The Role of RANTES (CCL5) In The Immune Response to Bacterial Infections

Shiying Tang

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Shiying Tang

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Declaration:

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Signed: Shiying Tang

Date: 11th September 2019

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Abstract

Background: Sepsis is a lethal condition that causes organ dysfunction due to a dysregulated host response to an infection. It is one of the leading causes of morbidity and mortality globally with approximately 6 million deaths occurring every year. Transfusion-associated sepsis (T-AS) can occur through the transfusion of contaminated blood components, commonly platelet concentrates (PCs). The "cytokine storm" is thought to be the main pathophysiology behind sepsis and the cytokines secreted within bacterial contaminated PCs could play a key role in the development of T-AS in recipients. This study investigated the effect of bacterially-primed platelet releasates on the activation status of neutrophils and monocytes, with the aim of understanding the link between platelet product contamination, the cytokine storm, and the severity of clinical symptoms in transfusion recipients. **Findings:** The cytometric bead array assay demonstrated that RANTES was significantly elevated in comparison with IL-1β, IL-6, and TNF-α when platelet rich plasma samples were incubated with planktonic and biofilm forms of *Staphylococcus epidermidis* and *Serratia marcescens*. Flow cytometric analysis of the surface markers CD54, CD11b and CD66b on U937 and HL-60 cell lines demonstrated no changes in expression when treated platelet releasates that had been primed with *S. epidermidis* and wild type (WT) *Escherichia coli*. Yet treating these cells with platelet releasates primed with multi-resistant *E. coli* (planktonic or biofilm form) caused a highly significant upregulation in CD54 expression. Platelet-free plasma alone was found to cause a degree of upregulation of all three surface markers in some cases suggesting minor immunomodulatory activity of plasma proteins. Despite its significant release from bacterially-primed platelets, recombinant RANTES (rhRANTES) at different concentrations did not induce significant changes in surface expression of the activation markers on U937 and HL-60 cells however, variable effects on CD54 and CD11b expression were seen on primary human neutrophils. Curiously, CD66b appeared to be downregulated with increasing exogenous rhRANTES concentration. **Conclusions:** The results may indicate that RANTES requires synergy with additional cytokines/chemokines in order to induce activation of neutrophils and monocytes and cannot act in isolation. Bacterial virulence appears to play a role in the resultant cytokine profiles of platelets evidenced by the stark difference in cell activation caused by multi-resistant *E. coli*-primed platelets in comparison with WT *E. coli* and *S. epidermidis*.

1.0 Introduction

1.1 Sepsis: Definition and Significance

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer *et al.,* 2016). It is estimated to affect over 30 million people globally with 6 million cases leading to death every year (Fleischmann *et al.,* 2016). This is more than the deaths caused by bowel, breast and prostate cancer combined (Sepsis Trust, 2019). Despite extensive research to elucidate the pathophysiology and potential treatments for sepsis, it remains as one of the leading causes of mortality and morbidity worldwide, affecting over 35% of hospitalised patients in Europe alone, and with increasing incidence (Vincent *et al.,* 2006). Increased incidence could also be due to the increased awareness within secondary healthcare settings and the improved disease coding for sepsis. However, many cases go unreported, especially in low economic countries, which bears the highest sepsis burden (Reinhart *et al.,* 2017), and so statistics on sepsis incidence are likely to be significantly underestimated.

It must be emphasised that although infection is the triggering event for sepsis, it is ultimately the dysregulated immune response involving concomitant pro- and anti-inflammatory responses that results in pathophysiology even after successful treatment of the infection (van der Poll *et al.,* 2017). The hyperinflammation of sepsis involves the activation of pathogen associated molecular patterns (PAMPs) and/or damage associated molecular patterns (DAMPs) which initiates the immune response, this is accompanied by the activation of the coagulation cascade and may result in disseminated intravascular coagulation (DIC) and multiple organ failure as well as leucocyte exhaustion (Boomer *et al.,* 2014). The extent of the hyperinflammatory phase is dependent on the patient's comorbidities, the causative agent, the bacterial load and genetic factors, both of the pathogen and host (Boomer *et al.,* 2014). Subsequent immunosuppression may develop in the efforts to control the excessive inflammatory response. However, this puts the patient at risk of developing reoccurring, secondary and nosocomial infections and difficulties in eradicating infection (Delano and Ward, 2016). The immunosuppressive phase is thought to be caused by T_{reg} cells (Hotchkiss *et al.,* 2013). A summary of the consequences of hyper- and hypo-inflammation in sepsis is described in Fig. 1.

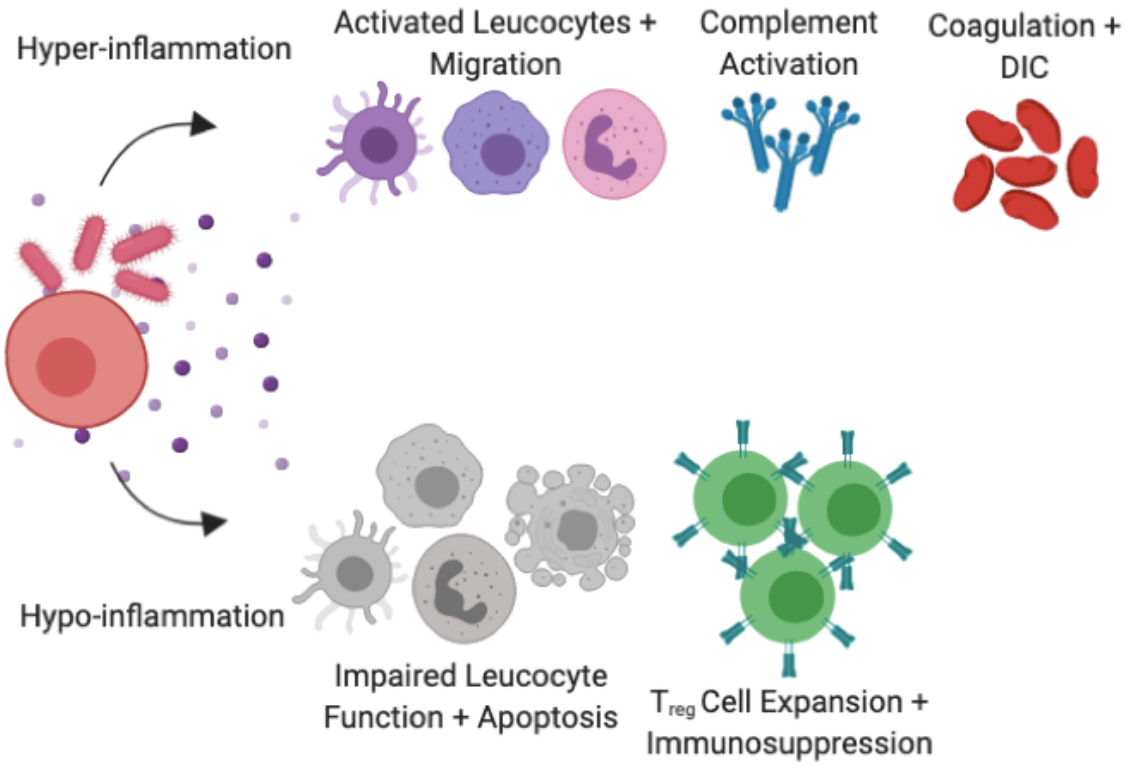


Figure 1: Sepsis is triggered by the recognition of bacteria by immune cells resulting in excessive secretion of proinflammatory cytokines and subsequent significant leucocyte activation and trafficking, complement activation and a pro-coagulatory state which may result in DIC tissue ischaemia thus organ failure. To counteract the effects of inflammation, anti-inflammatory cytokines may be secreted in an effort to return to homeostasis however, consequences of this results in impaired leucocyte effector function and apoptosis as well as the proliferation of T_{reg} cells to inhibit leucocyte activation. This predisposes patients to opportunistic infections. Adapted from Faix, 2013.

As a heterogenic syndrome, symptoms of sepsis vary from individual-to-individual and are nonspecific, but generally patients present with fever, tachycardia, altered mental status, hypotension and dyspnoea (Levy *et al.,* 2003). One study identified a link between the number of dysfunctional organs and mortality in emergency department patients leading to the development of the Sequential (sepsis-related) Organ Failure Assessment (SOFA) score (Table 1) to predict the clinical outcome and severity of organ failure in sepsis patients (Vincent *et al.,* 1996; Shapiro *et al.,* 2006). The SOFA has been validated as a good method for risk stratification and predictor of patient prognosis (Jones *et al.,* 2009). Around 15% of sepsis survivors die within a year of hospital discharge followed by 6-8% of patients dying per year over the subsequent 5 years (Shankar-Hari *et al.,* 2019). Those who do survive may suffer from post-sepsis syndrome which refers to the temporary or permanent physical and psychological complications that patients may experience (Tiru *et al.,* 2015). These include decreased cognitive function, chronic fatigue, difficulty sleeping, functional disability and poor concentration; this in turn, is associated with the development of mental health issues such as depression and post-traumatic stress disorder (Tiru *et al.,* 2015; Huang *et al.,* 2018).

Table 1. The Third International Consensus Definition for Sepsis and Septic Shock (Sepsis-3) presented the SOFA score as a mean to determine the extent of organ failure of patients with sepsis. Abbreviations: FIO_2 – fraction of inspired oxygen; MAP –mean arterial pressure; PaO_2 – partial pressure of oxygen. [a]Adapted from Vincent *et al.,* 1996. [b]Catecholamine doses are given as μ/kg/min for at least 1 hour. [c]Glasgow Coma Scale scores range from 3 – 15; higher score indicates better neurological function.

	Score				
Organ System	**0**	**1**	**2**	**3**	**4**
Respiration					
PaO_2/FIO_2, mm Hg (kPa)	≥ 400 (53.3)	< 400 (53.3)	< 300 (40)	< 200 (26.7) with respiratory support	< 100 (13.3) with respiratory support
Coagulation					
Platelets, x $10^3/\mu L$	≥ 150	< 150	< 100	< 50	< 20
Liver					
Bilirubin, mg/dL (μmol/L)	< 1.2 (20)	1.2 – 1.9 (20 - 32)	2.0 – 5.9 (33 - 101)	6.0 – 11.9 (102 - 204)	> 12.0 (204)
Cardiovascular					
	MAP ≥ 70 mm Hg	MAP < 70 mm Hg	Dopamine < 5 or dobutamine (any dose)[b]	Dopamine 5.1 – 15 or epinephrine ≤ 0.1 or norepinephrine ≤ 0.1[b]	Dopamine > 15 or epinephrine > 0.1 or norepinephrine > 0.1[b]
Central Nervous System					
Glasgow Coma Scale score[c]	15	13 – 14	10 – 12	6 – 9	< 6
Renal					
Creatinine, mg/dL (μmol/L)	< 1.2 (110)	1.2 – 1.9 (110 - 170)	2.0 – 3.4 (171 - 299)	3.5 – 4.9 (300) - 440	> 500 (440)
Urine Output, mL/d				< 500	< 200

1.2 Sepsis and the Cytokine storm

Cytokines are a group of proteins released by various cell types and function as intracellular signalling molecules. Cytokines can be grouped into several categories based on their function and structure but most cytokines have overlapping or duel functions (Tisoncik *et al.,* 2012). Some of these include tumour necrosis factor (TNF), interleukins (IL) and chemokines. TNF-α is one of the most extensively studied cytokines and is known for its prominent role within inflammation and is central in the occurrence of the "cytokine storm" and other inflammatory diseases (Idriss and Naismith, 2000). The main role of interleukins is in the differentiation and activation of leucocytes where they can exhibit pro- or anti-inflammatory roles (Tisoncik *et al.,* 2012). Chemokines can be divided into four subfamilies based on the placement of the first two cysteine residues (CXC, CX_3C, C and CC) and are vital in the recruitment of specific immune cells (Ono *et al.,* 2003).

The pathophysiology of sepsis remains to be fully elucidated but one of the main accepted ideas is the "cytokine storm" that is responsible for the immunopathology seen in sepsis. Currently, there is no clear definition of what exactly constitutes the "cytokine storm" but the general concept is an excessive or uncontrolled released of proinflammatory and anti-inflammatory cytokines (Tisoncik *et al.,* 2012). The initial phase of sepsis is acute inflammation in which the initial insult produces a local inflammatory response in which cytokines secreted may "spillover" into the systemic circulation leading to the priming of leucocytes and amplification of inflammation; this will rapidly become independent of any stimuli coming from the invading pathogen (Chousterman *et al.,* 2017). The later phase of sepsis is immunosuppression or compensated anti-inflammatory response syndrome, caused by the over secretion of anti-inflammatory cytokines such as interleukin-10 (IL-10) and as a consequence, many activated immune cells go through anergy and apoptose, leaving the patient vulnerable to secondary infections (Jacobi, 2002).

1.3 Transfusion-Associated Sepsis and Transfusion-Associated Bacterial Infection

Transfusion-associated bacterial infection (T-ABI) arises from the transfusion of bacterial contaminated blood products and is one of the biggest problems in transfusion medicine (Wilson-Nieuwenhuis *et al.,* 2017). Platelet concentrates (PCs) are particularly susceptible to bacterial contamination due to their storage conditions of high glucose and ambient (22^{o}C –

24°C) temperatures, which are ideal for bacterial growth; this in turn limits the shelf life of PCs to 5-7 days (Hong *et al.,* 2016). The source of contamination can be attributed to the venepuncture site, the presence of donor bacteraemia and whole blood processing (Blajchman *et al.,* 2005). The National Health Service Blood and Transplant (NHSBT) in England introduced routine screening of PCs in 2011 using an automated microbial detection system known as the BacT/ALERT® to ensure that contaminated PCs are effectively identified and withdrawn from stock (McDonald *et al.,* 2017). Screening is performed 6 h after donation and if the sample is culture negative, the blood product is released to hospitals labelled as "negative to date". The test aliquot remains within the Blood Centre for continuous BacT/ALERT® testing throughout the 7-day shelf life of the platelet product. If at a later time point, the aliquot flags as culture positive, the unit is either recalled, or if it has already been transfused, the patient is identified and treated with the most appropriate antibiotic regime. Like conventional sepsis, transfusion-associated sepsis (T-AS) occurs when there is a dysregulated host response to the bacterial infection, leading to organ dysfunction. The reported incidence of T-AS is likely to be an underestimate, as many PC recipients are leucopenic and/or immunosuppressed therefore, if the patient were to exhibit septic episodes, it is unlikely that the physician would suspect platelet transfusions as the cause (Blajchman and Goldman, 2001). In addition to this, symptoms of septic transfusion reactions broadly overlaps with other non-infectious transfusion reactions such as febrile non-haemolytic transfusion reactions, transfusion-associated circulatory overload and transfusion-associated acute lung injury (Hong *et al.,* 2016). If the recipient is also taking antibiotics, it could partially mask the symptoms associated with sepsis (Kuehnert *et al.,* 2001). Under-detection of bacterially contaminated platelet units within the NHSBT centres could also contribute to the low reported incidences of TA-S. The initial low bacterial inoculum or the formation of biofilms can both lead to false negative results from the BacT/ALERT® system (Murphy *et al.,* 2008), Biofilms are microbial communities adhered onto biotic or abiotic surfaces and are encased within an extracellular polymeric substance (EPS) (Hoffman *et al.,* 2005; Dong *et al.,* 2018). Bacterial cells within biofilms typically undergo phenotypic and genotypic changes, leading to altered cell growth which can bypass screening systems which rely on the production of CO_2 from respiring bacteria for detection (Benjamin and Wagner, 2007). Furthermore, the presence of biofilms in the original blood bag may decrease the

number of planktonic bacteria available for aliquoting into the test vials, resulting in false negatives and increased risk of T-BI and T-AS (Wilson-Nieuwenhuis *et al.,* 2017).

The severity of T-ABI is dependent on the causative bacteria with Gram negative species tending to cause more fatal reactions (60% of cases compared to Gram positive species) due to the presence of lipopolysaccharides in their cell wall (Kuehnert *et al.,* 2001; Védy *et al.,* 2009). Gram positive organisms on the other hand, are the predominant bacterial contaminants of blood products, especially commensals of the skin flora, with *Staphylococcus epidermidis* and *Propionibacterium acnes* being the most commonly isolated bacteria (Greco *et al.,* 2007). Gram negative microorganisms implicated in T-AS and T-ABI include *Serratia* spp., *Salmonella* spp., *Pseudomonas* spp., *Enterobacter* spp. and *Escherichia coli* (Védy *et al.,* 2009). The conditions of collection, preparation and storage of the different blood components constitute a broad range of environments that may select a specific bacterial species, subpopulation or even distinct multispecies consortia (Desroches *et al.,* 2018).

1.4 *Staphylococcus epidermidis*

S. epidermidis is a Gram positive, coagulase-negative, normal skin flora bacterium and is a major causative agent in T-ABI and T-AS (Greco *et al.,* 2007). Despite the use of disinfectant wipes to sterilise the phlebotomy site, *S. epidermidis* has still been found adhered firmly onto the donors' arm hairs enabling it to cause infection after wound entry (Mase *et al.,* 2000). The opportunistic pathogen was shown to display a slow-growth rate in PCs compared to other bacteria resulting in false negatives during screening (Brecher *et al.,* 2000; Mohr *et al.,* 2006). The pathogenesis of *S. epidermidis* is largely related to its ability to form biofilms, several studies have shown that even non-biofilm forming strains are able to switch to a biofilm phenotype within PCs (Greco *et al.,* 2007; Greco-Stuwart *et al.,* 2012; Loza-Correa *et al.,* 2019). The switch between strains exhibiting a biofilm-negative phenotype to a biofilm-positive phenotype was hypothesised to be due to antimicrobial peptides (AMPs) present within the PCs triggering a survival mechanism in *S. epidermidis* cells (Loza-Correa *et al.,* 2019). It is proposed that the trigger initiates pathways in which metabolism and cell growth are significantly reduced to reserve energy and, in the process, there is upregulation of virulence genes such as those involved in biofilm formation, as biofilms confer protection from antimicrobial agents (Hodgson *et al.,* 2014; Ali *et al.,* 2014). Platelets themselves are also

thought to play a role in biofilm formation as they can act as a scaffold for the primary attachment of *S. epidermidis* cells through the polysaccharide intracellular adhesin (PIA) (Ali *et al.,* 2014; Loza-Correa *et al.,* 2019). A study found that strains that have a fast growth rate produced less biofilms compared to strains with a slow growth rate (Ali *et al.,* 2014). This observation could be interpreted as that slow growing strains are not slow-growing *per se*, but rather have more growth associated with the biofilm than planktonic cells (Ali *et al.,* 2014).

1.5 *Serratia marcescens*

S. marcescens is a facultative anaerobe found in various environments such as soil, bodies of water, plants and animals (Cristina *et al.,* 2019). It is also a commensal of the human gastrointestinal tract and was once thought to be non-pathogenic to humans due to its low virulence (Ramírez-Acros *et al.,* 2006). Colonies of this bacterium can be identified by production of the pigment prodigiosin (red) and is thought to be associated with increased growth compared to non-prodigiosin producing strains (Haddix and Shanks, 2018). The role of *S. marcescens* as an opportunistic pathogen was brought to light after being found as the causative agent for the T-AS epidemic in 1991 in Denmark and Sweden (Heltberg *et al.,* 1993; Högman *et al.,* 1993). The epidemic occurred as a result contamination of the blood bags during the cooling step of the manufacturing process, this led to the subsequent transfusion of *S. marcescens* resulting in three fatalities (Heltberg *et al.,* 1993; Högman *et al.,* 1993; Szewzyk *et al.,* 1993; Roth *et al.,* 2000). The material of the blood bags, polyvinylchloride, was thought to contribute to the survival of *S. marcescens* as it permits the evaporation of fluids creating a humid environment in which the pathogen thrives (Heltberg *et al.,* 1993). Countermeasures were implemented to prevent such contaminations through the use of oxygen and water reduction technologies and ventilated with nitrogen to prevent the growth of aerobic microorganisms (Högman *et al.,* 1993). However, this technique has no effect on *S. marcescens* as it is a facultative anaerobe (Högman *et al.,* 1993).

The ubiquitous nature of *S. marcescens* contributes to its adaptability and its survival within healthcare environments and as such, it can be found in sinks, soap dispensers, disinfectants and medical apparatuses making it difficult to fully eradicate (Dessi *et al.,* 2009). As a consequence, transmission of *S. marcescens* can occur through the hands of healthcare workers leading to nosocomial infections; most noticeably 16% of Gram negative bacteraemia

cases within the neonatal intensive care unit (Stock *et al.,* 2003; Arslan *et al.,* 2010; Voelz *et al.,* 2010; Greco-Stewart *et al.,* 2012). In addition to its ability to survive within hostile environments, studies have shown that strains of *S. marcescens* possess R factors which are plasmids that encode for the resistance to multiple antibiotics, adding to the difficulty of eradicating this pathogen (Stock *et al.,* 2003; Dessi *et al.,* 2009). Other studies have also demonstrated that *S. marcescens* is able to resist phagocytosis which may explain its survival within blood components (Högman *et al.,* 1993). *Serratia* spp. has also been reported to remain culture negative even after 9 days of storage in PCs (Ramírez-Acros *et al.,* 2006).

1.6 *Escherichia coli*

The main reservoir of the Gram negative *E. coli* is the gastrointestinal tract of humans and animals in which horizontal transfer of plasmids occur, leading to high numbers of genetically variable populations and pathogenicity (Desroches *et al.,* 2018). *E. coli* can be divided into four phylogenetic groups: A, B1, B2 and D in which groups B2 and D are the most highly virulent strains, whilst groups A and B1 are generally considered commensals (Jauréguy *et al.,* 2007). The opportunistic pathogen is able to translocate from the intestines to the bloodstream causing bacteraemia and extra-intestinal infections which often precedes sepsis; donors may often present with an asymptomatic bacteraemia at the time of donation (Conceição *et al.,* 2012; Mosavie *et al.,* 2019). The origin of *E. coli* bacteraemia in donors is most commonly caused by urinary tract infections as inflammation of the urinary tract promotes the translocation of *E. coli* from the bladder to the bloodstream (Desroches *et al.,* 2018). In 20% of cases of bacteraemia, *E. coli* was the most prevalent isolate and accounted for 17% of septic episodes in 2001 in the USA, resulting in 40, 000 deaths (Jauréguy *et al.,* 2007).

Within PCs, the abundance of AMPs (mostly released by the platelets themselves), antibodies and complement proteins provides a harsh environment for bacteria to survive in (Taha *et al.,* 2016). As *E. coli* continually alternates between its niches, i.e. the intestines of vertebrates and the outside environment, its versatility enables it to colonise new niches and generates a population that survive optimally in PCs (Bleibtreu *et al.,* 2013). These strains when transfused have often gained serum resistance, which proves to be highly advantageous for it to cause infection within the recipient, and thus T-ABI/T-AS occurs (Desroches *et al.,* 2018).

Iron availability was also thought to be partly responsible for the selection of T-ABI/T-AS strains as it was demonstrated that implicated strains grew rapidly in the anticoagulant citrate solution of PCs where iron was limited since iron sustains bacterial growth during infection (Desroches *et al.,* 2018; Johnsen *et al.,* 2019). Another issue with citrate solutions is that a low abundance of iron within the solution selects for strains that over express siderophores, this characteristic is commonly associated with many T-ABI strains, including *E. coli* (Desroches *et al.,* 2018; Gravemann *et al.,* 2019). As *E. coli* is one of the leading causes of Gram negative bacteraemia due to its ability to translocate from the host's intestines, the health status of donors along with follow-ups post donation should be implemented to detect clinical signs of bacteraemia to minimise the risks of PC contamination (Desroches *et al.,* 2018).

1.7 Innate Immunity

Innate immunity is the first line of defence against infection, consisting of anatomic barriers, humoral factors and cellular components, until the more specific adaptive immunity is activated. Harmful stimuli will trigger an inflammatory response involving the migration of leucocytes to the injured or infected area, vasodilation of the blood vessels and secretion of proinflammatory mediators. Initiation of the innate response requires the recognition of PAMPs or DAMPs by pathogen recognition receptors (PRRs) found on various innate cells (neutrophils, monocytes, macrophages, dendritic cells etc.). Recognition of PAMPs and/or DAMPs will trigger downstream signalling pathways for pathogen clearance and wound repair (Jiang *et al.,* 2007). However, excessive inflammation can lead to a number of chronic inflammatory diseases and SIRS. On the other hand, inadequate inflammation will result in persistent infections and compromised wound healing. Therefore, a balance between hyper- and hypo-inflammation is necessary in the development and resolution of a normal immune response to infection.

