mL.