Activation of the inflammatory response is mediated by a multiprotein complex, called the inflammasome, which promotes the secretion of IL-1β and IL-18 by activated macrophages, monocytes and platelets (Martinon *et al.,* 2002). The inflammasome is composed of several NOD-like receptor (NLR) proteins (NALP1, NLRP3 and NLRC4), the ASC adaptor molecule, and caspase-1 and -5 (Martinon *et al.,* 2002). Upon stimulation by microbial factors or endogenous signals through the recognition by the NLR family proteins, there is an increased

production of pro-IL-1β and pro-IL-18 (inactive forms of IL-1β and IL-18) (Kumar, 2018). Caspase-1 is self-activated *via* self-cleavage leading to proteolytic activity on pro-IL-1β and pro-IL-18 and subsequent secretion of IL-1β and IL-18 (Guo *et al.,* 2015). Both proinflammatory cytokines are then able to exert their effect and recruit inflammatory cells to the site of injury or infection leading to a positive feedback mechanism in which various other proinflammatory cytokines and chemokines are secreted (Martinon *et al.,* 2002).

Migration of circulating leucocytes to the chemotactic gradient established involves tightly coordinated interactions between the leucocyte and the endothelium in three steps. The first step is margination and rolling which involves the low-affinity interaction between L-selectin constitutively expressed on leucocytes and P-selectin glycoprotein ligand-1 (PSGL-1), and E- and P-selectin on endothelial cells upregulated by inflammatory stimuli. This reversible adhesion allows the leucocytes to roll along the vasculature towards the inflamed site (Alves-Filho *et al.*, 2010). The next step is the firm adhesion between the leucocytes and the endothelial cells *via* β_2 integrin (CD11b/CD18) and their respective receptors; intracellular adhesion molecule 1 (ICAM-1/CD54) and vascular cell adhesion molecule 1 (VCAM-1/CD106) (von Hundelshausen and Weber, 2007). This initiates the activation of leucocytes and L-selectin shedding for the transition from rolling to firm adhesion (Hafezi-Moghadam *et al.,* 2001; Shen *et al.,* 2017). Following firm adhesion, the cytoskeleton of the leucocyte is rearranged to an amoeba-like state allowing leucocytes to pass through the tight junctions of endothelial cells and extravasate into the interstitial fluid (Alves-Filho *et al.,* 2010).

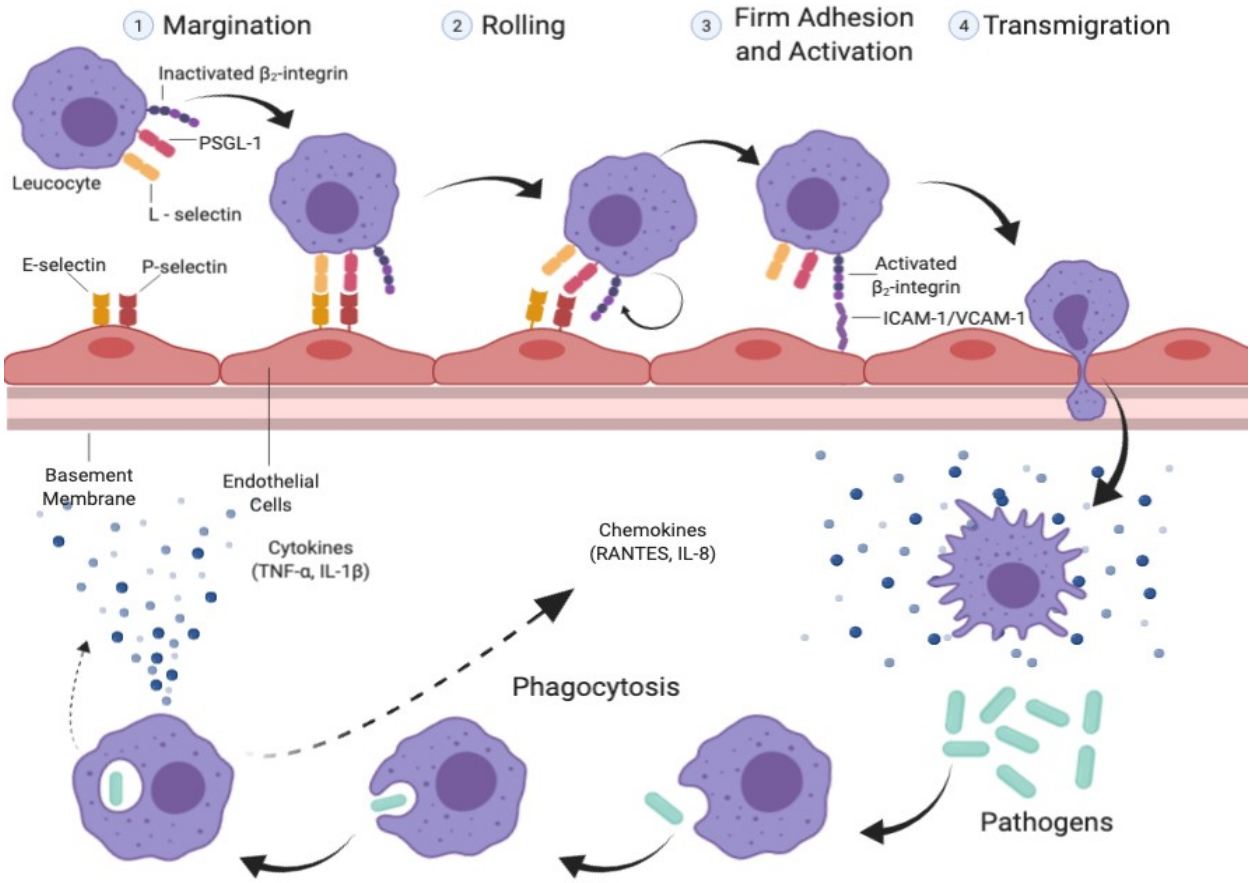


Figure 2: Steps involved in leucocyte transmigration. 1) Inflammation activates the endothelium to express adhesion molecules, E- and P-selectin, ICAM-1 and VCAM-1 along with secretion of cytokines and chemokines for leucocyte recruitment. 2) Rolling involves weak interactions between L-selectin and PSGL-1 on leucocytes to E- and P-selectin on endothelial cells. 3) The β_2 integrin on leucocytes binds to ICAM-1 or VCAM-1 on endothelial cells with a strong affinity causing arrest and subsequent crawling along the vasculature before extravasation. 4) The leucocyte releases proteolytic enzymes to digest through the basement membrane allowing it to enter the site of inflammation to perform its effector functions. Subsequent cytokine and chemokine release (dashed arrows) by the leucocyte sustain recruitment of leucocytes. Adapted from Schooner and Parkos, 2008.

1.7.1 Neutrophils

Neutrophils are phagocytic, polymorphonuclear cells characterised by their distinctive multi-lobed nuclei and are the most abundant circulating leucocytes. They possess an arsenal of antimicrobial factors such as neutrophil extracellular traps (NETs), proteolytic enzymes and reactive oxygen species (ROS) to aid in pathogen clearance (Shen *et al.,* 2017). Playing an essential role in innate immunity, they are the first cells to respond to inflammatory stimuli and the control of an infection depends on their efficiency to transmigrate (Fig. 2). Failure to eradicate the invasive pathogen would lead to its systemic spread resulting in sepsis. In septic conditions, neutrophil effector functions are impaired along with altered chemotactic activity (Sônego *et al.,* 2016). Nitric oxide (NO), synthesised by inducible-nitric oxide synthase (iNOS) in leucocytes, is a molecule with cardiovascular, immunomodulatory and neuronal properties and is responsible for the bactericidal activity of neutrophils (Wolkow, 1998). However, its overproduction has been shown to contribute to the pathogenesis of septic shock, demonstrated by significant vasodilation and subsequent hypotension, hyporesponsiveness to vasopressors, and organ damage (Petros *et al.,* 1994). Several studies have also demonstrated the inhibitory effects of NO on neutrophil chemotaxis along with the proinflammatory cytokines, TNF-α, macrophage-derived neutrophil chemotactic factor (MNCF), IL-1 and IL-8 by decreasing the adhesiveness of neutrophils to the endothelium (Tavares-Murta *et al.,* 1998; Benjamim *et al.,* 2000; Benjamim *et al.,* 2002).

The IL-8 chemokine receptor, CXCR2, found on the surface of neutrophils is also critical for neutrophil migration and its expression is regulated by the PRR, toll-like receptor-2 (TLR-2) and TLR-4 (Eash *et al.,* 2010). Recognition of bacterial peptidoglycan and lipopolysaccharide by TLR-2 and TLR-4 respectively leads to the activation of the GRK2 pathway and resultant internalisation of CXCR2 on neutrophils; this mechanism is important to keep the neutrophils within the infectious loci to eliminate invasive pathogens (Alves-Filho *et al.,* 2009). However, in septic conditions CXCR2 is significantly downregulated which may be due to the effect of simultaneous release of pro- and anti-inflammatory cytokines as well as the overstimulation of the TLRs leading to desensitization of CXCR2 thus defective neutrophil migration (Van Der Meeren *et al.,* 1999; Benjamim *et al.,* 2000; Souto *et al.,* 2011). In addition to this, patients with sepsis have been observed to have significant serum soluble L-selectin levels, likely due to the increased L-selectin shedding in response to the uncontrolled systemic inflammation

(Ferri *et al.,* 2009). Consequently, neutrophils are strongly adhered onto the endothelium and sequester within the vascular compartment causing tissue ischaemia and organ dysfunction (Lerman and Kim, 2015).

Other effector functions of neutrophils observed to be suppressed are ROS production and phagocytosis (Kaufmann *et al.,* 2006). Impaired phagocytosis could be explained by desensitisation of CD64 (human IgG receptor FcγRI) under excessive inflammatory conditions, a receptor that is responsible for phagocytic activity (Shen *et al.,* 2017). On the contrary, NET release appears to be upregulated in sepsis due to metabolic changes induced in sepsis and is correlated with severe organ dysfunction. It is thought that NET can mediate the vascular endothelium damage *via* myeloperoxidase activity and promote thrombus formation, as it can provide a scaffold for platelets and erythrocytes to attach (Shen *et al.,* 2017).

1.7.2 Monocytes/Macrophages

Monocytes are mononuclear, phagocytic cells which can differentiate into macrophages, osteoclasts or dendritic cells depending on their microenvironment (Cavaillon and Adib-Conquy, 2005). The main role of monocytes in the absence of infection is the removal of apoptotic cells and cellular debris. During infection, they are the one of the first responders, alongside neutrophils, in the control of pathogens and are a source of inflammatory cytokines such as TNF-α, IL-1α and IL-1β (Cavaillon and Adib-Conquy, 2005). Macrophages can act as a bridge between the innate and adaptive immune response through antigen presentation.

Similarly to neutrophils, the effector functions of monocytes/macrophages are also impaired in sepsis in which their capacity to produce proinflammatory cytokines are diminished along with decreased antigen presentation (Sinistro *et al.,* 2008). Monocyte recruitment was also observed to be reduced as a consequence of downregulation of CCR5 (receptor for the monocyte chemotactic protein) expression through TLR-2 signalling (Souto *et al.,* 2011) (Fig. 2). As patients progress into the immunosuppressive phase of sepsis, monocytes adopt the immunosuppressive phenotype where they are unable to respond to secondary infections, and patients are at increased risk of morbidity and mortality (Drewry *et al.,* 2018). This phenomenon could be explained by monocytes gaining lipopolysaccharide (LPS) tolerance following challenge induced by the transcription factor, hypoxia inducible factor-1α (Ertel *et*

al., 1995; Salomao *et al.,* 2002; Shalova *et al.,* 2015). However, studies have shown that monocytes still possess phagocytic abilities and other antimicrobial activities as well as tissue repair in septic patients (Shalova *et al.,* 2015; Santos *et al.,* 2016). This suggests that rather than suppression of genes involved in effector functions, monocytes are "reprogrammed" due to the multitude of stimuli occurring in sepsis (Shalova *et al.,* 2015). In support of this, Tang et al. failed to identify a strict pro- or anti-inflammatory phenotype at a genomic level and suggested that leucocytes exhibit both pro- and anti-inflammatory phenotypes simultaneously.

Secretion of proinflammatory cytokines and activation of the adaptive immunity requires the ligation between CD40 and CD40 ligand (CD40L) leading to the increased expression of surface CD80 and CD86 for T cell activation (Sinistro *et al.,* 2008). Monocytes isolated from patients with sepsis displayed an inability to activate CD40L and therefore failure to induce T cell effector functions (von Müller *et al.,* 2006). Instead, monocytes undergo spontaneous apoptosis or anergy due to the absence of costimulatory signals through T cell interactions (Sinistro *et al.,* 2008). In addition, isolated monocytes from sepsis patients show a markedly reduced expression of Human Leucocyte Antigen – DR isotype (HLA-DR), a complex critical in antigen presentation. This reduced expression is associated with poor prognosis in sepsis (Hotchkiss *et al.,* 2013). The mechanism behind this event is currently still under investigation however, one theory suggests that sustained secretions of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β1 (TGF-β1) may inhibit the expression of HLA-DR (Monneret *et al.,* 2004).

1.8 Platelets

Platelets are anucleate fragments of megakaryocytes essential for the maintenance of haemostasis. Deviation from the norm will trigger the coagulation cascade to prevent blood loss and to preserve vascular integrity. Individuals with haematological or oncological disorders may suffer from thrombocytopenia as a complication of the disease and would require platelet transfusions to alleviate symptoms (Wilson-Niewenhuis *et al.,* 2017). Recently, significant attention has focused on the role of platelets in innate immunity, rather than solely considering these cells as "blood clotters". The discovery that platelets express all ten TLR transcripts implies that platelets have the ability to recognise PAMPs on invading

microorganisms (Akinosoglou and Alexopoulos, 2014). It has since been shown that recognition of PAMPs leads to platelet activation, release of AMPs and secretion of a variety of cytokines and chemokines from platelet α-granules that act to coordinate immune responses. In addition to this, platelets were also shown to have phagocytic properties in which immunohistochemical staining revealed *Staphylococcus aureus* were contained within a vacuole, similar to a phagosome in phagocytes, after incubation (Youssefian *et al.,* 2002). However, the outcome of the internalised bacteria remains to be elucidated but it has been suggested that merging of the α-granule and vacuole may facilitate bacterial killing (Youssefian *et al.,* 2002; Hamzeh-Cognasse *et al.,* 2018). It is possible that the combination of these abilities allows contaminated platelet units to self-sterilise as a result of bactericidal action during storage but their efficacy is still not clearly understood (Brecher, 2018).

As one of the major sources of cytokine release, it is believed that platelets are a major contributor of the "cytokine storm" and uncontrolled activation in sepsis can lead to DIC and tissue ischaemia resulting in considerable platelet consumption and thus thrombocytopenia (Venkata *et al.,* 2013). Production of non-specific, anti-platelet antibodies by B cells as well as cytokine-driven haemophagocytosis of platelets can also contribute to sepsis-induced thrombocytopenia (Venkata *et al.,* 2013). Thrombocytopenia was shown to impair host defence leading to increased bacterial loads, which further implies that platelets have a role in innate immunity (Zhou *et al.,* 2018). Administration of anti-platelet drugs such as aspirin and $P2Y_{12}$ inhibitors, both of which are used extensively in patients to reduce the haemostatic function of hyperactive platelets, was shown to be beneficial in reducing mortality in sepsis patients (Thomas *et al.,* 2015). Downregulation of P-selectin, inhibition of platelet-leucocyte aggregates, diminished prothrombotic activity and, most importantly, decreased secretion of cytokines and chemokines was observed following anti-platelet treatment in a human model of sepsis (Thomas *et al.,* 2015).

1.9 RANTES

RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) is a proinflammatory chemokine responsible for activation and trafficking of T cells, dendritic cells, basophils, natural killer cells, eosinophils and monocytes (Bacon *et al.,* 1995). The effects of RANTES on neutrophils is currently still under investigation as some studies have shown that

RANTES is able to activate neutrophils (Appay *et al.,* 1999; Ajuebor *et al.,* 2001; Ramakrishna and Cantin, 2018) while others reported that RANTES had no effect on neutrophil function (Schall *et al.,* 1990; Rot *et al.,* 1992; Proudfoot *et al.,* 1996). It was first discovered that T cells and platelet α-granules were the major sources of RANTES but it is now known to be secreted by other cells at lower levels such as endothelial cells, epithelial cells and osteoclasts (Mohs *et al.,* 2017). Contrary to the evidence for a pro-inflammatory role for RANTES, other data suggest that RANTES inhibits the release of histamine and therefore halts the inflammatory response, instead shifting the cellular environment towards wound repair and healing (El-Sharkawy *et al.,* 2007). Nonetheless, these conflicting studies all suggest that platelets, as major contributors to the RANTES pool, have an important role in regulating the immune response.

Pilot work from our research group (Wilson-Niewenhuis *et al.,* 2018) has found that artificially contaminating platelets with bacteria (particularly in biofilm form) leads to the secretion of high concentrations of RANTES whilst other cytokines and chemokines (such as IL-1β, TNF-α and IL-6) remained relatively unaffected. Reasons for this selective upregulation and the significance of it remain to be elucidated. Elevation of RANTES was also reported in sepsis, correlating with infection severity, tissue damage and mortality (Cavaillon *et al.,* 2003; von Hundelshausen *et al.,* 2007; Koupenova *et al.,* 2015).

This upregulation of RANTES in response to the bacteria is in addition to the increased release of this chemokine due to storage of the platelets in the blood bank. Indeed, factors secreted by platelets during storage will accumulate over time with high levels of RANTES providing an indication of platelet activation or destruction (Boehlen and Clemeston, 2001). There is also a possible correlation between platelet counts and the level of RANTES present in stored platelet units (Klinger et al., 1995; Fujihara et al., 1999). The shelf life of platelet concentrates in the blood bank is 5-7 days, and the accumulation of RANTES within these products over this time period is sufficient to trigger febrile reactions in recipients. Furthermore, RANTES may work synergistically with other chemokines to promote the severity of transfusion reactions (Boehlen and Clemeston, 2001).

It is clear that contaminated platelet units can lead to the development of sepsis in the transfusion recipient. However, what is not clear is what pushes the recipient's immune system in to overdrive, to produce such an exaggerated response to the infection. Since RANTES is released in large quantities from platelets in response to bacteria (Wilson-Nieuwenhuis *et al.,* 2018), and platelets are vital in promoting activation of other components of the innate immune system (von Hundelshausen and Weber*,* 2007), the role of RANTES in direct activation of such components warrants further investigation. From 2015 to 2018, there were 422 cases of suspected bacterial infections with 273 of these cases found to be negative for bacterial contamination (upon follow-up testing of the test aliquot), inconclusive, or due to late detection of bacteria upon further testing (SHOT, 2015; SHOT, 2016; SHOT, 2017; SHOT, 2018). This indicates that sepsis is largely an immunological pathology rather than bacterially driven. Research within this area would clarify the significance of RANTES in immunological responses to bacterial infections, and may add further weight to the use of RANTES as a marker for monitoring severity of immunological reactions. Further, it may highlight its use as a therapeutic target in patients with T-ABI/T-AS.

1.10 Aim

The aim of this project is to establish the effects of platelet releasates on neutrophil and monocyte responses, thereby contributing to a deeper understanding of the mechanism behind the link between bacterially contaminated platelet transfusions and infection/sepsis severity in transfusion recipients.

1.11 Objectives

- Determine the cytokine/chemokine profile being released from platelets in response to different microorganisms.
- Determine the effects of the platelet releasate from bacterially-contaminated platelets on the activation status of primary human neutrophils, HL-60 neutrophils and U937 monocytes, by flow cytometric assessment of established activation markers (CD11b, CD54 and CD66b).

- Determine the effects of recombinant human RANTES (rhRANTES) on the activation status of primary human neutrophils, HL-60 neutrophils and U937 monocytes, by flow cytometric assessment of established activation markers (CD11b, CD54 and CD66b).

2.0 Methods

2.1 Media and Bacterial Preparation

All media used was prepared in accordance the manufacture's guidance and sterilised using an autoclave at 121^{o}C and 100 kPa for 20 min. The media was cooled in a water bath at 50^{o}C for 30 min. Stock cultures of *S. epidermidis* DSM28319, *S. marcescens* NCTC9741*, E. coli* NCIMB10213 (wild-type/WT) and multi-resistant *E. coli* 82268 were streaked onto tryptone soy agar (TSA) (Oxoid, UK), stored in a chiller (4°C) and replaced every 4 weeks. A 10 mL volume of tryptone soy broth (TSB) (Oxoid, UK) was added to a sterile universal, and a single colony of *S. epidermidis, S. marcescens, E. coli* and multi-resistant *E. coli* was added and the culture was incubated for 24 h at 37^{o}C. The culture was centrifuged at 1731 *g* for 10 min and the supernatant discarded. The cells were washed with 10 mL of sterile distilled water and centrifuged again. The supernatant was again discarded and the pellet resuspended with 10 mL of sterile distilled water, vortexed and adjusted to an optical density (OD) of 1.0 ± 0.05 at 540 nm using a spectrophotometer (Jenway 3605, UK); sterile distilled water was used as the blank. The cell concentration corresponded to approximately 1.00 x 10^{9}, 2.41 x 10^{8}, 1.3 x 10^{9} and 5.73 x 10^{9} colony-forming units per mL (CFU mL) for *S. epidermidis*, *S. marcesens, E. coli* and multi-resistant *E. coli* respectively. This was calculated after performing serial dilutions and plate counts (n = 3).

2.2 Zone of Inhibition (ZoI) Assay

One hundred microliters of cell suspension were pipetted and spread evenly across 25 mL TSA plates. An antibiotic testing ring (Gram negative and Gram positive specific) (Mast Group Ltd., UK) was placed on the agar and incubated at 37^{o}C for 24 h. The zones of inhibition were measured in mm and an average mean value was determined (n = 3). Antibiotic susceptibility was determined by comparing ZoIs to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Version 9.0 database.

2.3 Cytometric Bead Array (CBA)

2.3.1 CBA Standard Preparation

The standards for CBA were prepared according to the manufacturer's guidance. A vial of lyophilized standard from each BD CBA Human Soluble Protein Flex Set were pooled into one

15 mL falcon tube and was labelled as the 'Top Standard'. The standards were reconstituted with 4 mL of Assay Diluent and was equilibrated for 15 min at room temperature then mixed with a pipette. A dilution of 1:2 of the standard was made by pipetting 500 μL of the top standard with 500 μL of the Assay Diluent and was thoroughly mixed with the pipette. Further dilutions were made to obtain 1:4, 1:8, 1:16, 1:128 and 1:1256 concentrations with the assay diluent as the blank.

2.3.2 Capture Beads and PE Detection Reagent Preparation

The capture beads stock vials for TNF-α, IL-1β, IL-6, and RANTES were vortexed for 15 s to ensure thorough resuspension of the beads. Undiluted capture beads were provided so that 1 μL was used for one test. The volume required for each capture beak stock was scaled according to the number of tests run and the volume of capture bead diluent required was calculated as follows:

Capture Bead Diluent (μL) = (Number of Tests x 50 μL) – (Number of Cytokines x Number of Tests) [1]

The capture beads were pipetted into a tube containing capture bead diluent labelled 'Mixed Capture Beads'. The 'Mixed PE Detection Reagent' was prepared following the same procedure, using the PE detection reagent provided in each kit and the detection reagent diluent. The mixed PE detection reagent was then stored at 4°C and protected from light until ready for use.

2.3.4 Performing the Human Soluble Protein Flex Set Assay

A volume of 50 μL of either a standard or a test sample was added to the appropriate tubes. The mixed capture beads were vortexed for 5 s and 50 μL was added to all tubes and mixed with a pipette. The tubes were incubated at room temperature for 1 h. After this incubation, 50 μL of the mixed PE detection reagents were added to each tube, gently mixed with a pipette and incubated at room temperature for 2 h in the dark. After incubation, 1 mL of wash buffer was added to each tube, mixed and the tubes were centrifuged at 200 g for 5 min. The supernatant was carefully aspirated with a pipette and 300 μL of wash buffer was added to each tube followed by a brief vortex to resuspend the beads. The BD Accuri C6F1 flow cytometer was set up with the 2 blue, 2 red instrument configuration and the FL3 laser filter

was changed to the 780/60 filter. The beads were run through a 2000 event limit in the R1 gate at medium flow rate. The standards were run first, starting at the lowest concentration first, followed by the samples. The data was exported to FCAP Array software (Version 3.0) to be analysed.

2.4 Cell Culture and HL-60 Differentiation

U937 and HL-60 cells were cultured in RPMI-1640 media (Lonza, Belgium) supplemented with 10% foetal bovine serum (Sigma-Aldrich, UK) and 2% penicillin streptomycin (Lonza, Slough, UK) and incubated at 5% CO_2 for 24 h at 37°C. The media was changed every 72 h and cells were cultured at a concentration of 0.5 x 10^6 cell/mL. A ratio of 1:1 of cell suspension and trypan blue (Sigma-Aldrich, UK) were used to assess cell viability using a TC10 automated cell counter (BioRad, Singapore).

A concentration of 0.5 x 10^6 cells/mL of HL-60 were differentiated into neutrophils with the addition of 1.3% dimethyl sulfoxide (DMSO) (Fisher). The cells were incubated for 48 h and 72 h at 37°C and 5% CO_2. Confirmation of HL-60 cells differentiation into neutrophils were assessed by measuring the expression of CD11b. Untreated differentiated HL-60 and U927 cells were used as negative controls. PRP treated cells were used as positive controls for the protocols described in 2.7.3 and 2.7.4.

2.5 Isolation of Human Primary Neutrophils

Ethical approval for the use of primary human neutrophils was granted. Healthy adult volunteers gave written, informed consent to donate whole blood. Whole blood was collected in ethylenediaminetetraacetic acid (EDTA) coated vacutainers (BD Bioscience, New Jersey, USA). Lysing solution was made using 0.8% of ammonium chloride (Sigma-Aldrich, Dorset, UK) and 0.1 mM of EDTA (Sigma-Aldrich, Dorset, UK) in sterile distilled water buffered with potassium bicarbonate (Sigma-Aldrich, Dorset, UK) to a pH of 7.2 to 7.6. Neutrophils were isolated by adding lysing solution to whole blood in a 1:10 ratio and was gently mixed with a pipette and placed in ice for 10 min. The mixture was centrifuged for 5 min at 500 *g* with the supernatant discarded, and the cells were washed twice with 45 mL of Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza, Belgium). Cells were counted using the automated counter and ready for experimentation.

2.6 May-Grüwald Stain

HL-60 promyelocytes, HL-60 DMSO-treated neutrophils and primary human neutrophils were visually confirmed through May-Grüwald stains (VWR, UK). A volume of 200 µL of neutrophils were taken and smeared onto glass slides using the cytospin (Sigma-Aldrich, UK) at 500 *g* for 10 min. The samples were then fixed with methanol (Fisher Scientific, Leicestershire, UK) for 5 min and dried at room temperature. The May-Grüwald stain was diluted at a ratio of 1:10 with distilled water and samples were immersed with the diluted stain for 4 min. The stain was rinsed off by pouring distilled water and dried at room temperature in the class II airflow cabinet ready to be observed under the light microscope.

2.7 Bacterial Contamination of Platelet-Rich-Plasma (PRP)

2.7.1 Biofilm Growth

Plasticized poly(vinyl chloride) (p-PVC) platelet bags (Terumo BCT, USA) were hole punched into coupons with a diameter of 11 mm. The p-PVC coupons were sterilised in 70% ethanol for 10 min and rinsed in distilled water and dried in the class II airflow cabinet. A sterilised p-PVC coupon with the rough surface up, was placed in each well of a 12-well plate (Nunc Non-Treated Multidishes, Thermo Fisher Scientific, UK) along with 1.8 mL of TSB. The coupons were pushed to the bottom of well using the reverse end of a sterile swab. The 12 well plate was left at room temperature overnight on the bench to determine if the coupons were fully sterile and to identify any contaminant growth present. An overnight culture of the test microorganism was prepared and diluted to obtain a concentration of 1.0×10^8 CFU mL. A bacterial suspension of 200 µL was added to the appropriate wells to give a working concentration of 1.0×10^7 CFU mL. The 12 well plate was parafilmed to prevent the media from evaporating. The plates were incubated at 22^{o}C for 7 days with gentle shaking (68 *rpm*). Following incubation, all the media in each well was removed via pipetting, leaving the coupons and biofilm behind. The wells were all washed with 2 mL of Tyrode's buffer, carefully at the side of the well to not disturb the biofilm and the plate was gently shaken manually for 5 s. The buffer was pipetted out to remove any unbound planktonic cells. The 12 well plates and coupons were air dried in a class II cabinet for 1 h.

2.7.2 PRP Isolation

A volume of 20 mL of whole blood from healthy volunteers was collected into 3.2% sodium citrate vacutainers (BD Biosciences, New Jersey, USA). The vacutainers were centrifuged at 180 *g* for 20 min to compact the red blood cells to the bottom of the tube whilst the PRP was left as the supernatant. The PRP was pipetted out without disturbing the compacted red blood cells into sterile 50 mL falcon tubes.

Note: The total volume of PRP obtained will vary from donor to donor, as this is dependent on their haematocrit.

2.7.3 PRP Treatment of Biofilms

The PRP was diluted with Tyrode's buffer in a ratio of 1:1 in 50 mL falcon tubes prior to being added to the biofilms. Two millilitres of diluted PRP were added to each well. These were incubated at 22^{o}C for 4 h with gentle agitation (68 *rpm*) prior to experimental work. Following incubation, the bacterial contaminated PRP was removed from the wells and placed in sterile 15 mL falcon tubes. They were spun down at 1200 *g* for 10 min and the resultant supernatant was pipetted into sterile 15 mL falcon tubes to remove the platelets. The supernatant was centrifuged at 1721 *g* for 5 min to remove the remaining bacteria from the sample. A hundred microliters of platelet and bacteria free supernatant was added to the appropriate wells of a 96 well plate (Sarstedt, Germany) containing 100 μL of U937 cells or HL-60 neutrophils. The 96 well plate was incubated at 37^{o}C, 5% CO_2 for 24 h for flow cytometry.

Note: Experiments with S. marcescens were discontinued as the production of coagulase interfered with the experiments.

2.7.4 PRP Treatment of Planktonic Bacteria

An overnight culture of *S. epidermidis*, *E. coli* and multi-resistant *E. coli* was made up to a concentration of 2 x 10^7 CFU mL with Tyrode's buffer. The culture was diluted to 1 x 10^7 following the addition of PRP at a ratio of 1:1 in sterile eppendorf tubes. The samples were incubated at 22^{o}C for 4 h with gentle agitation (68 *rpm*) prior to flow cytometric analysis.

2.8 Recombinant RANTES Treatment

A stock solution of RANTES was prepared by mixing 200 µL of sterile DPBS and 20 µg of RANTES powder (Pepro Tech, UK). Working solution 'A' was made using a 1 in 100 dilution of the stock solution and sterile RPMI-1640 media i.e. 2 µL of RANTES stock solution to 198 µL RPMI-1640 media with the final concentration of working solution 'A' as 1 µg/mL. The working solution 'A' was diluted by 1 in 50 to achieve a final concentration of 20 ng/mL, further dilutions was made to obtain concentrations of 2 ng/mL, 0.2 ng/ 4 mL, 0.02 ng/mL and 0.002 ng/mL. A hundred microliters of the appropriate concentrations were added to wells containing 100 µL of either U977 monocytes, HL-60 neutrophils or human primary neutrophils in a 96 well plate. The 96 well plate was incubated at 37^{o}C, 5% CO_2 for 24 h before flow cytometry.

2.9 Flow Cytometry

After incubation, the cells were transferred into 1.5 mL eppendorf tubes and centrifuged for 5 min at 500 *g* with the supernatant discarded. The cells were washed with 500 µL of RPMI-1640 media and centrifuged again. The supernatant was removed and the cell pellet was re-suspended with 200 µL of paraformaldehyde (Sigma-Aldrich, UK) and was left to incubate for 10 min at room temperature. This was followed by an addition of 200 µL of DPBS and was centrifuged immediately. The number of samples that were required to be stained with anti-CD11b, anti-54 and anti-66b (BD Pharmingen, USA) was determined and the antibodies were diluted in a 1:20 ratio with washing buffer (10% FBS in DPBS). A volume of 50 µL of the antibody mixture was added to the appropriate samples and was incubated in the fridge in the dark for 30 min. The samples were washed with 200 µL of washing buffer and was centrifuged at 500 *g* for 5 min. The supernatant was removed and the samples were re-suspended with 500 µL DPBS. CD54, CD11b and CD66b expression was assessed through 10,000 events (live cells) using flow cytometer with the BD Accuri C6 software (Biosciences, USA) after gating in the forward scatter/side scatter and fluorescence parameter 1, 2 and 4 (FL1, FL2, FL4) windows. Data was presented as average percentage $CD54^{+}$, $CD11b^{+}$ and $CD66b^{+}$ cells (%) and median fluorescence intensity (MFI) relative to unstained U937, HL-60 neutrophils and primary human neutrophils from three independent experiments.

2.10 Statistical Analysis

All statistical analysis was performed in GraphPad Prism version 7.00. Results were presented with standard error of the mean (SEM) as error bars on graphs. As data One-Way ANOVA and Tukey post-hoc test was used to compare significance between mean values at 95% confidence.

3.0 Results

3.1 Zones of Inhibition of Transfusion-Associated Sepsis/Transfusion-Associated Bacterial Infection Relevant Bacteria

Zones of inhibition (ZoI) assays were carried out to determine the antibiotic resistance of the four pathogens relevant to transfusion-associated sepsis/transfusion-associated bacterial infection (T-AS/T-ABI) used in this work, which are *S. epidermidis, S. marcescens, E. coli* and multi-resistant *E. coli*.

Multi-resistant *E. coli* displayed the greatest antibiotic resistance (hence its name) with resistance found in 5 out of 8 antibiotics tested (ampicillin, kanamycin, nalidixic acid, nitrofurantoin and tetracycline) (Fig. 3). Chloramphenicol appears to be the only antibiotic to have antimicrobial activity to multi-resistant *E. coli* with a ZoI of 20 mm. The EUCAST breakpoints for colistin sulphate, kanamycin and streptomycin have not been previously established therefore the sensitivity of *E.* coli and multi-resistant *E. coli* to these antibiotics cannot be determined. However, multi-resistant *E. coli* appear to be weakly susceptible to both antibiotics.

S. marcescens had the second greatest antimicrobial resistance as no zones were observed for ampicillin, colistin sulphate, nitrofurantoin and streptomycin (Fig. 4). According to EUCAST, chloramphenicol is the only antibiotic with antimicrobial activity against *S. marcescens*. Data for nalidixic acid, and tetracycline was not found on the EUCAST database for Enterobacterales therefore, no comments can be made on their antimicrobial activity.

S. epidermidis showed intermediate antibiotic susceptibility out of the four pathogens tested in which susceptibility was found for chloramphenicol, fusidic acid and tetracycline. *S. epidermidis* was the most sensitive to fusidic acid with an average ZoI of 29.5 mm followed by tetracycline (28.3 mm) and chloramphenicol (19.7 mm) (Fig. 5). Resistance for *S. epidermidis* was found for erythromycin, penicillin G, streptomycin and tetramycin as no zones were observed. According to the European Committee on Antibiotic Susceptibility Testing (EUCAST), most *Staphylococcus spp.* are resistant to penicillin class antibiotics through the production of penicillinase therefore, it is assumed that *S. epidermidis* is resistant to

oxacillin even though a zone of 14.7 mm was observed. Data on the susceptibility of *S. epidermidis* for novobiocin was not found on the EUCAST database therefore, no comments on its antimicrobial activity could be made.

The pathogen that showed the highest antibiotic susceptibility was *E. coli*. Sensitivity was found in ampicillin, chloramphenicol, nitrofurantoin and tetracycline in accordance to EUCAST breakpoints. Tetracycline showed the highest activity against *E. coli* with a zone of 31 mm followed by chloramphenicol (26.7 mm), nitrofurantoin (20.7 mm) and ampicillin (19.5 mm). Nalidixic acid was the only antibiotic observed to have no antimicrobial effect on *E. coli* indicating its resistance (Fig. 3).

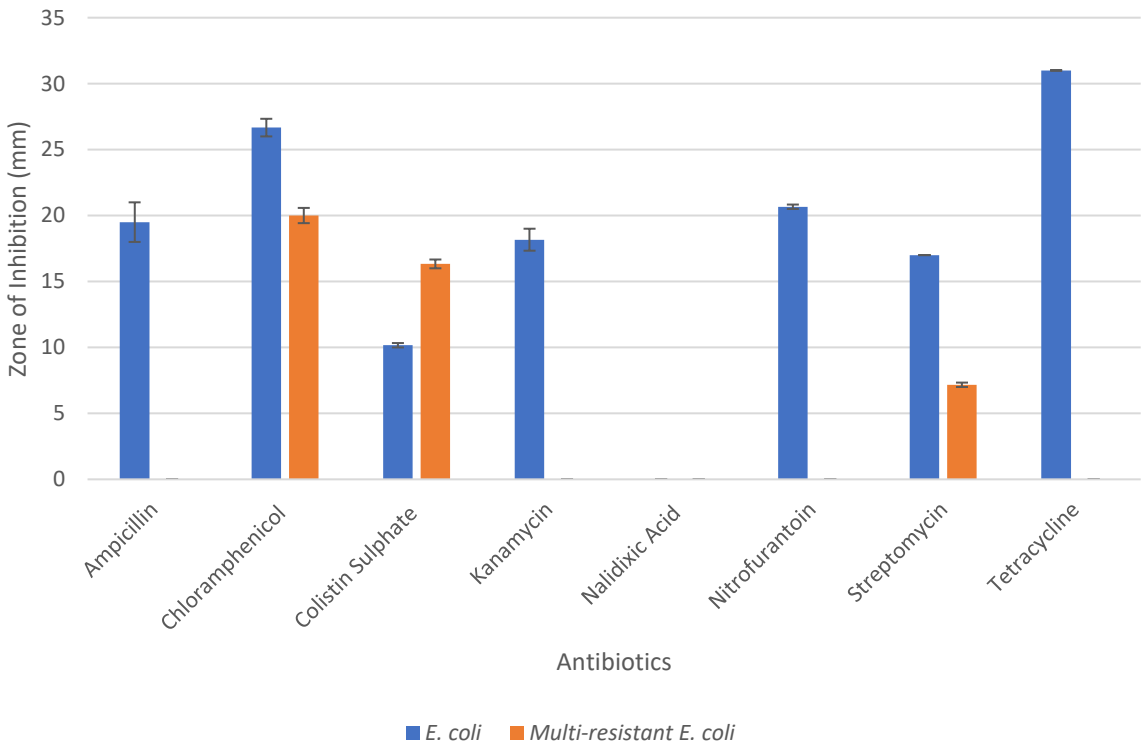


Figure 3. Zones of inhibition of the Gram-negative *E. coli* and multi-resistant *E. coli*. Data are presented as Mean ± SEM, where n = 3. Resistance for *E. coli* was only found with nalidixic acid while multi-resistant *E. coli* was found to be resistant to ampicillin, kanamycin, nalidixic acid, nitrofurantoin and tetracycline.

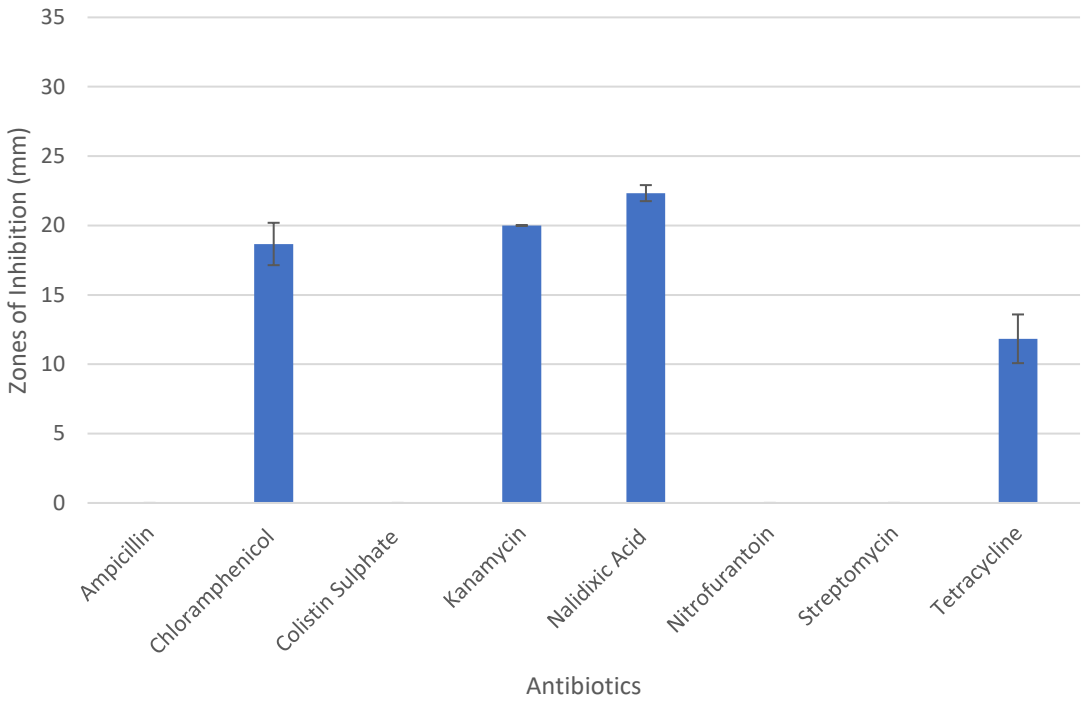


Figure 4. Zones of inhibition of Gram-negative *S. marcescens* showing the second greatest antimicrobial resistance between all bacteria tested. Data is presented as Mean ± SEM, where n = 3.

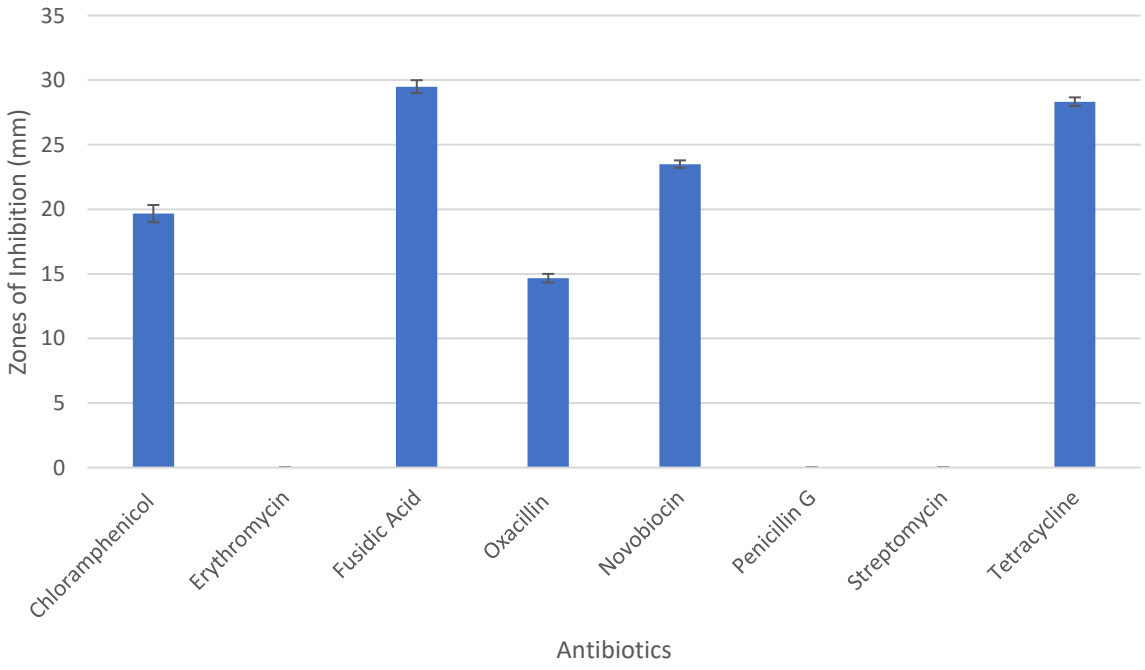
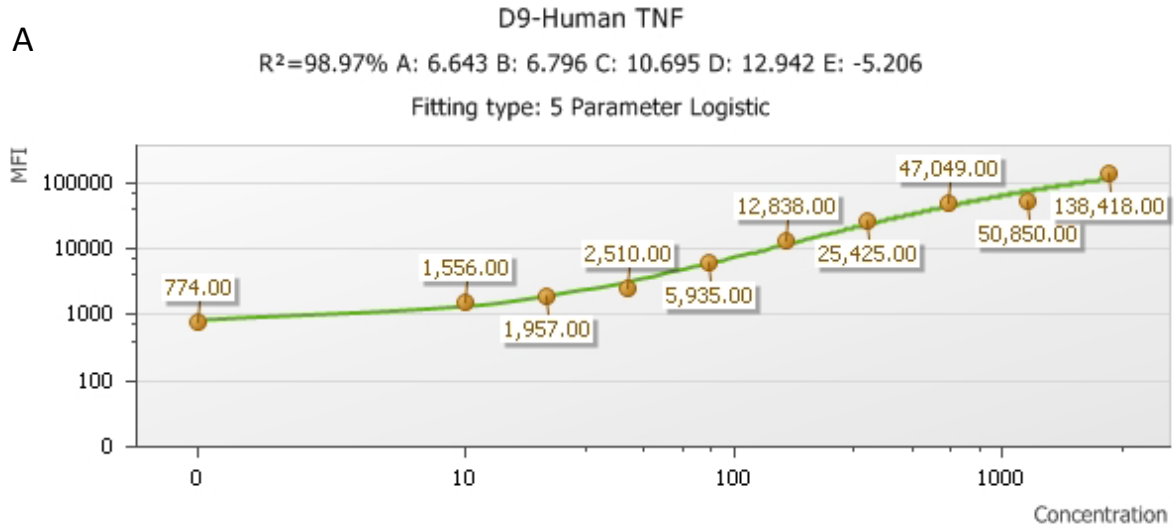


Figure 5. Zones of inhibition of the Gram-positive *S. epidermidis*. Data is presented as Mean ± SEM, where n = 3. Fusidic acid was the most effective antibiotic whilst erythromycin, penicillin G and streptomycin demonstrated no antimicrobial effect on *S. epidermidis*.

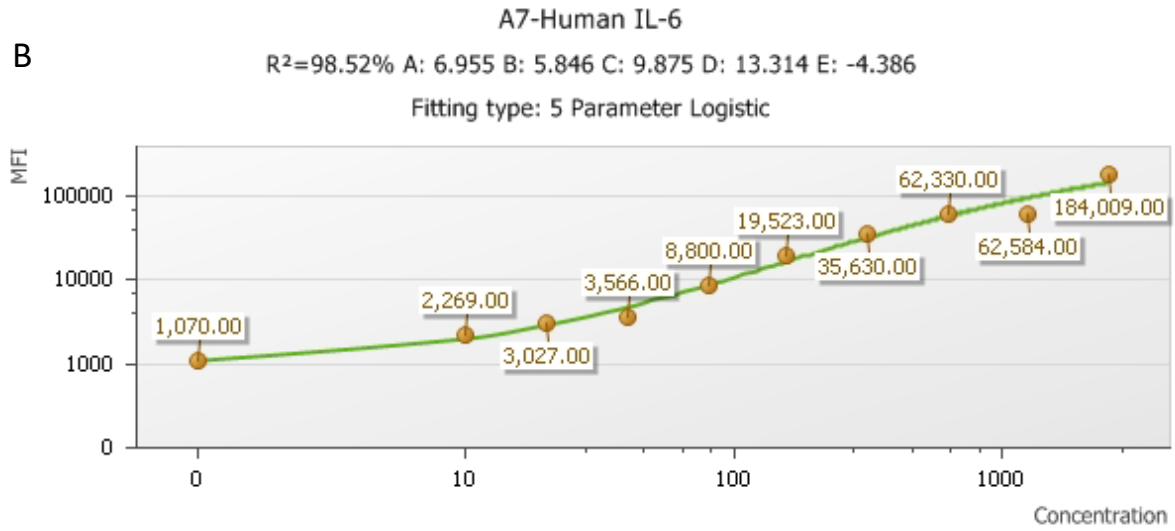
3.2. Analysis of bacterially-contaminated platelet releasates

Platelet-rich plasma (PRP) from healthy participants was contaminated with two key bacterial species frequently implicated in T-ABI/T-AS, namely *S. epidermidis* and *S. marcescens*. The PRP samples were exposed to the bacteria in both planktonic and biofilm forms for four and twenty-four hours, and supernatants (free from bacteria and platelets) were collected for analysis of major cytokines/chemokines (IL-1β, IL-6, TNF-α and RANTES) by Cytometric Bead Array. Standard curves (Fig. 6) of the four cytokines tested were constructed to allow for quantification of the relevant cytokine/chemokine in the test samples.

A



B



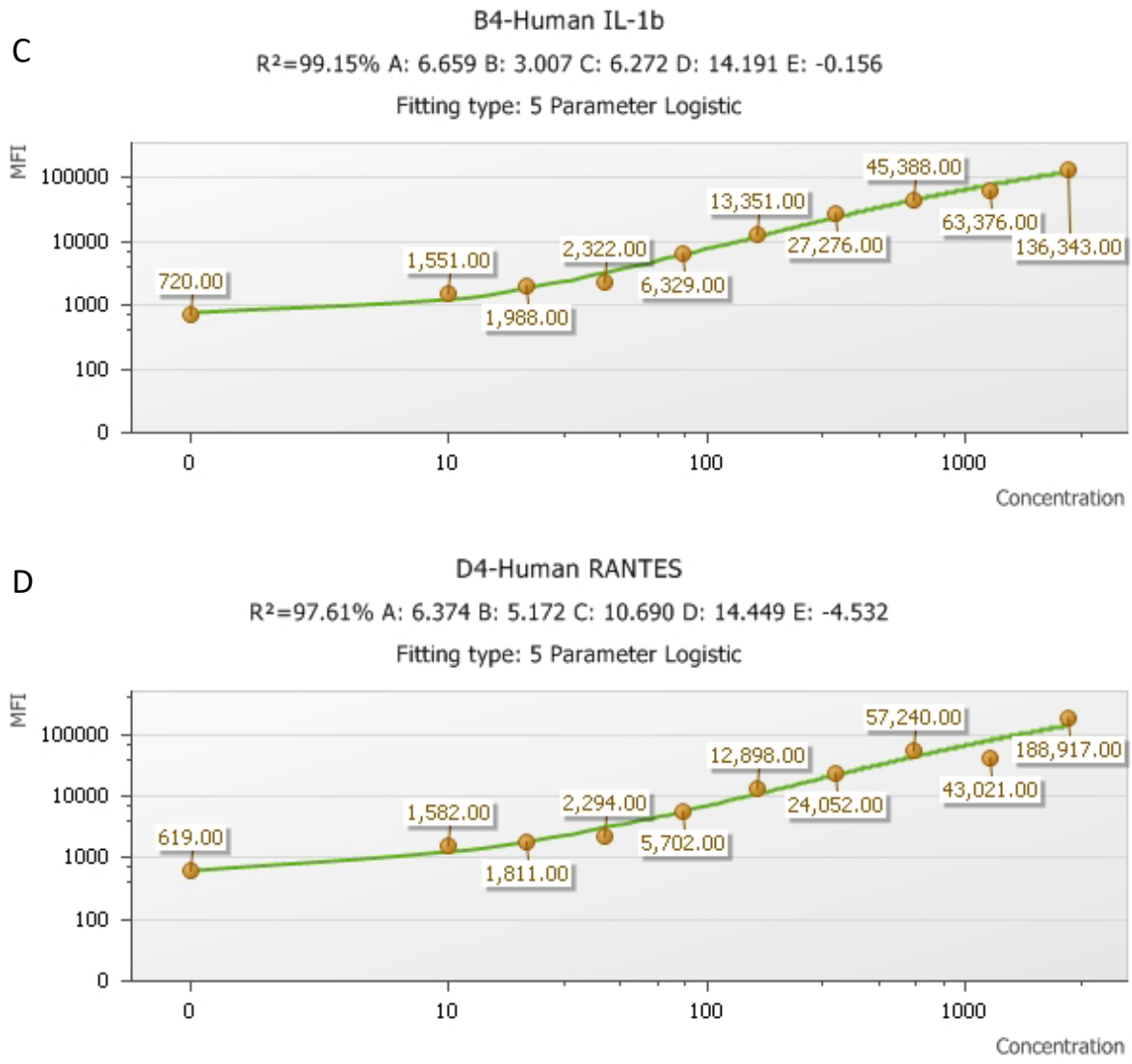
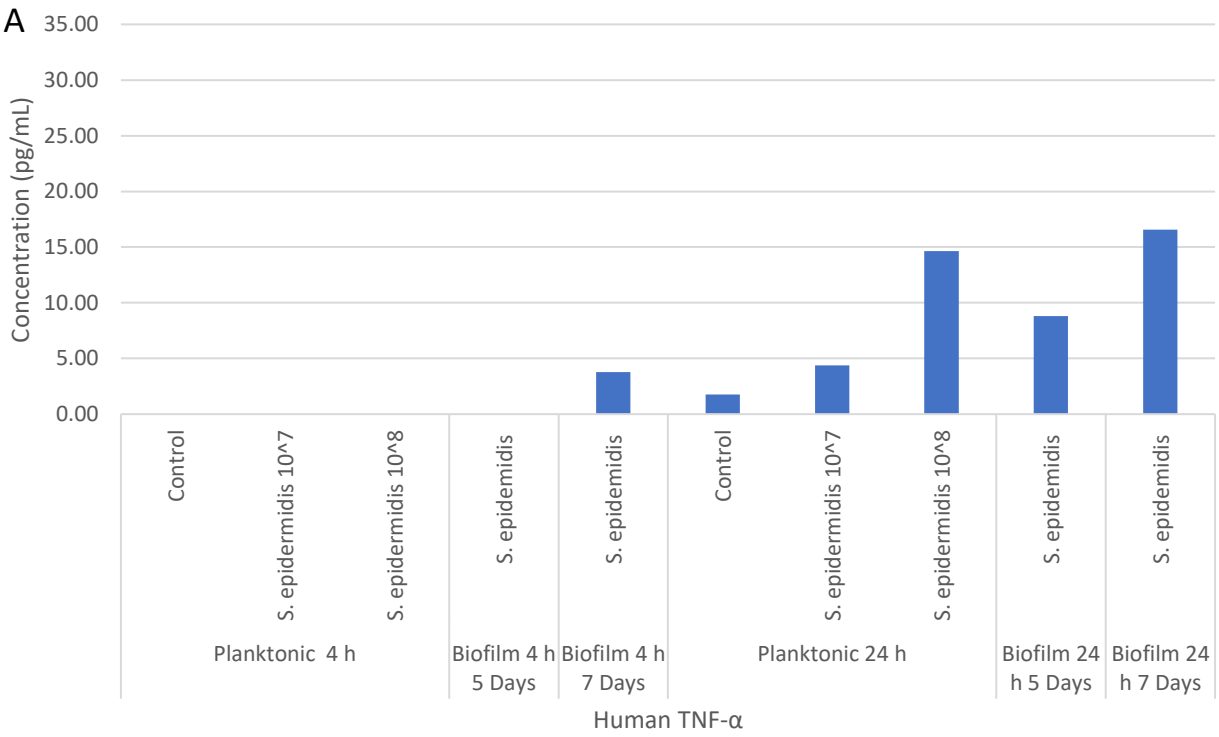


Figure 6. Standard curves of the four cytokines tested: A) Human TNF-α, B) Human IL-6, C) Human IL-1β and D) Human RANTES. MFI = mean fluorescent intensity, concentration is measured in pg/mL. Data is presented as 10,000 data points per individual reads.

Levels of IL-1β, IL-6, and TNF-α were not significantly altered by incubation with *S. epidermidis* or *S. marcescens* in either planktonic or biofilm forms, and in fact, the levels of these cytokines in the platelet supernatant were negligible. However, the level of RANTES was significantly increased (Fig. 7 – Fig. 10) in comparison to the concentrations of IL-1β, IL-6, and TNF-α for both planktonic and biofilm forms of *S. epidermidis* and *S. marcescens*. Overall, the planktonic forms of both species incubated with PRP for 4 h did not elicit any concentrations of IL-1β, IL-6, and TNF-α. There is a general trend of increased cytokine production when PRP had been treated with a higher concentration (1 x 10^8 CFU/mL) of planktonic *S. epidermidis* and *S.*

marcescens for 24 h with the exception of RANTES with *S. epidermidis* and IL-1β with *S. marcescens*. The same trend can also be seen when PRP was incubated with more mature biofilms (established over 7 days compared to 5 days) of *S. epidermidis* and *S. marcescens* for 24 h in which bacterial counts were higher. RANTES appeared to be constitutively secreted within the plasma by platelets as its concentration was measured at 7598.65 pg/mL and 38, 224.51 pg/mL for the controls for the planktonic form of *S. epidermidis* and *S. marcescens* incubated at 4 h and 24 h respectively. These concentrations are far greater than the concentrations obtained for IL-1β, IL-6, and TNF-α where the concentration was found to be 0 pg/mL. The cytokine/chemokine response does not appear to be dependent on the contaminating bacterial species.



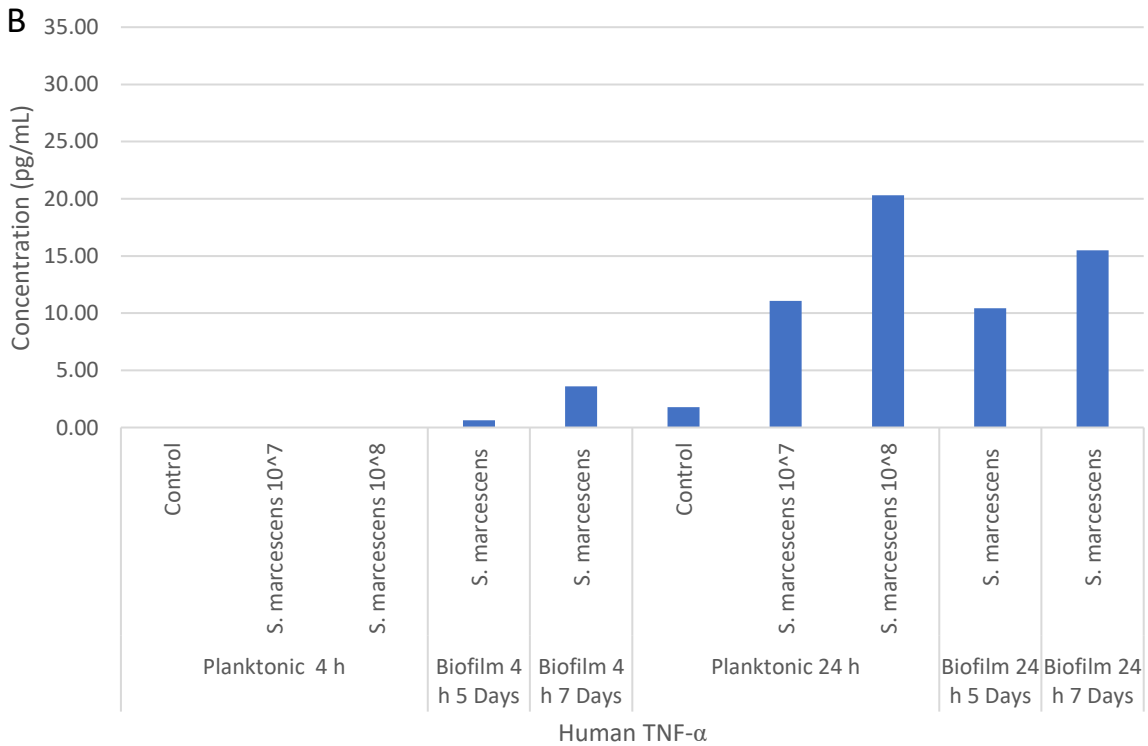
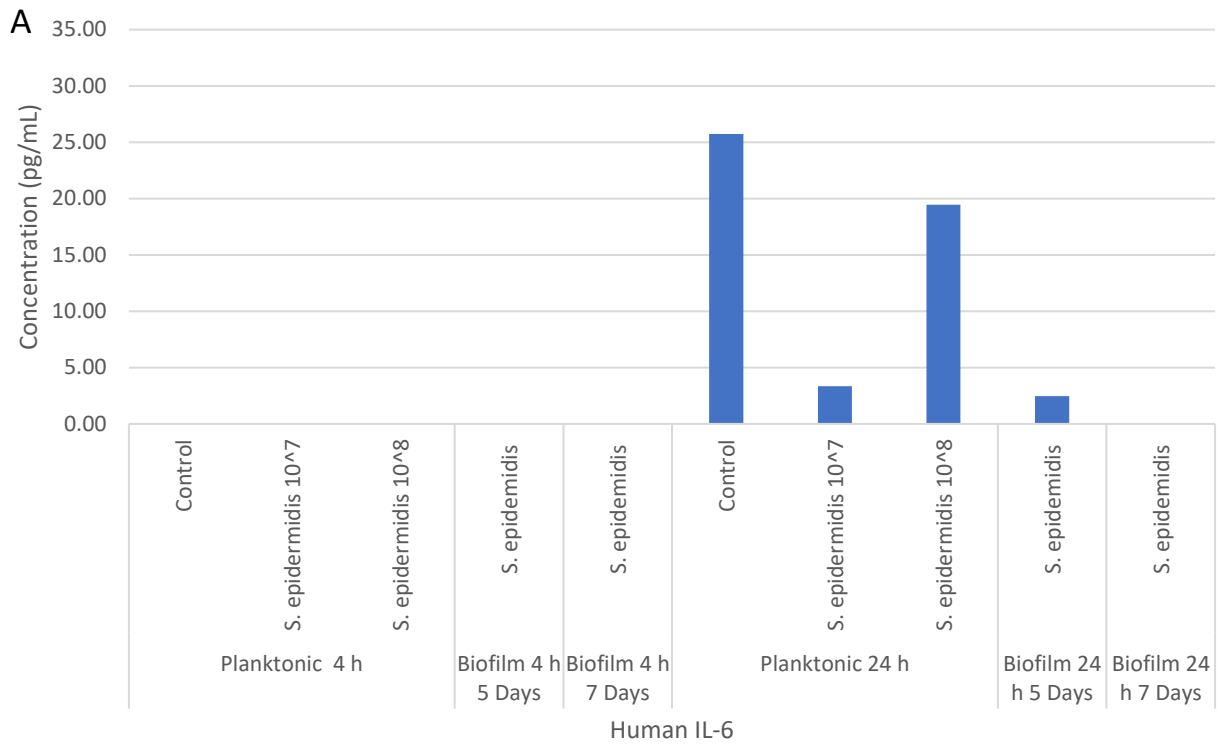


Figure 7. Concentrations of human TNF-α planktonic and biofilm forms of A) *S. epidermidis* and B) *S. marcescens*. Data are presented as 10,000 data points acquired per individual read.



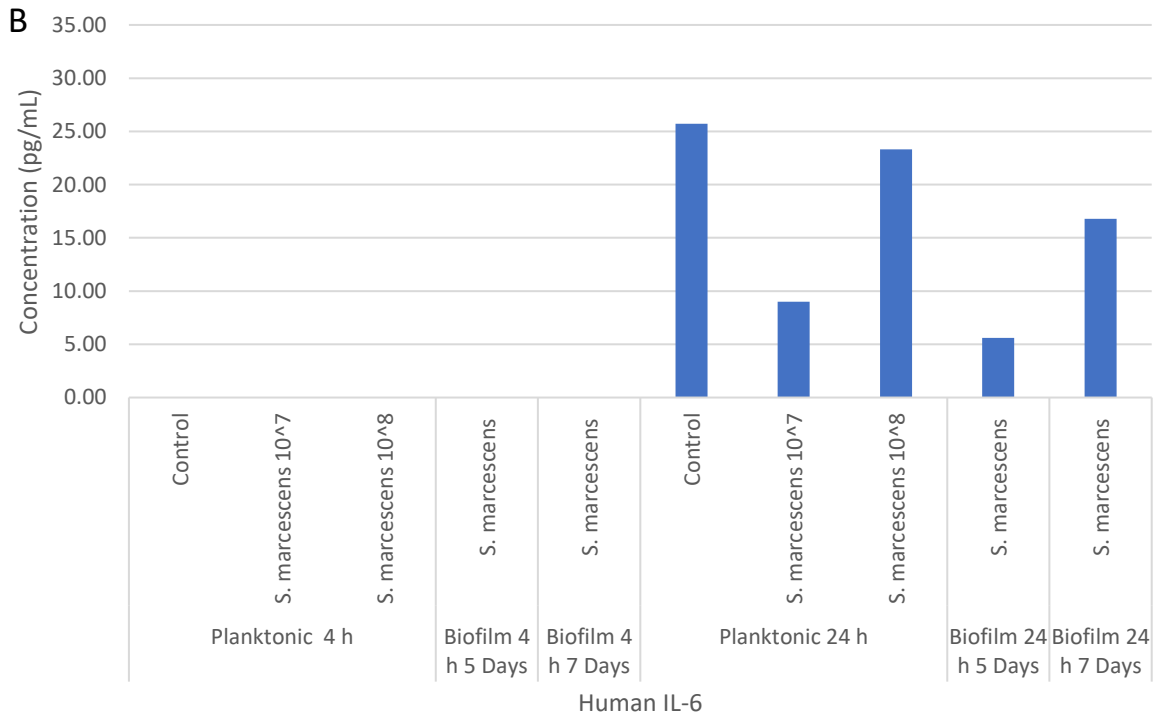
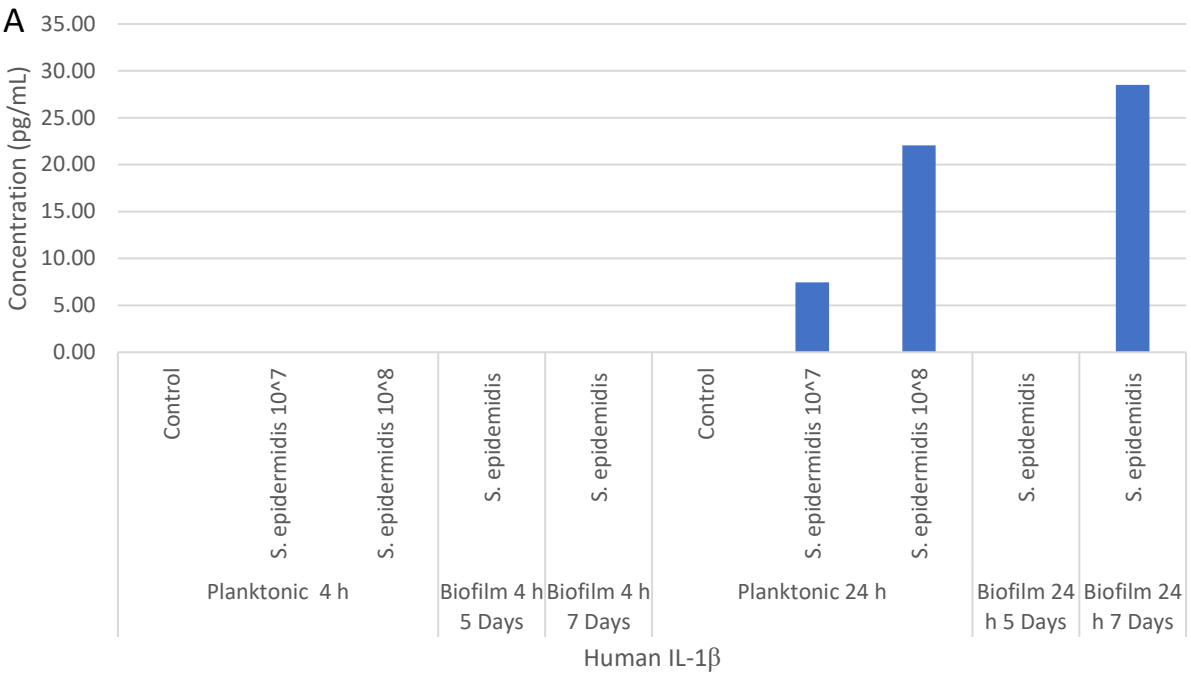


Figure 8. Concentrations of human IL-6 planktonic and biofilm forms of A) *S. epidermidis* and B) *S. marcescens*. Data are presented as 10,000 data points acquired per individual read.



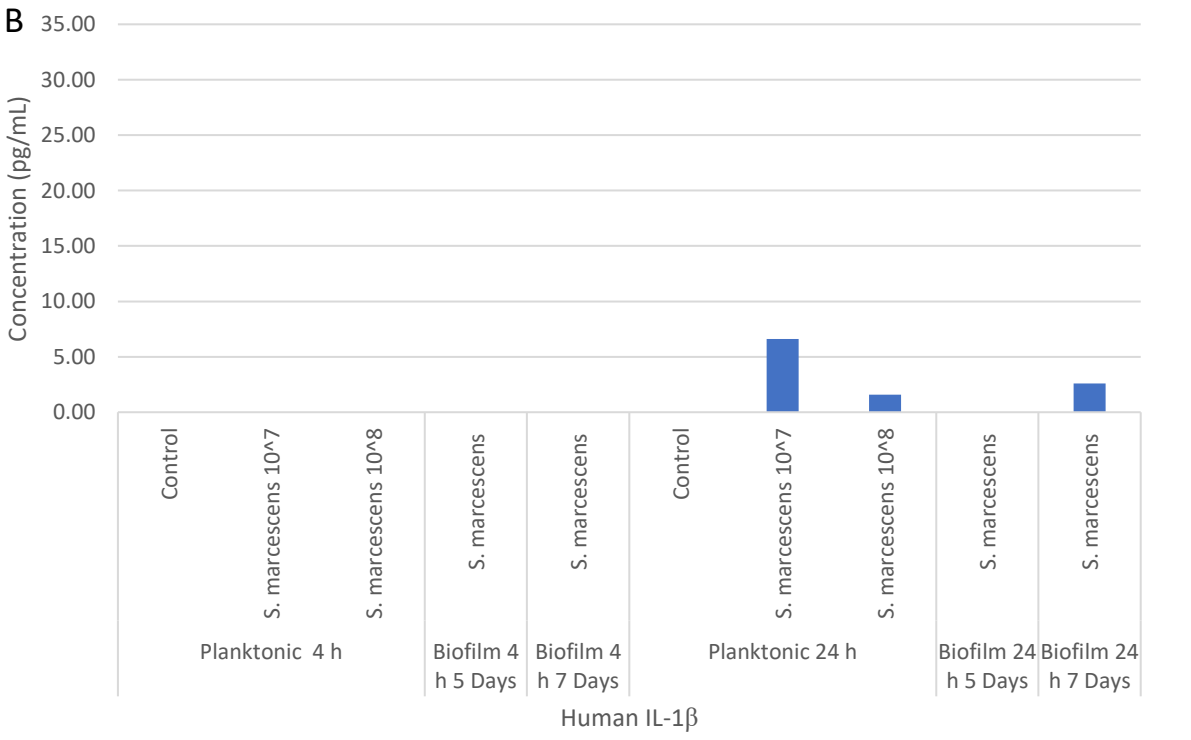
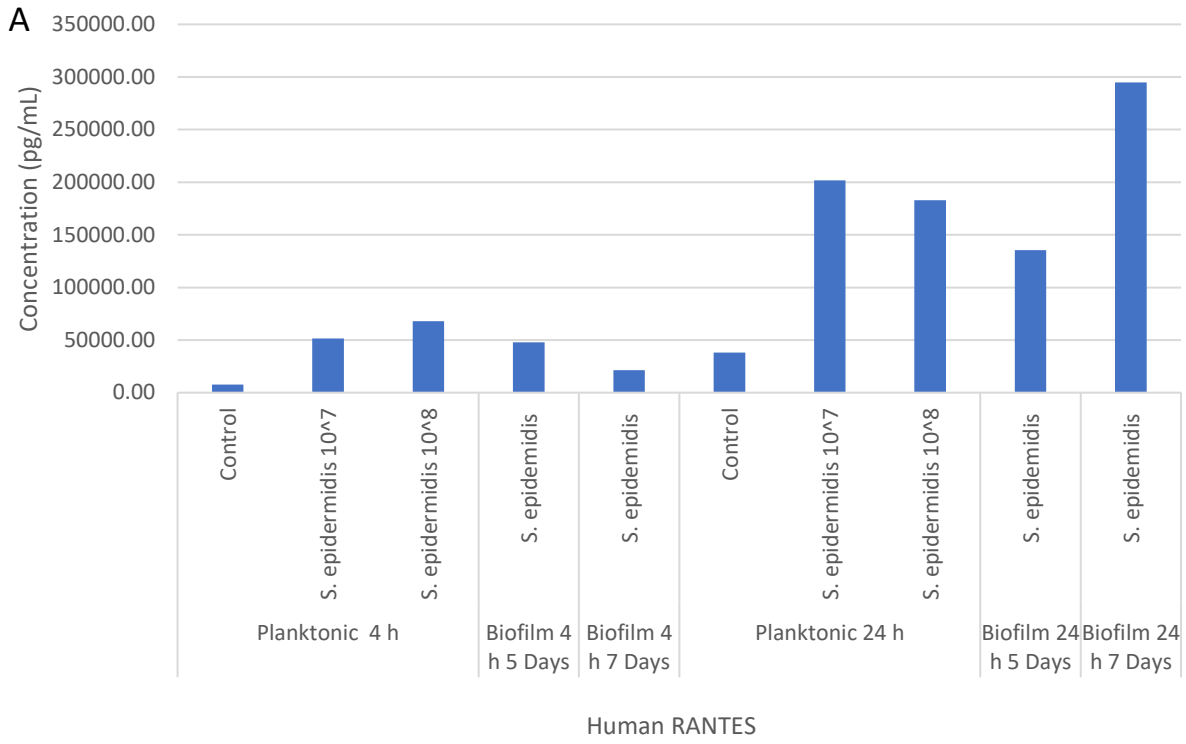


Figure 9. Concentrations of human IL-1β planktonic and biofilm forms of A) *S. epidermidis* and B) *S. marcescens*. Data are presented as 10,000 data points acquired per individual read.



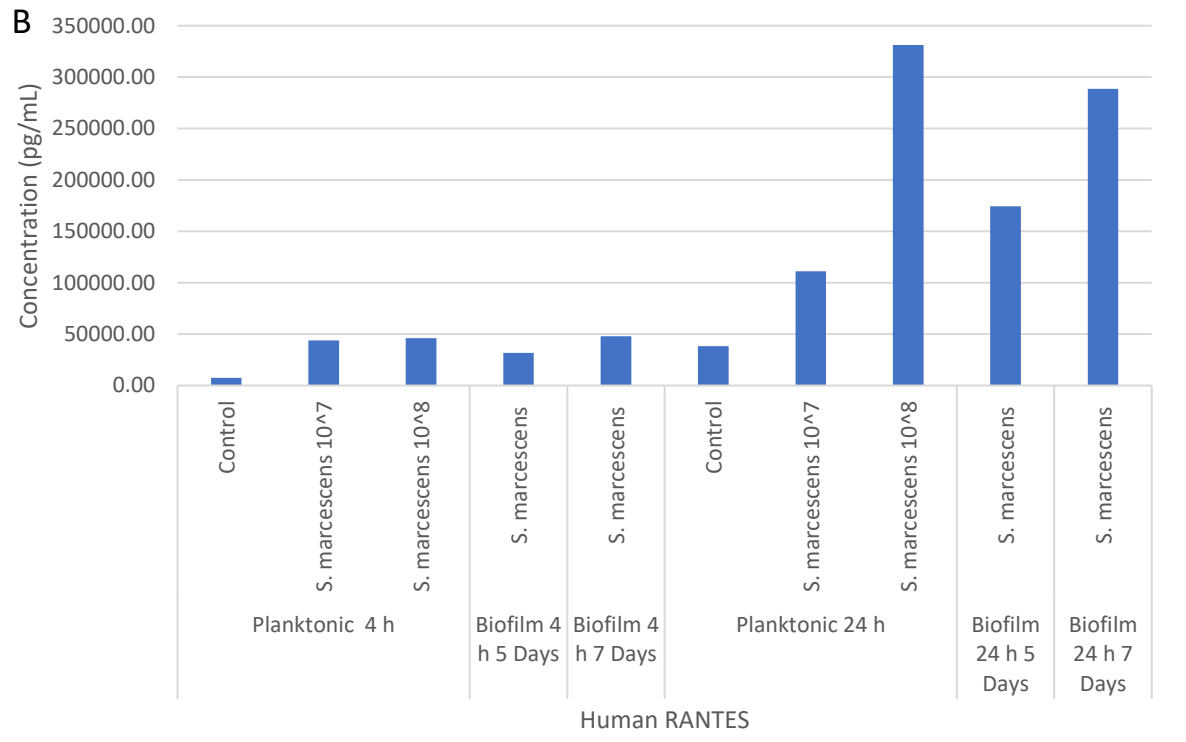


Figure 10. Concentrations of human RANTES planktonic and biofilm forms of A) *S. epidermidis* and B) *S. marcescens.* Data are presented as 10,000 data points acquired per individual read.

3.3 Preparation of leucocyte populations for testing the effects of bacterially contaminated platelet releasates on immune responses.

The transformed cell lines U937 and HL-60 represent monocytes and promyelocytes respectively, and the bacterially-contaminated platelet releasate can be applied directly to these cells to determine the effects on monocytes and promyelocytes (immature myeloid cells). However, in order to test such effects on neutrophils, the HL-60 cells must be stimulated to differentiate down the neutrophil differentiation pathway. Low concentrations (1.3%) of DMSO during 72 h culture have been reported to induce such differentiation (Rincón *et al.,* 2018).

The differentiation of HL-60 cells into neutrophils after treatment with 1.3% DMSO was confirmed via May-Grüwald staining and increased expression of the marker CD11b using flow cytometry (Fig. 16 and Fig. 17 respectively).

The incubation of DMSO with HL-60 cells at time points 24 h, 48 h, 72 h, 96 h and 120 h induced morphological changes that were similar to those found in primary neutrophils such as segmented nuclei, chromatin condensation and decreased nucleocytoplasmic ratio (Fig. 15). The segmentation of the nuclei was most prominent at 72 h. Post-72 h with DMSO, the cells began to show prominent signs of apoptosis.

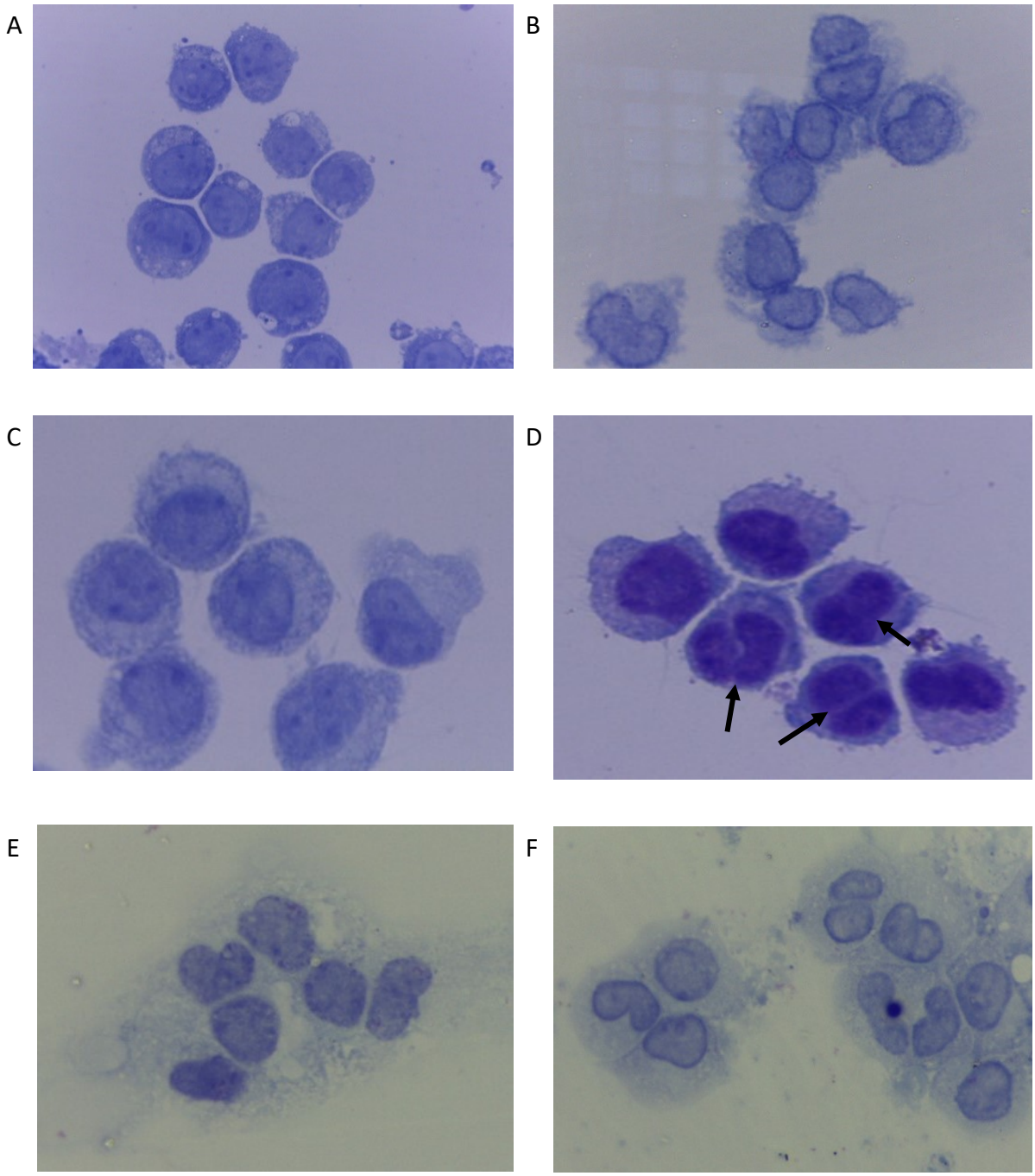


Figure 11. May-Grünwald stained cytosmears of HL-60 cell line. A) Control – HL-60 not treated with DMSO. B) 24 h treatment with DMSO. C) 48 h treatment with DMSO. D) 72 h treatment with DMSO; arrows indicate the segmented nuclei that is characteristic of neutrophils. E) 96 h treatment with DMSO. F) 120 h treatment with DMSO.

DMSO-treated HL-60 were stained with anti-CD11b as CD11b is an early differentiation marker for neutrophils (Drayson *et al.,* 2001). Flow cytometric analysis of CD11b expression was shown to increase when HL-60 cells were treated with DMSO for both time points tested (48 h and 72 h) (Fig. 12 A and B respectively). Statistical analysis showed there was significance ($p < 0.05$) between the control and 48 h DMSO treated HL-60 cells but no significance ($p > 0.05$) was found for the 72 h DMSO treated HL-60 cells. It must be noted that although no statistical significance is found for the 72 h DMSO treated HL-60, CD11b expression was increased in comparison to the control and untreated HL-60.

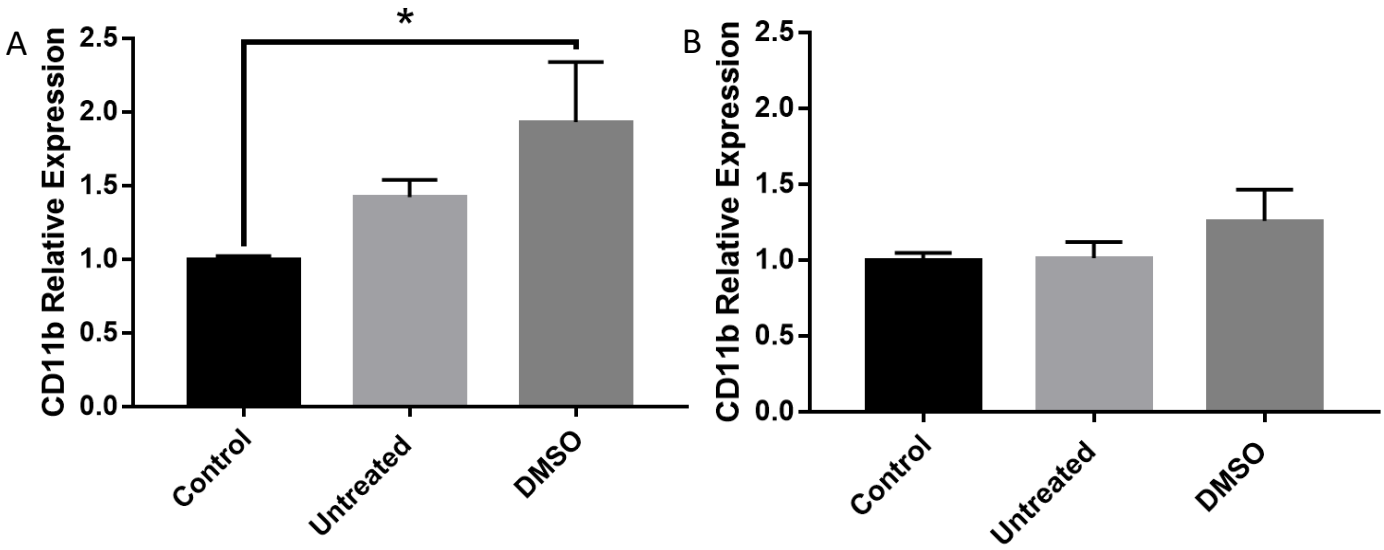


Figure 12. CD11b expression of HL-60 cells treated with DMSO for A) 48 h and B) 72 h. Significance was found between the control and DMSO treated HL-60 in terms of CD11b expression ($p < 0.05$), $*p = 0.0304$ for 48 h. No significance was found in terms of CD11b expression ($p > 0.05$) for 72 h. Data are presented as Mean ± SEM, where n = 3.

Primary human neutrophils obtained from healthy donors were also used in this work as a comparison for the HL-60 neutrophils. To confirm that a population of primary human neutrophils were isolated from the healthy donors, samples were stained with May-Grüwald and showed definitive lobed nuclei indicating that the neutrophils were mature (Fig. 13).

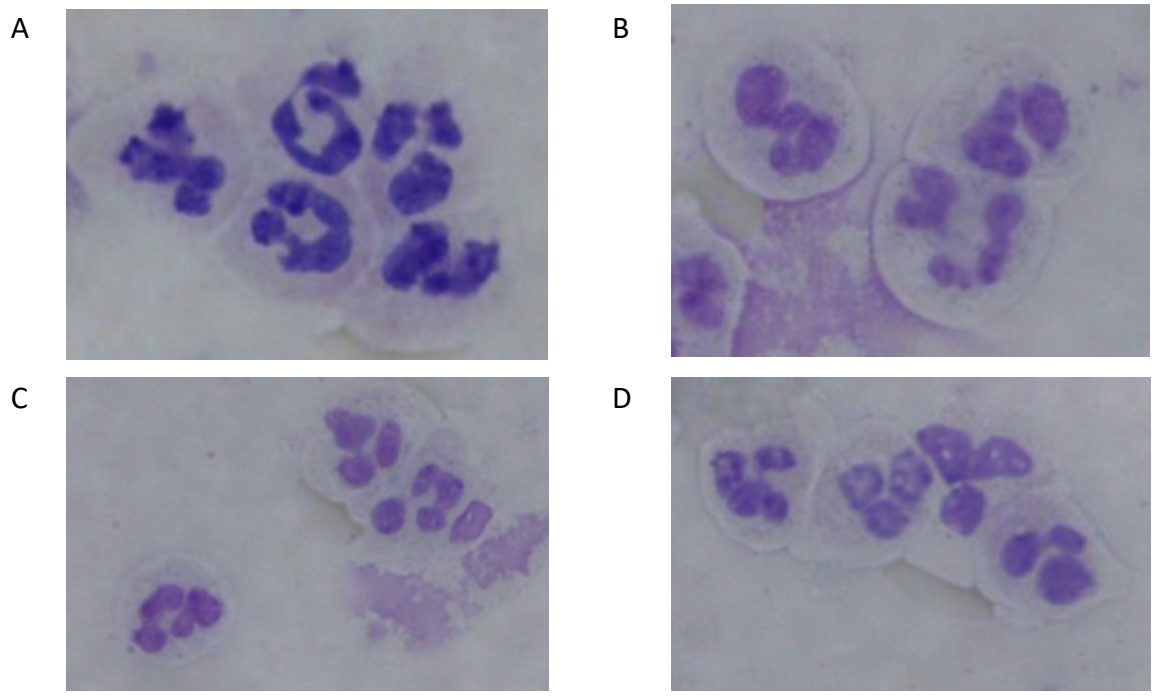


Figure 13. May-Grüwald staining of primary human neutrophils obtained by whole blood lysing of EDTA-anticoagulated blood from a healthy donor nuclei indicating that the neutrophils were mature.

3.4 Effect of bacterially-contaminated platelet releasates on leucocyte surface activation markers.

To determine the effects of the bacterially-contaminated platelet-releasates on leucocyte subsets, transformed cell lines (U937 monocytic cell line, HL-60 promyelocytic cell line, and HL-60-derived neutrophils) as well as primary leucocytes were used. Surface activation markers CD11b, CD54 and CD66b were analysed by flow cytometry. CD66b was not analysed in U937 monocytes as the marker is neutrophil-specific (Lakschevitz *et al.,* 2016).

It must be noted when both cell lines were treated with the biofilm-contaminated platelet-releasates coagulation had occurred despite the pathogens tested being coagulase-negative; which may have affected the results of this study.

3.4.1 Effect of bacterially-contaminated platelet releasates on U937 monocyte surface activation markers.

U937 monocytes was treated with platelet releasates from PRP incubated with *S. epidermidis, E. coli* and multi-resistant *E. coli* in planktonic and biofilm forms. No statistical differences were seen in both CD54 and CD11b expression ($p > 0.05$) when U937 monocytes were incubated with releasate from planktonic *S. epidermidis*-contaminated PRP (Fig. 14 A and B). CD11b appeared to be slightly down regulated when U937 cells were treated with releasate from planktonic *S. epidermidis*-contaminated PRP. This would indicate that platelets primed by *S. epidermidis* did not affect the activation status of U937 monocytes.

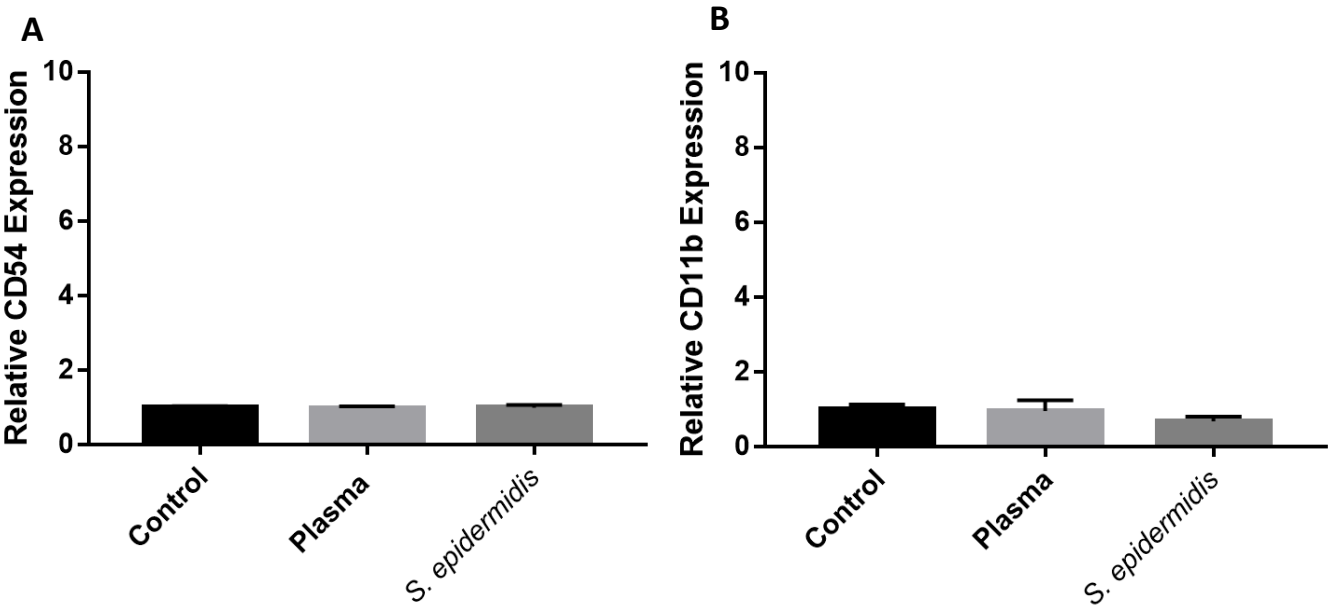


Figure 14. A) CD54 and B) CD11b expression of U937 cells treated, or not, with platelet releaseate collected from platelets contaminated with planktonic *S. epidermidis* for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Significance was not found for CD54 and CD11b expression ($p > 0.05$).

Statistical significance was not found when comparing expression of CD54 or CD11b between untreated cells and those exposed to *E. coli*-treated platelet releasate (Fig. 15 A and B), again indicating that *E. coli*-primed platelets do not have an effect on the activation status of U937 monocytes.

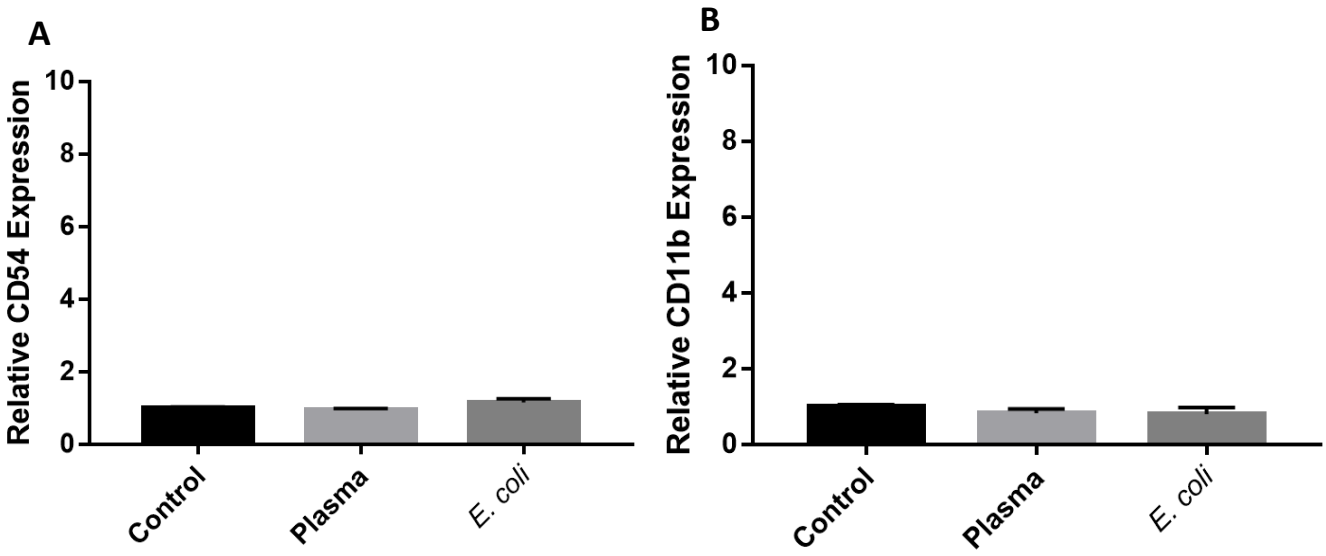


Figure 15. A) CD54 and B) CD11b expression of U937 cells treated, or not, with platelet releaseate collected from platelets contaminated with planktonic *E. coli* for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Significance was not found for CD54 and CD11b expression ($p > 0.05$).

In contrast to these results, incubation of planktonic multi-resistant *E. coli* induced a highly significant upregulation of CD54 in U937 monocytes with *p* values of less than 0.0001 when compared to the control and plasma treatment group (Fig. 16 A). As planktonic *S. epidermidis* and WT *E. coli* contaminated platelet releasates had no effect on U937 monocyte CD54 expression, it can be interpreted that the multi-resistant strain was more effective in inducing expression of CD54 and therefore activating monocytes more strongly than *S. epidermidis* and the non-resistant *E. coli* strain. Although no statistical difference can be found when analysing the CD11b data (Fig. 16 B), a different trend can be seen with multi-resistant *E. coli* compared to *S. epidermidis* and non-resistant *E. coli*, in which CD11b activation increased rather than decreased as seen in the other two pathogens.

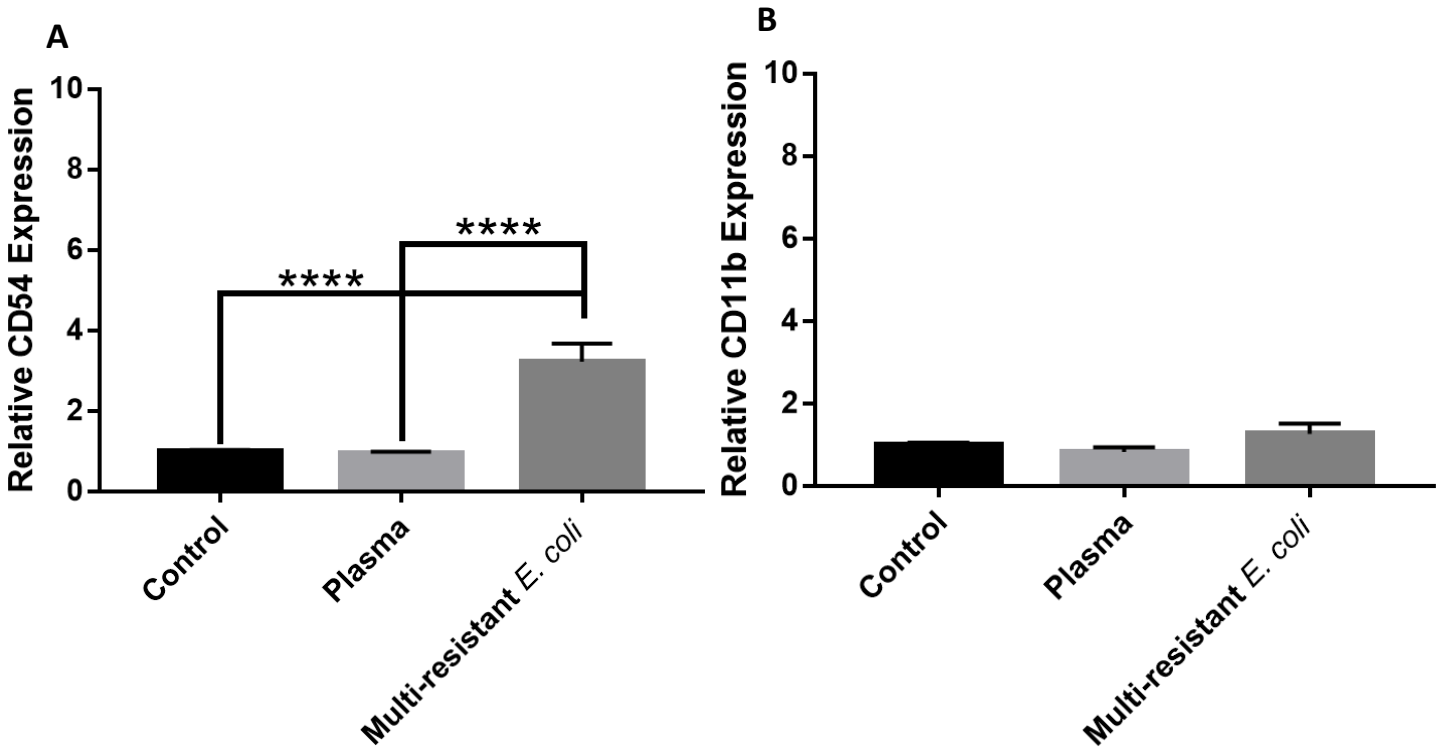


Figure 16. A) CD54 and B) CD11b expression of U937 cells treated, or not, with platelet releaseate collected from platelets contaminated with planktonic multi-resistant *E. coli* for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Significance was found for CD54 expression in which ****$p = < 0.0001$ while CD11b expression had no statistical significance ($p > 0.05$).

No changes in CD54 expression was observed following the treatment of platelets with planktonic *S. epidermidis* (Fig. 14). However, with the biofilm *S. epidermidis* platelet releasate, a minor decrease in CD54 expression was demonstrated ($p = 0.0209$) (Fig. 17 A). In contrast, CD11b expression appeared to be increased when U937 monocytes were treated with this same platelet releasate, but no statistical significance was observed ($p > 0.05$) (Fig. 17 B). Taken together, the results may suggest that planktonic and biofilm forms of pathogens induced different cytokine profiles *via* alternative mechanisms leading to contrasting results in surface marker expressions on leucocytes.

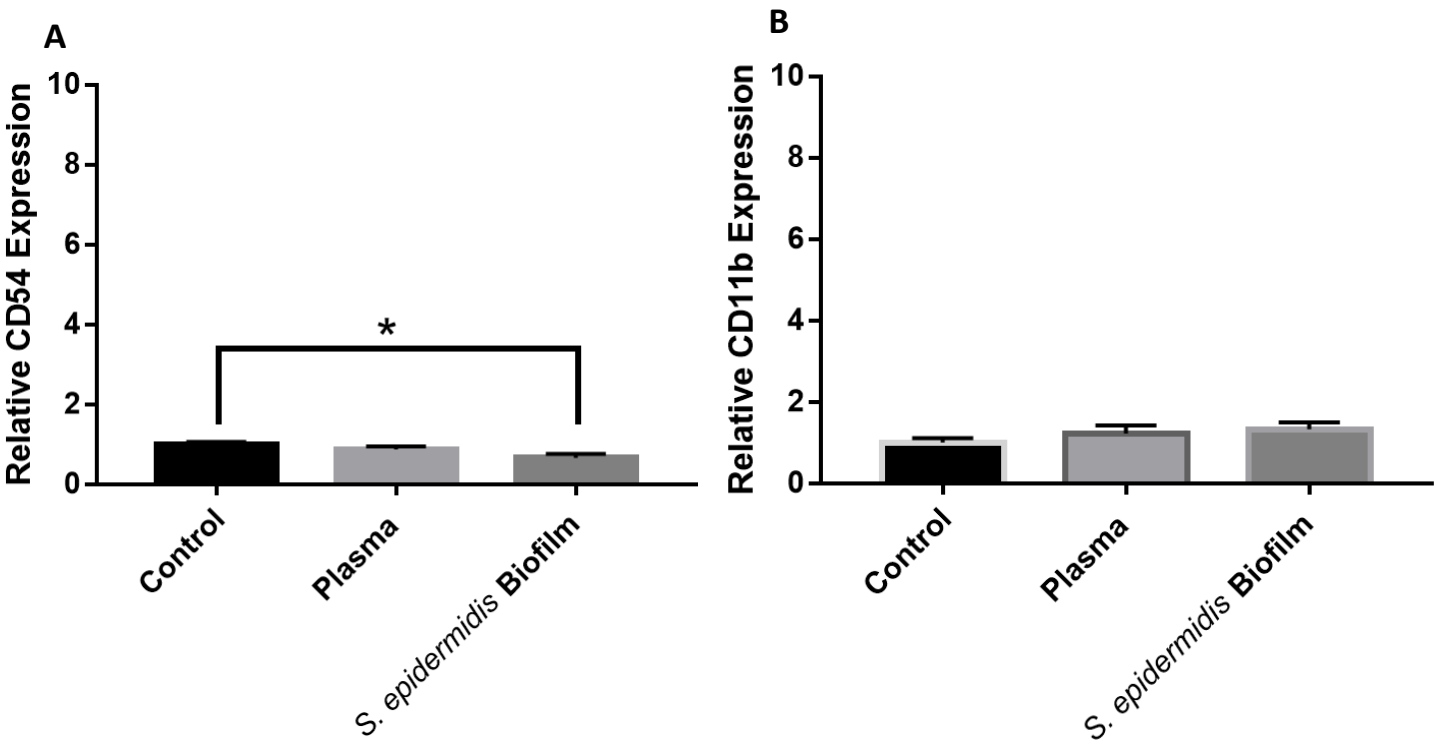


Figure 17. A) CD54 and B) CD11b expression of U937 cells treated, or not, with platelet releaseate collected from platelets contaminated with *S. epidermidis* biofilm for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Significance was for CD54 *$p = 0.0209$, but no significance was found for CD11b expression ($p > 0.05$).

The expression of CD54 was upregulated when U937 monocytes were incubated with releasate from *E. coli* biofilm-contaminated PRP when compared to the control and plasma treatments however, no statistical significance could be seen (Fig. 18 A). On the other hand, CD11b expression was significantly upregulated following treatment of the monocytes with releasate from *E. coli* biofilm-contaminated PRP ($p = 0.0025$) (Fig. 18 B). However, the CD11b expression on monocytes treated with platelet releasate that had not been in contact with bacteria (plasma) was also significantly elevated in comparison with control samples ($p = 0.0004$), and the levels were not different from those following treatment with bacterially-primed platelets. This indicates that something in the plasma was causing the upregulation of CD54 and not the pathogen itself.

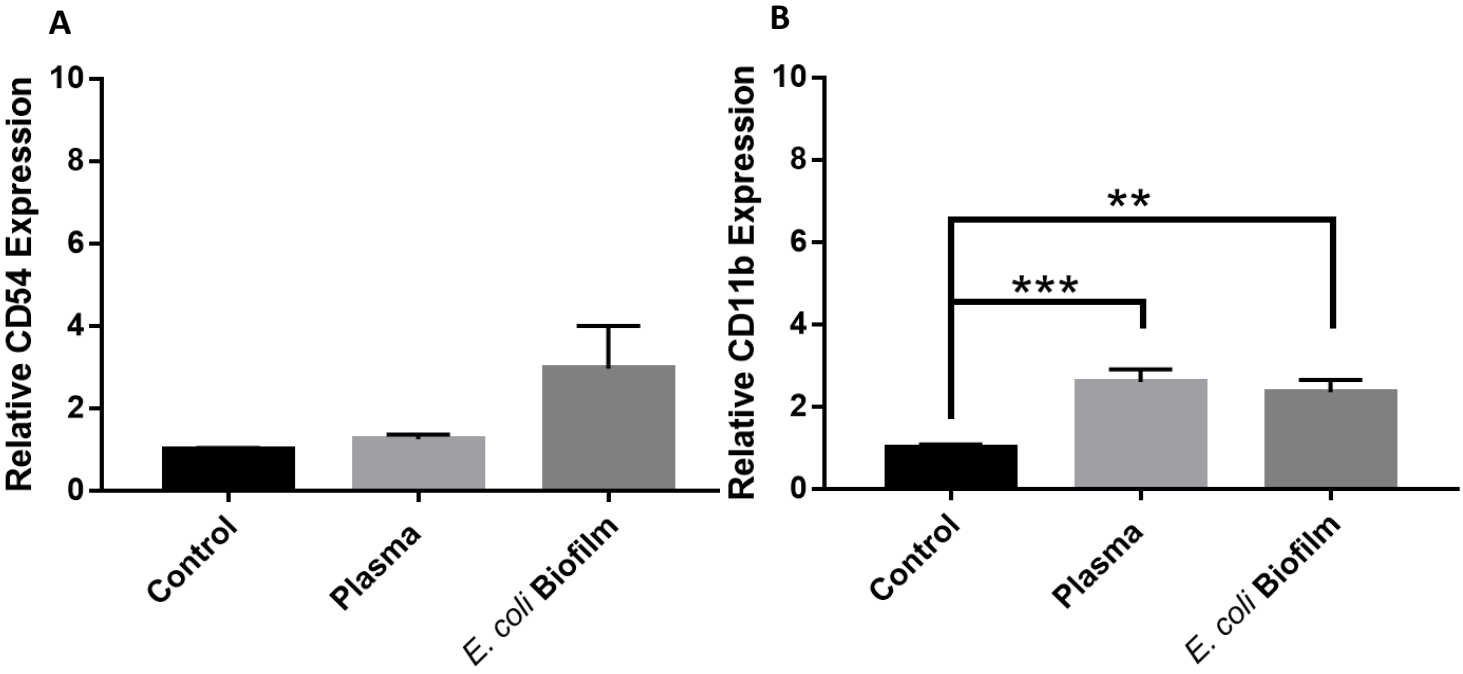


Figure 18. A) CD54 and B) CD11b expression of U937 cells treated, or not, with platelet releaseate collected from platelets contaminated with *E. coli* biofilm for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. No statistical significance for CD54 expression ($p > 0.05$) while significance was found for CD11b expression, ***$p = 0.0004$, **$p = 0.0025$.

CD54 surface expression was found to be highly upregulated ($p < 0.001$) in response to treatment of monocytes with releasate from platelets contaminated with the biofilm form of multi-resistant *E. coli* (Fig. 19 A). Although this was also the case for CD11b expression, this transmembrane protein was also found to be upregulated in response to platelet releasate that had not been contaminated with bacterial biofilm (Fig. 19 B). Overall, the planktonic and biofilm form of multi-resistant *E. coli* displayed the same trend, with the exception of the plasma treatment in the planktonic form, where both surface markers were increased.

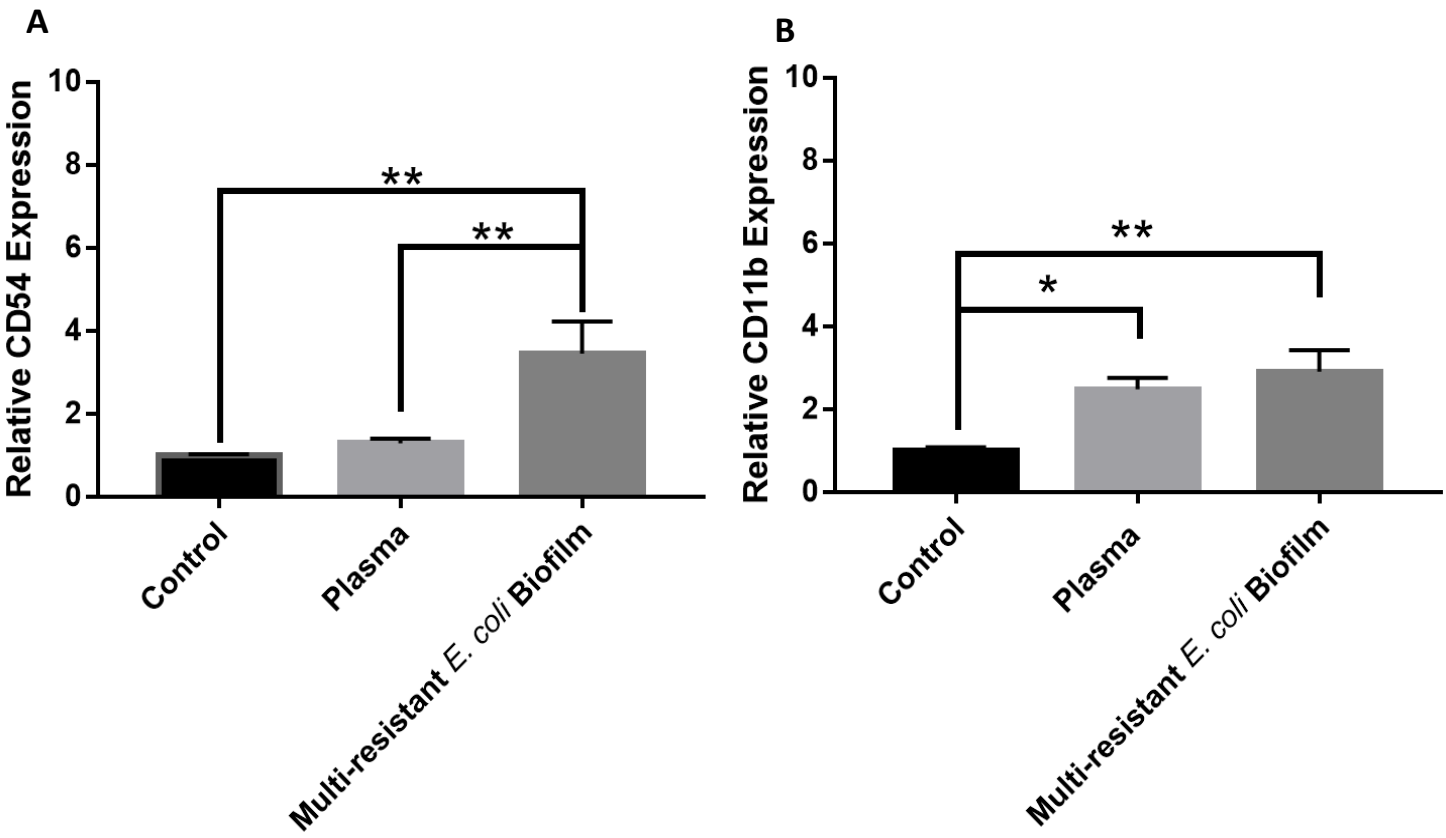


Figure 19. A) CD54 and B) CD11b expression of U937 cells treated, or not, with platelet releasate collected from platelets contaminated with multi-resistant *E. coli* biofilm for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Statistical significance was found for CD54 expression with $**p$ values of 0.0023 (control vs multi-resistant *E. coli*) and 0.007 (plasma vs multi-resistant *E. coli*). CD11b expression was found to be statistically significant, $*p = 0.014$ and $**p = 0.0016$.

3.2.2 Effect of bacterially-contaminated platelet releasates on HL-60 neutrophils cell surface activation markers.

The expression of CD54, CD11b and CD66b were measured on HL-60 neutrophils *via* flow cytometry following stimulation with various platelet releasates to compare whether surface expression were similar or different to those observed with U937 monocytes. Statistical significance were seen with CD54 and CD66b but not CD11b ($p > 0.05$) with planktonic *S. epidermidis* (Fig. 20). All the surface markers tested appeared to increase after incubation with planktonic *S. epidermidis* suggesting that the platelets primed with the pathogen had an effect on HL-60 neutrophil activation status.

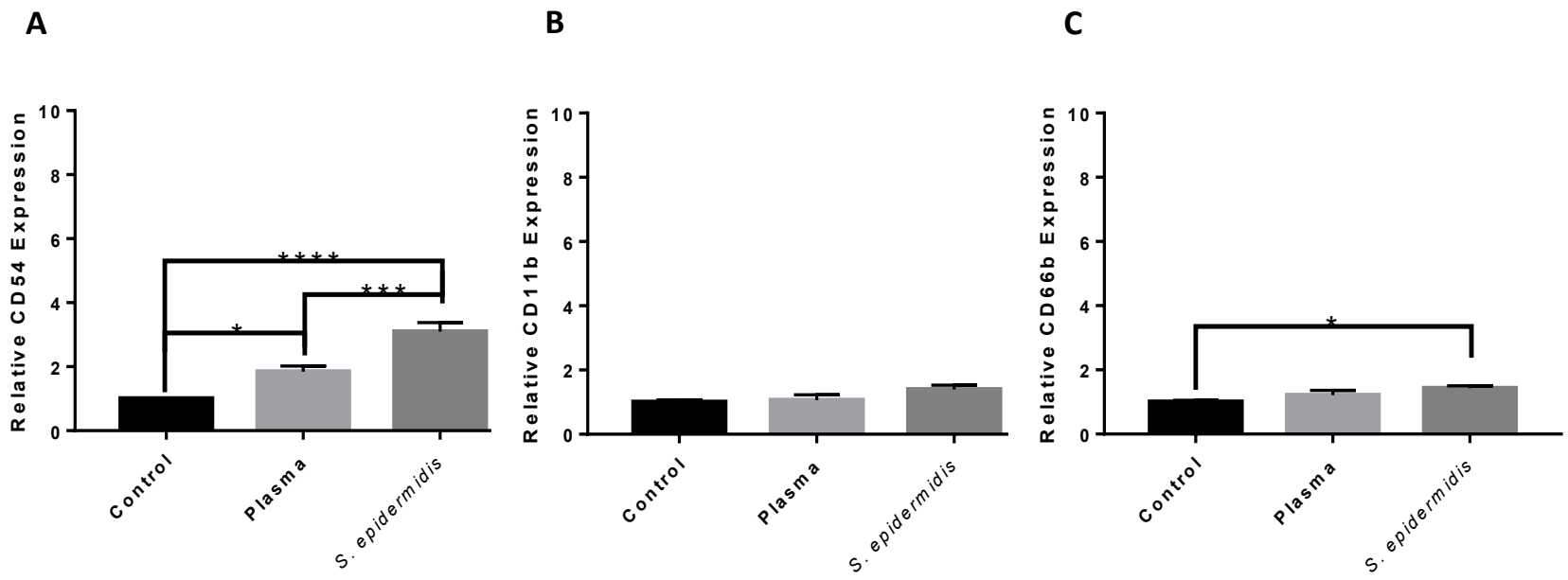


Figure 20. A) CD54, B) CD11b and C) CD66b expression of HL-60 cells treated, or not, with platelet releaseate collected from platelets contaminated with planktonic *S. epidermidis* for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Significance was found for CD54 expression on HL-60 cells between all three treatment groups, *$p = 0.129$, ***$p = 0.0003$ and ****$p = <0.0001$. No significance was found for CD11b expression ($p > 0.05$). Significance was only found between the control and planktonic *S. epidermidis* treatments for CD66b, *$p = 0.018$.

Expression of CD54 and CD11b was increased after treatment of planktonic WT *E. coli* contaminated PRP while no changes in CD66b expression were observed ($p > 0.05$) (Fig. 21 C). As a significant difference can be seen between the control and the plasma ($p = 0.0009$) it is implicated that the upregulation of CD54 was caused by the factors released by the platelets prior to priming or from factors that were already present in the plasma (Fig. 21 A). A significant difference was also found between the plasma and planktonic WT *E. coli* ($p = 0.195$). This suggests that the platelet releasate primed with planktonic *E. coli* has a greater effect on upregulating CD54 than with plasma alone. Significant differences can be found between the control and planktonic WT *E. coli* contaminated PRP (**$p = 0.0012$), and the plasma and planktonic WT *E. coli* contaminated PRP samples (**$p = 0.003$) for the expression of CD11b (Fig. 21 B). As no significance difference was found between the control and plasma treatments in reference to CD11b, it may be suggested that platelets primed with planktonic *E. coli* contaminated releasate has an effect on the activation status of HL-60 neutrophils.

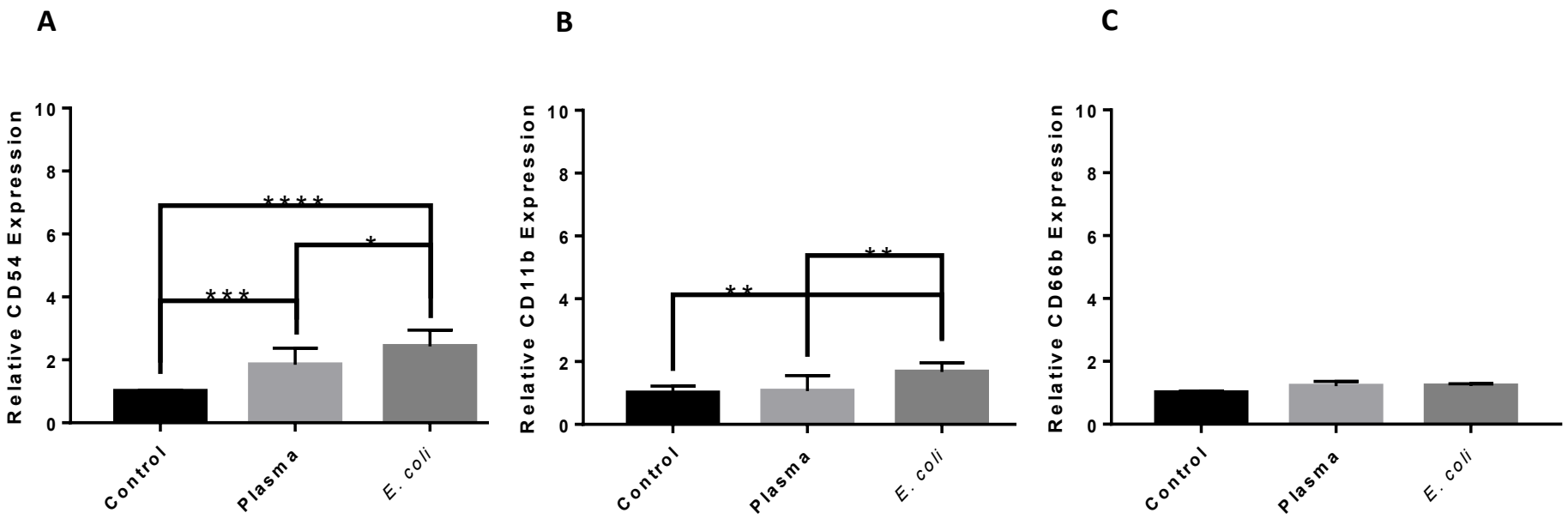


Figure 21. A) CD54, B) CD11b and C) CD66b expression of HL-60 cells treated, or not, with platelet releaseate collected from platelets contaminated with planktonic *E. coli* for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Significance was found for CD54 expression on HL-60 cells between all three treatment groups, *$p = 0.195$, ***$p = 0.0009$ and ****$p = < 0.0001$. Significance was also found for CD11b, **$p = 0.0012$ for control and *E. coli*, **$p = 0.003$ for plasma and *E. coli*. Significance was not found for CD66b ($p > 0.05$).

CD54 was significantly upregulated when HL-60 neutrophils were treated with multi-resistant *E. coli* contaminated PRP (Fig. 22 A) compared to all the other bacteria, surface markers and cell lines tested. As no significance was detected between the control and plasma treatment on CD54 expression, it may be suggested that upregulation of CD54 was mainly caused by platelet releasate contaminated with planktonic multi-resistant *E. coli*. A slight increase in CD11b expression was observed but this was not significant ($p > 0.05$) (Fig. 22 B). CD66b expression was also upregulated after treatment. However, expression of CD66b was not as high as that of CD54 (Fig. 22 C).

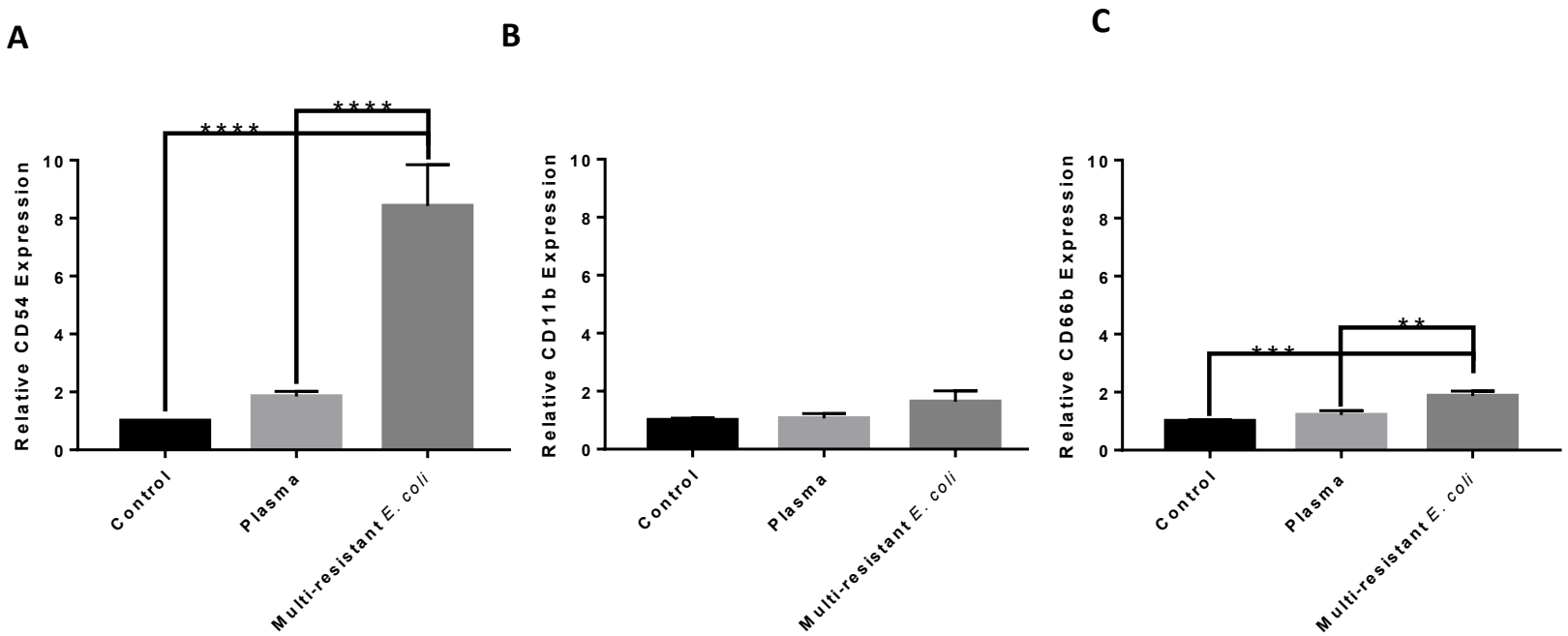


Figure 22. A) CD54, B) CD11b and C) CD66b expression of HL-60 cells treated, or not, with platelet releaseate collected from platelets contaminated with planktonic multi-resistant *E. coli* for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Significance was found for CD54 expression on HL-60 cells ****p = < 0.0001. No significance for CD11b ($p > 0.05$). Significance was found for CD66b **p = 0.0063, ***p = 0.0004.

HL-60 neutrophil CD54 expression was found to increase when the cells were treated biofilm-contaminated platelet releasates for *S. epidermidis*, *E. coli* and multi-resistant *E. coli* biofilms respectively (Fig. 23 A, Fig. 24 A and Fig. 25 A). However, in the case of the *S. epidermidis,* there was no significant difference in the CD54 expression between neutrophils treated with platelet releasates that had not been in contact with bacteria, and those that had, indicating that the small observed increase in expression is due to components within the blood plasma that are independent of bacterial contamination. In contrast to this *E. coli* (WT or multi-drug resistant) appeared to have a significant effect on neutrophil activation status with multi-drug resistant *E. coli* having the biggest effect. CD11b and CD66b expression did not appear to be affected by incubation with *S. epidermidis* biofilm contaminated platelet relesates, as significant differences were not observed between these samples and those treated with non-contaminated platelet releasates (Fig. 23 B and C).

CD11b and CD66b expression of HL-60 cells treated with WT *E. coli* biofilm were weakly upregulated (Fig. 24 B and C). Statistical significance was not found between the plasma and biofilm treatment for both surface markers therefore the increased expression observed is likely to be caused by plasma components rather than the bacterial priming of platelets.

Similarly, to the WT *E. coli* biofilm treatment, expression of CD11b was increased to an extent of the multi-resistant *E. coli* biofilm contaminated PRP treatment when compared to the control therefore, the same can be said about plasma components are causing these changes (Fig. 25 B). As for CD66b expression, it was upregulated when HL-60 neutrophils were treated with plasma however, expression was downregulated when treated with biofilm contaminated platelet releasate (Fig. 25 C). Although no significance was found for the biofilm treatment, the multi-resistant *E. coli* may have an antagonistic effect on platelets or an antagonistic effect on plasma factors.

Overall, multi-resistant *E. coli* had the most significant effect on the upregulation of CD54 in comparison to *S. epidermidis* and WT *E. coli* in both planktonic and biofilm forms. CD11b and CD66b expression was either moderately increased or not changed for all three pathogens in both planktonic and biofilm forms.

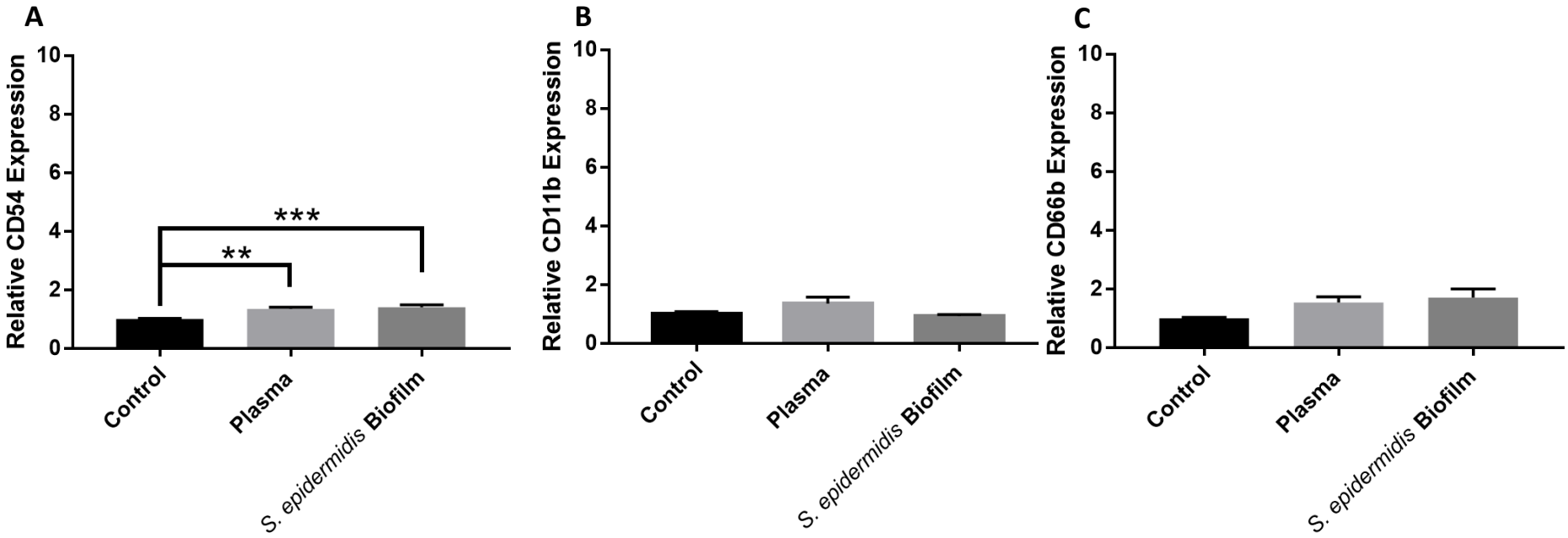


Figure 23. A) CD54, B) CD11b and C) CD66b expression of HL-60 cells treated, or not, with platelet releaseate collected from platelets contaminated with *S. epidermidis* biofilm for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Significance was found for CD54 expression on HL-60 cells only with control vs. plasma $^{**}p = 0.0014$ and control vs. biofilm $^{***}p = 0.0002$. No significance was found for CD11b and CD66b expression ($p > 0.05$).

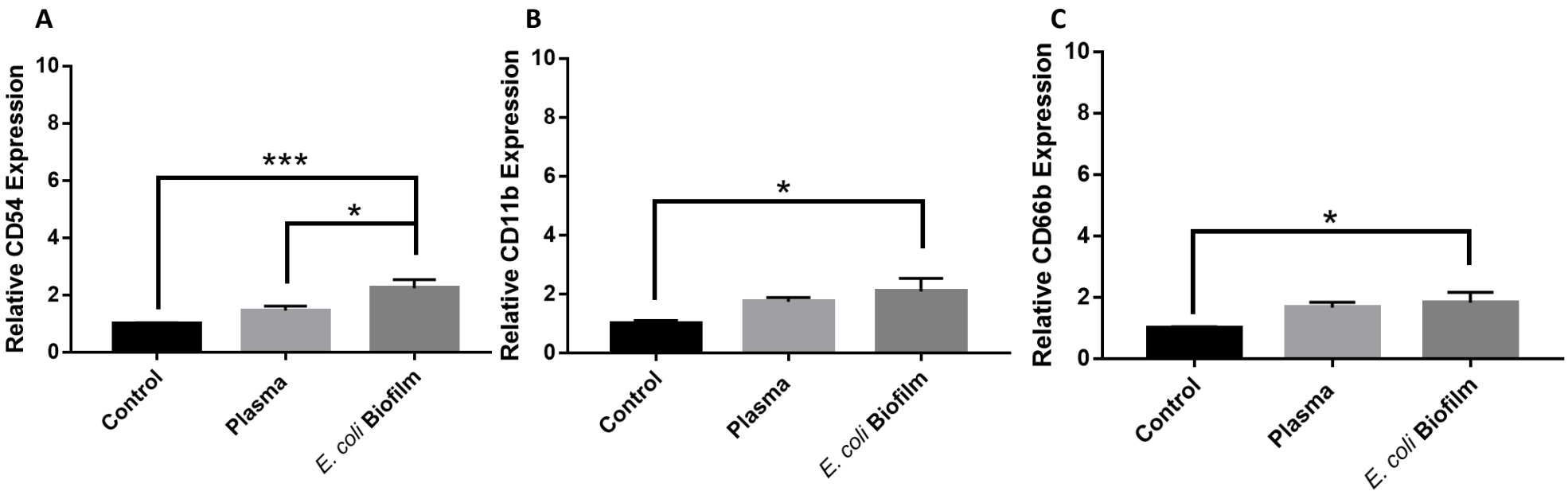


Figure 24. A) CD54, B) CD11b and C) CD66b expression of HL-60 cells treated, or not, with platelet releaseate collected from platelets contaminated with *E. coli* biofilm for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Significance was observed for CD54, $*p = 0.282$ and $***p = 0.0006$. Significance was only found between the control and E. coli biofilm for CD11b and CD66b, $*p = 0.0249$ and $*p = 0.423$ respectively.

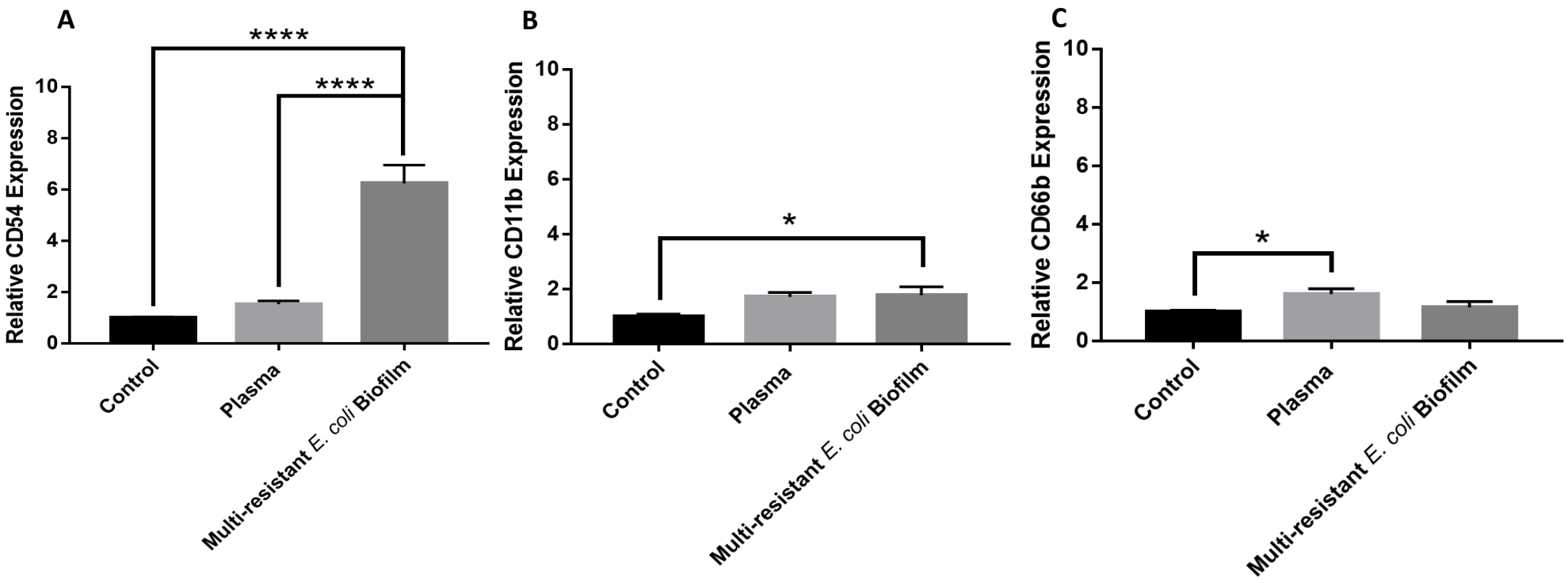


Figure 25. A) CD54, B) CD11b and C) CD66b expression of HL-60 cells treated, or not, with platelet releaseate collected from platelets contaminated with multi-resistant *E. coli* biofilm for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA. Statistical significance was defined as $p < 0.05$. Stastical significance was observed for CD54, ****p = < 0.0001, CD11b *p = 0.0360 and CD66b *p = 0.0309.

3.5 Effect of recombinant human RANTES (rhRANTES) on leucocyte surface activation markers.

As RANTES was found to be the most significant chemokine within the bacterially contaminated platelet releasates (Fig. 10), the effect of recombinant human RANTES (rhRANTES) on the activation status of these leucocyte subtypes was evaluated.

3.5.1 Effect of rhRANTES on U937 surface activation markers.

For U937 cells, no significant difference was found ($p > 0.05$) between the expression of both CD54 and CD11b markers when U937 cells were incubated for 24 h with recombinant RANTES at varying concentrations (Fig. 26).

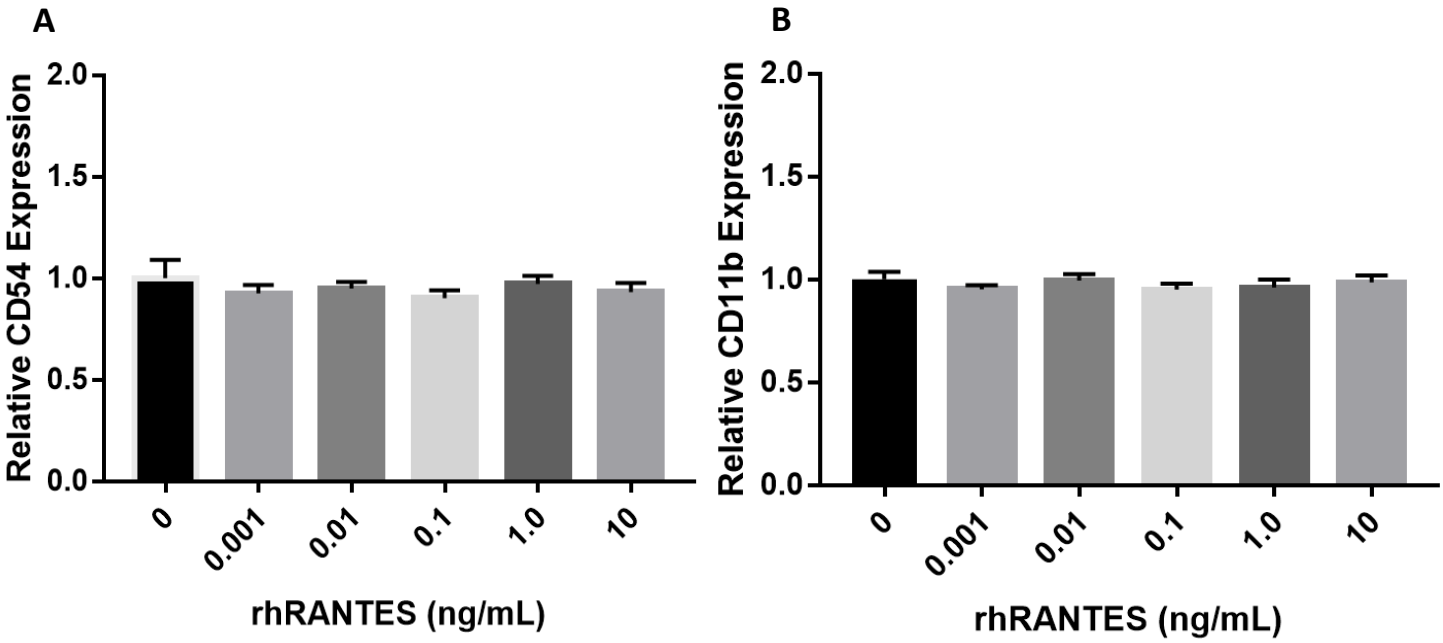


Figure 26. A) CD54 and B) CD11b expression on U937 cells after 24 h treatment with rhRANTES. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc tests. Statistical significance was defined as $p < 0.05$. No significance was found for either surface markers ($p > 0.05$).

3.5.2 Effect of rhRANTES on HL-60 Neutrophils Surface Activation Markers.

The incubation of rhRANTES and HL-60 neutrophils appeared to have no effect on surface marker expression for CD54 and CD66b (Fig. 27 A and C respectively). A trend can be seen with CD11b in which the marker was down regulated as the concentration of rhRANTES increased (0.001 ng/mL and 0.01 ng/mL) (Fig. 27 B). CD11b expression peaked at 0.1 ng/mL and gradually decreased again as concentration increased. No statistical significance was found for all three surface markers.

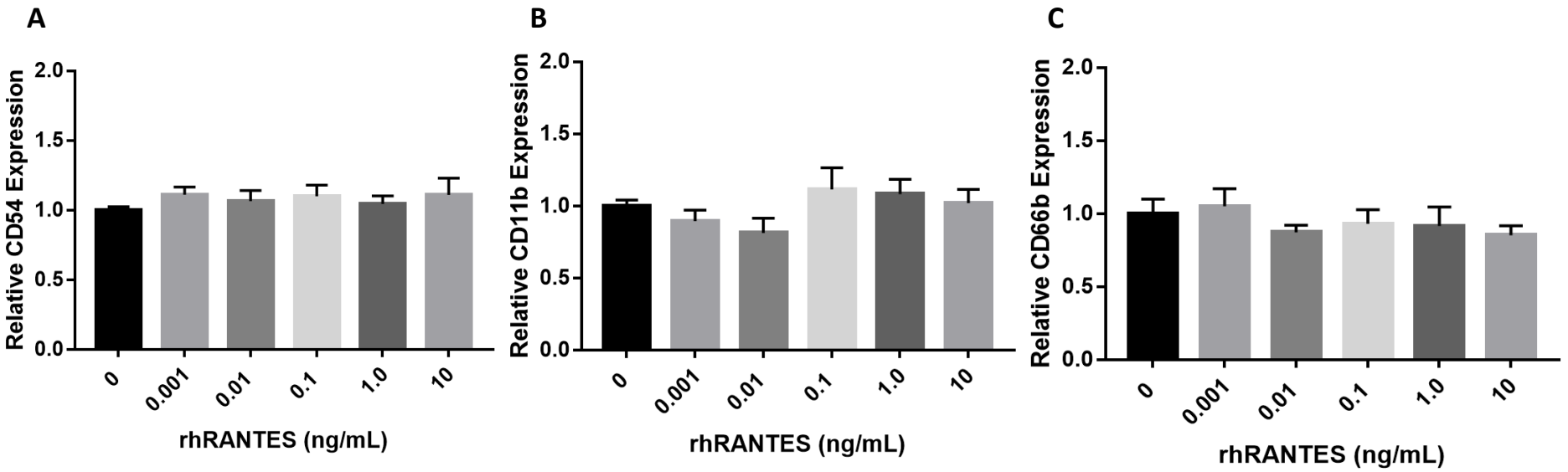


Figure 27. A) CD554, B) CD11b and C) CD66b expression on HL-60 differentiated neutrophils after 24 h incubation with rhRANTES. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc tests. Statistical significance was defined as $p < 0.05$. No significance was found for all surface markers at all concentrations of rhRANTES tested ($p > 0.05$).

3.5.3 Effect of rhRANTES on primary human neutrophils.

Treatment of rhRANTES with primary human neutrophils had variable effects on surface marker expression at different concentrations. However, no trend was observed with the exception of CD66b in which expression appeared to gradually decrease as the concentration of rhRANTES increased (Fig. 28 C). No statistical significance was found for CD54 expression and although expression peaked at 0.001 ng/mL it may be an anomaly (Fig. 28 A). Significance for CD11b expression was only found at 0.1 ng/mL in which CD11b levels were downregulated (Fig. 28 B). CD66b expression declined as concentration of rhRANTES increased and significance was found for all concentrations with the exception of 0.001 ng/mL (Fig. 28 C).

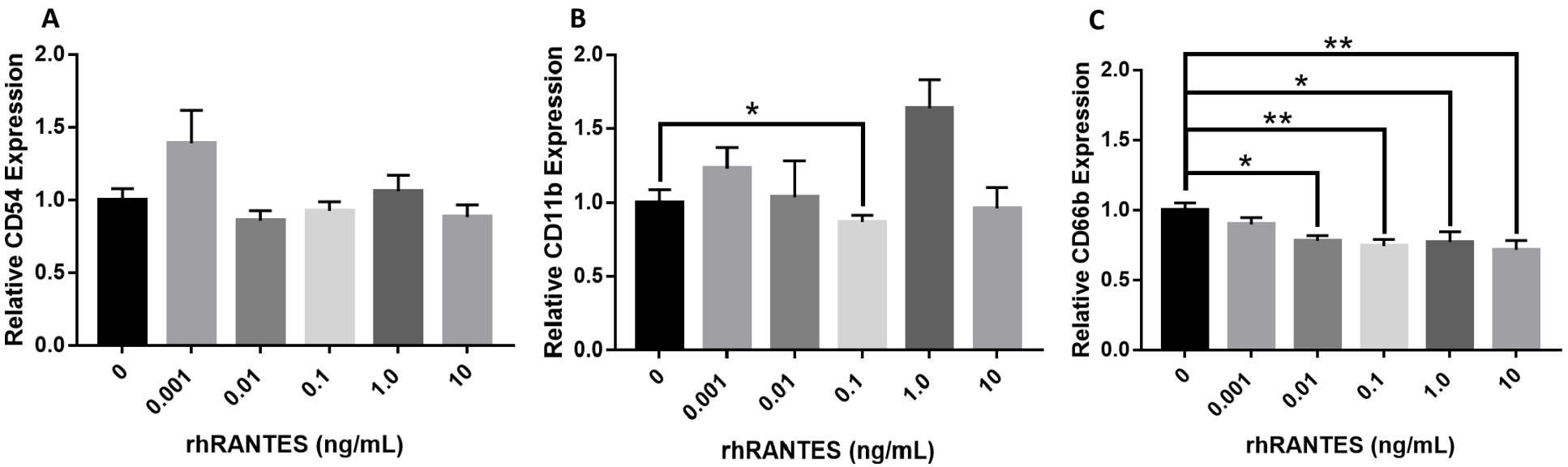


Figure 28. A) CD54, B) CD11b and C) CD66b expression on primary human neutrophils after treatment of recombinant RANTES for 24 h. Data are presented as Mean ± SEM, where n = 3 for each treatment group. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc tests. Statistical significance was defined as $p < 0.05$. No significance was found for CD54. Significance was found for CD11b expression, *p = 0.0247. Significance was found for CD66b expression, 0.01 ng/mL *p = 0.0303, 0.1 ng/mL **p = 0.0086, 1.0 ng/mL *p = 0.0231, 10 ng/mL **p = 0.0033.

In summary, planktonic and biofilm forms of *S. epidermidis* had minimal to no effect on the activation status of U937 cells. Multi-resistant *E. coli* was the most potent pathogen to upregulate surface marker expression in U937 cells, WT *E. coli* on the other hand, showed the same effects as *S. epidermidis* in its planktonic form but does appear to potentially supress CD11b expression in its biofilm morphology. As for Hl-60 cells, all pathogens were capable to exert changes in surface marker expression (mainly CD54) in both planktonic and biofilm forms, with the biofilms appearing to be more effective in upregulating CD54. The variable activation status of both cell lines towards bacterially primed platelet releasate could be indicative of a differential platelet response to different bacteria, which in-turn resulted in a differential response by elements of the innate immune system. In general, treatment of rhRANTES on both cell lines and human neutrophils did not show any notable changes in the surface markers tested with the exception of CD66b on human neutrophils where increasing the concentration of rhRANTES decreases the levels of CD66b.

4.0 Discussion

Blood products undergo strict regulations during collection and processing to ensure the quality of the product is maintained throughout its shelf-life with the overall aim of preventing the occurrence of adverse transfusion reactions. Implementation of risk reduction strategies and surveillance systems, more notably bacterial detection technologies and active surveillance, has reduced the number of reported incidences of transfusion reactions (Hong *et al.,* 2016). However, episodes of T-AS remains as one of the greatest concerns of transfusion medicine, particularly since many immunocompromised patients that receive platelet products which are the most susceptible blood component to bacterial contamination. Despite the universal testing systems (BacT/ALERT®) in place to detect bacterial contamination in platelet units, false negatives sometimes occur, mainly due to the presence of slow-growing species that fail to produce the required level of CO_2 required for detection (Benjamin and Wagner, 2007).

However, the question remains as to how the bacterial species or their products interact with components of the recipient's immune system following transfusion of the contaminated unit. The approach to this problem, presented in this thesis, was to determine the platelet secretory response to bacterial species commonly-implicated in T-AS, and investigate the effect of this platelet releasate on neutrophils and monocytes (the primary cells involved in the initial stages of sepsis).

4.1 Zones of Inhibition of Relevant T-AS Bacteria

It was important to firstly fully characterise the antibiotic susceptibility of the relevant T-AS bacteria being used in the study. *S. epidermidis* is considered one of the most clinically significant coagulase-negative Staphylococci as it is one of the most commonly isolated nosocomial pathogens (Eladli *et al.,* 2019). Therefore, it would not be unexpected to find resistance to multiple antibiotics, as *S. epidermidis* is part of the natural skin flora and resistant strains may be selected during antibiotic therapy (Eladii *et al.,* 2019). The results demonstrated, *S. epidermidis* was resistant to macrolides (erythromycin and streptomycin) which is encoded by the *erm*(C) gene (Sjölund *et al.,* 2005). The product of the *erm*(C) gene is a methylase that acts on the adenine residue on the 23S RNA, resulting in a conformational change in the 50S ribosomal subunit and thus macrolides can no longer bind to its specific

site (Sjölund *et al.,* 2005; Juda *et al.,* 2016). It has been frequently reported that *Staphylococcus* spp. were resistant to penicillin conferred through the production of β-lactamase (Baldwin *et al.,* 1969) which may explain the absence of a zone of inhibition with penicillin G. In addition, a study reported over 70% of *S. epidermidis* strains were resistant to methicillin classed drugs (oxacillin). This may occur since methicillins share a similar structure to penicillins which β-lactamase could also act upon (Farrell *et al.,* 2011). An implication of the antibiotic susceptibility profile for this strain of *S. epidermidis* would be that patients should be administrated with fusidic acid, tetracycline or chloramphenicol if found, to have been transfused with a *S. epidermidis*-positive platelet unit. Although data for the susceptibility of *S. epidermidis* to novobiocin was not found it is commonly used to distinguish it from *Serratia saprophyticus* (Eladli *et al.,* 2019).

S. marcescens has been recently recognised as a nosocomial pathogen after it was thought to be non-pathogenic to humans (Ramírez-Acros *et al.,* 2006). Like *E. coli*, *S. marcescens* is abundant in the environment which provides plenty of opportunity for it to gain antimicrobial resistance through horizontal gene transfer. For example, aminoglycoside (e.g. kanamycin) resistance is conferred through the transfer of the acquisition of a plasmid coding for a 16S rRNA methylase from *Pseudomonas aeruginosa* (Doi *et al.,* 2004). The susceptibility of the *S. marcescens* strain used in the study to kanamycin however, cannot be commented as the breakpoint was not available on EUCAST. Chloramphenicol would be the best antibiotic to use against infection with this specific strain of *S. marcescens.*

As a highly versatile bacterium, being able to survive within the gastrointestinal tract of its host and the outside environment, *E. coli* will exhibit a variety of antibiotic susceptibility profiles. *E. coli* WT was highly susceptive to a wide range of antibiotics tested with the exception of nalidixic acid, indicating that this strain did not possess a wide range of antibiotic resistant mechanisms. Therefore, treatment with this particular strain can be easily accomplished using tetracycline, chloramphenicol, nitrofurantoin or ampicillin. Nalidixic acid is a quinolone-classed antibiotic that targets DNA gyrase and topoisomerase IV, both of which are essential for DNA, RNA and protein synthesis respectively (Sáenz *et al.,* 2003). At low concentrations, nalidixic acid is bacteriostatic whilst high concentrations of the drug are bactericidal (Greenwood and O'Grady, 1978). Resistance to nalidixic acid could be conferred

through mutations of the *gyr*A and *gyr*B genes for DNA gyrase or mutations in the *par*C and *par*E genes for typoisomerase IV (Sáenz *et al.,* 2003). Mutations in either of the genes result in the loss of negatively charged amino acid residues important for the interactions between the enzymes and nalidixic acid (Niemira, 2005). Another explanation for resistance is that nalidixic acid activity is known to be affected by the inoculum density (Greenwood and O'Grady, 1978).

For a pathogen to be classified as multi-drug resistant, they must be non-susceptible to one agent in three or more classes of antibiotics (Arana *et al.,* 2017). The multi-resistant *E. coli* tested in this study was found to be resistant to five antibiotics all in different classes such as penicillin, polymyxin, aminoglycoside, quinolone and tetracycline. Acquisition of co-resistance is usually through mobile genetic elements such as plasmids, gene cassettes and transposons all in which would code for efflux pumps, enzymes that modify drugs, changes in target sites and changes in membrane permeability to antibiotics (Sáenz *et al.,* 2004). Chloramphenicol would be the recommended antibiotic to treat infection with this particular strain of multi-resistant *E. coli.* Although EUCAST does not have data on the susceptibility of *E. coli* to streptomycin, it has been reported that strains are highly resistant to the drug (van den Bogaard *et al.,* 2001).

4.2 Cytometric Bead Array Analysis of Bacterially Contaminated Platelet Releasates

Bacterial infections typically result in the production of specific pro-inflammatory cytokines and chemokines, leading to clearance of invading microbes. Platelets are now known to be a critical player in the initial recognition of bacteria, and the orchestration of subsequent immune responses. Cytometric Bead Array assays were used to determine the concentration of cytokines and chemokines released by platelets after bacterial contamination. TNF-α, IL-6, IL-1β and RANTES were measured as these cytokines and chemokines are commonly implicated in sepsis in the proinflammatory phase (Rigato *et al.,* 1996; Capoulade-Métay *et al*., 2006; Bester and Pretorius, 2016). Platelets primed with *S. epidermidis* and *S. marcescens* induced very similar cytokine profiles in which incubation with both forms of bacteria generated little to no TNF-α, IL-6 or IL-1β after 4 h incubation. Platelets primed with *S. epidermidis* (seven days) and *S. marcescens* (five and seven days) biofilms induced detectable TNF-α concentrations whilst their planktonic counterparts did not. Literature reported that

biofilm-released bacteria demonstrated higher virulence potential than their planktonic and biofilm-associated cells which may be one reason as to why TNF-α was detected in the biofilm samples (França *et al.,* 2016). Another reason could be that TNF-α is pre-formed in the α-granules of platelets (Limb *et al.,* 1999) and would be immediately detected in the samples. IL-6 and IL-1β on the other hand, may require longer an incubation period to allow synthesis and detection. This was supported by the detection of IL-6 and IL-1β after 24 h.

Both bacteria induced in very similar cytokine profiles after incubation with PRP for 24 h. However, *S. marcescens* produced higher concentrations of TNF-α and IL-6 compared to *S. epidermidis.* This may be due to the presence of lipopolysaccharide (LPS) in the cell wall of the Gram negative *S. marcescens* being a more potent mediator of TNF-α and IL-6 secretion. Another explanation for this may be that lipoteichoic acid (LTA), a cell wall component of Gram positive bacteria i.e. *S. epidermidis*, and LPS are recognised by TLR2 and TLR4 respectively and may activate different pathways for cytokine secretion resulting in variable cytokine profiles. In support of this, a study demonstrated that LTA was able to induce a modest inflammatory response in human lungs thus indicating that a different inflammatory profile was generated (Hoogerwerf *et al.,* 2008). Surprisingly, no IL-6 was detected following *S. epidermidis* biofilm treatment (established over seven days) but IL-6 was detected with the five-day biofilm. It was expected that the more mature biofilm would provoke a higher concentration of IL-6 due to higher bacterial counts thus increasing the chances of interaction between platelets and bacteria. This might be an anomaly or may be better explained by the development of a thicker extrapolymeric substance (EPS) in the seven day biofilm which provided better protection from immune recognition as bacterial ligands become inaccessible to platelets (Thurlow *et al.,* 2011). Indeed, it is known that more established biofilms are effective at evading immune recognition (Le *et al.,* 2018).

It was also unexpected to find a lack of IL-1β secretion following 24h incubation with *S. epidermidis* or *S. marcescens* biofilms. As one of the key mediators of the inflammatory response, IL-1 β would be expected to be released at similar concentrations as TNF-α. Previous data has shown that biofilms elicited lower levels of proinflammatory cytokines by 5- to 10-fold compared to planktonic cells (Spiliopoulou *et al.,* 2012). It was hypothesised that biofilm phase bacteria weakly stimulate Th_1 cells which are one of the major proinflammatory

cytokine secretor cells (Spiliopoulou *et al.,* 2012). In addition, it was also observed that production of immunosuppressive cytokines such as IL-13 and IL-10 were increased in biofilm-stimulated leucocytes (Spiliopoulou *et al.,* 2012). Generally, *S. epidermidis* (Gram positive) stimulated a higher concentration of IL-1β from platelets than *S. marcescens* (Gram negative). It has been shown that Gram negative bacteria possess chemoreceptors for specific chemokines to enhance their growth (Di Domenico *et al.,* 2018). The same study also demonstrated that *Staphylococcus aureus* (Gram positive) in both planktonic and biofilm forms was able to increase its own growth through the uptake of IL-1β and interferon-γ (IFN-γ) in a concentration dependent manner which may explain the low concentrations of IL-1β for *S. marcescens* (Di Domenico *et al.,* 2018). However, the validity of this in the present setting would require further investigation.

Interestingly, levels of RANTES were significantly higher than TNF-α, IL-6 and IL-1β following incubation of either bacteria in both planktonic and biofilm forms. RANTES has been established as a chemokine therefore its upregulation is expected during inflammation to promote the chemotaxis of leucocytes (Bacon *et al.,* 1995). Platelets are also known to be a major reservoir of RANTES (Ellis *et al.,* 2005), therefore, this could be a potential explanation for its high concentration in comparison to the other cytokines measure. Another suggestion for this observation is that IFN-γ has been shown to increase RANTES expression (Pan *et al.,* 2000). Therefore, other cytokines present within the platelet secretome may work in synergy with RANTES to promote its function. Erythrocytes have been shown to inhibit RANTES-mediated activation on a number of cells (Appay *et al.,* 1999), thus, the absence of erythrocytes may result in the uncontrolled release of RANTES from platelets.

Due to time constraints, CBA analysis of both *E. coli* strains were not carried out in this study.

4.3 U937 Monocytes Surface Expression After Incubation with Bacterially Contaminated PRP

The consequences of platelet and bacterial interactions leads to platelet degranulation of cytokines and chemokines to activate the immune response (Yeaman, 2010). As a result of this, surface expression of adhesion molecules and activation markers on leucocytes will be upregulated to allow transmigration of these cells towards the inflamed loci to exert their

effector functions (Lintomen *et al.,* 2018). CD54 and CD11b are adhesion molecules that are constitutively expressed on many leucocytes (Shin *et al.,* 2018). They mediate the adhesion between leucocytes and the endothelium and their upregulation is associated with leucocyte activation during infection and other clinical conditions (Latger-Cannard *et al.,* 2004; Sheikh and Jones, 2008). Therefore, changes in the expression of these markers can indicate the potential immune response within the recipient to bacterially contaminated PCs.

Collective interpretation of the data obtained when analysing the activation marker expression on U937 monocytes following contamination with planktonic bacteria showed that the platelet releasate from platelets contaminated with *S. epidermidis* or WT *E. coli* did not induce the upregulation of CD54 or CD11b on this cell type. However, the platelet releasate secreted in response to the multi-drug resistant strain of *E. coli* was found to vastly upregulate CD54, but not CD11b. Studies have shown that LPS on Gram negative bacteria is more potent in activating the inflammatory response compared to LTA on Gram positive bacteria by 100-fold (Yipp *et al.,* 2002; Finney *et al.,* 2012), which may provide a possible explanation for the difference in U937 monocyte activation status caused by *S. epidermidis* and multi-resistant *E. coli*. In addition to this, *S. epidermidis* lack typical elements that are usually recognised as virulence factors such as toxins (Yao *et al.,* 2005). Its main virulence is characterised by its ability to form biofilms (Greco *et al.,* 2007) therefore, this may explain the lack of changes in U937 monocyte activation. Some strains of *S. epidermidis* also possess a capsule which may prevent its recognition by platelet TLR2, thus there would be a lack of proinflammatory cytokine production to generate an immune response (Yipp *et al.,* 2002). To add to this, different species of Gram positive bacteria possess LTA with different efficacies in stimulating TLR2, for example, *Streptococcal pneumoniae* LTA is 100-fold less effective than *Staphylococcus aureus* LTA in stimulating TLR2 on Chinese hamster ovary cells (Han *et al.,* 2003); thus suggesting that *S. epidermidis* LTA lack the potency to elicit U937 cell activation. Strain variation between WT and multi-resistant *E. coli* could affect the potency of LPS in inducing upregulation of CD54 and CD11b, in which the LPS of multi-resistant *E. coli* appeared to be a more effective activator. However, despite demonstrating a significant increase in CD54 expression, levels of CD11b remains unchanged with multi-resistant *E. coli* contaminated releasate. The precise mechanism behind this observation is unknown but some reasons for this may include secretion of cytokines that specifically upregulate CD54

and not CD11b or that bacterial components of multi-resistant *E. coli* may work synergistically with plasma factors to regulate CD11b expression.

When looking at the effect of bacterial biofilm-stimulated platelets, again CD54 expression was found to be significantly elevated on U937 monocytes in response to stimulation with platelet-releasate from platelets contaminated with the multi-resistant *E. coli* strain. Stimulation of the platelets with biofilms of *S. epidermidis* or WT *E. coli* did not have the same effect. CD11b expression on U937 monocytes remained unaffected following stimulation by platelets that had been in contact with any of the biofilm species tested. One study had shown that biofilms causes lower level of cell activation through a sustained release of cytokines at reduced concentrations compared to their planktonic counterparts (Spiliopoulou *et al.,* 2012), which could be a possible explanation for the results observed for *S. epidermidis* and WT *E. coli*. Other potential reasons as to why there was a lack of changes in activation status with *S. epidermidis* and WT *E. coli* biofilms could be that previous studies have established that biofilms are capable of skewing the host's immune response towards immunosuppression to allow its persistence (Hanke *et al.,* 2013). In addition to this, it was shown that *S. epidermidis* was able to elicit a cytokine profile that encourages the polarisation of monocytes towards a M2 macrophage phenotype which possess anti-inflammatory properties and therefore, a decreased in the activation of U937 monocytes can be observed (Hanke *et al.,* 2013).

The reason for the upregulation of CD54 with contaminated multi-resistant *E. coli* releasate is again, not fully understood but the same reasoning with planktonic multi-resistant strain could be applied here. Genotypic and phenotypic changes, such as the exchange and upregulation of virulence factors, can occur when bacteria are within a biofilm (Jefferson, 2004). These changes may include the ability to produce toxins by multi-resistant *E. coli* and act as a potent platelet activator induce CD54-mediated aggregation in platelets through specific cytokine production (Watson *et al.,* 2016). Therefore, the cytokines produced by multi-resistant *E. coli* primed platelets could induced very similar effects on U937 monocytes. The ability of bacteria to form biofilms does not appear to cause any significant effect on the activation markers on U937 cells, instead, the antimicrobial resistance, species and strain of the bacteria seem to have a bigger impact.

4.4 HL-60 Neutrophils Surface Expression After Incubation with Bacterially Contaminated PRP

Results for HL-60 neutrophils showed that planktonic *S. epidermidis* was able to have a more significant effect on the activation status than its biofilm form and more so than on U937 monocytes. There may be some platelet activation due to the isolation protocol but the planktonic *S. epidermidis* contaminated releasate had a more significant effect on CD54 than plasma. Expression of CD66b was analysed in addition to CD54 and CD11b as it is a known granulocyte specific marker (Lakschevitz *et al.,* 2016). Neutrophil activation was observed for planktonic *S. epidermidis* but not the biofilm. This could be attributed to the anti-inflammatory effect of the biofilm as mentioned previously. As for the upregulation of CD54, both forms of *S. epidermidis* may work synergistically with factors in the plasma and/or platelet releasate to cause this effect.

In contrast to U937 monocytes, WT *E. coli* contaminated PRP was able to activate neutrophils in both forms. The biofilm appeared to have a more significant effect as both CD11b and CD66b activation markers were upregulated.

In line with the results observed for the U937 monocytes, the multi-resistant *E. coli* had the most significant effect on platelet-induced neutrophil CD54 expression, whilst CD11b and CD66b expression was comparable to that induced by other bacteria tested and their effects seen on U937 monocytes. This particular multi-resistant *E. coli* strain may possess the α-haemolysin toxin which could induce apoptosis, necrosis or lysis of cells (Nazareth *et al.,* 2007). In agreement with this study, α-haemolysin could cause the lysis of platelets and therefore, sudden release of its granule contents, resulting in subsequent variable surface marker expression.

4.5 U937 Monocytes, HL-60 Neutrophils and Primary Human Neutrophil Surface Expression After Incubation with rhRANTES

RANTES is classified as a C-C chemokine which was described to have a preferential effect on mononuclear cells, T cells and eosinophils but recent evidence has demonstrated that RANTES may also be involved in neutrophil migration in murine models (Ramos *et al.,* 2005). The receptors for RANTES are CCR1, CCR2, CCR3, and CCR5 and have been reported to be present

on many leucocytes including monocytes and neutrophils (Reichel *et al.,* 2006; Øynebråten *et al.,* 2015). From this, it may be speculated that RANTES would have a biological effect on neutrophils.

Flow cytometric analysis was carried out to investigate whether RANTES alone affected the activation status of the three cell lines tested. From the results, it was determined that rhRANTES had no effect on U937 monocytes and HL-60 neutrophils at all concentrations tested. However, it appeared to have some variable effect on primary human neutrophils but no correlation was shown between rhRANTES concentration and surface expression with the exception of CD66b. The effects of rhRANTES on HL-60 neutrophils also did not correlate to human neutrophils. These results are surprising as literature had reported that RANTES is a chemokine for monocytes and therefore, upregulation of CD54 and CD11b should be observed (Schall *et al.,* 1990; Kameyoshi *et al.,* 1992; Conklyn *et al.,* 1996). There is still an ongoing debate whether RANTES is a chemotactic agent for neutrophils as some studies have reported that neutrophil activation was absent in the presence of RANTES (Rot *et al.,* 1992; Conklyn *et al.,* 1996). Others authors have described that RANTES may be a weak chemoattractant for neutrophils as chemotaxis was observed but only moderately (Schall *et al.,* 1990; Zarbock *et al.,* 2007*;* Houard *et al.,* 2009). Others have reported an influx of neutrophils infiltration after RANTES expression (Pan *et al.,* 2000; Lim *et al.,* 2006).

One potential reason for the discrepancies between literature and the results of this study could be that RANTES exists in variable forms which can exert different biological activities. Natural RANTES and rhRANTES exist in the form of 1-68 but it was discovered that it can be processed by proteases associated on the surface of monocytes and neutrophils (Proost *et al.,* 1998; Lim *et al.,* 2006). The 3-68 RANTES variant is produced by cathepsin G by removing two NH_2-terminal residues and has been shown to have poor chemotactic activity on monocytes (Lim *et al.,* 2006). CD26 in contrast, converts 1-68 RANTES into 4-68 RANTES through the removal of three residues which exhibit a 10-fold lower affinity for CCR5 and 100-fold weaker chemotactic effect compared to the 3-68 variant (Lim *et al.,* 2005). As such, U937 and HL-60 cells may express high levels of CD26 and cathepsin G on their surfaces leading to high concentration of variant RANTES with reduced chemotactic properties. In contrast, human neutrophils were isolated from different donors therefore, heterogenetic expression

of these proteases may affect the number of available functional RANTES therefore significance can be found at variable concentrations of rhRANTES with no trends (Lim *et al.,* 2006). This may also explain the decrease in CD66b expression as the variant RANTES may cause an anti-inflammatory response in human neutrophils (Braunersreuther *et al.,* 2010). Another possibility is that the isolation protocol cannot guarantee the complete removal of erythrocytes which was demonstrated to have an inhibitory effect on RANTES (Appay *et al.,* 1999). In addition to this, human neutrophils can rapidly degranulate during isolation (Hanke *et al.,* 2013) which may cause an increase in CD66b activation but the presence of rhRANTES cause an anti-inflammatory effect and subsequent downregulation of CD66b.

Proteolysis of RANTES is thought to regulate the inflammatory response as both truncated RANTES are weak chemotactic agonists and/or inhibits the function of unprocessed RANTES through competitive binding of CCR5 leading to immunosuppression (Struyf *et al.,* 1996; Proost *et al.,* 1998; Lim *et al.,* 2006). In addition, it was discovered that the 3-68 variant was the most abundant form of RANTES and that intact RANTES may be a minor component of the total RANTES pool (Proost *et al.,* 1998; Lim *et al.,* 2006). This could suggest that intact RANTES may not be causing the effects observed in this study and/or that both truncated forms of RANTES may have more of an effect than what was originally thought.

Another factor that may affect the bioactivity of RANTES is that it has been recognised that RANTES is able to self-associate in a concentration-dependent manner and that polymorphisms of the E26 and E66 residues abolishes aggregation (Appay *et al.,* 1999; Czaplewski *et al.,* 1999). The functional properties of RANTES are dependent upon its self-aggregation as disaggregation renders RANTES non-inflammatory (Appay *et al.,* 1999; Pan *et al.,* 2000). Oligomerization of RANTES is promoted at high concentrations and by its interactions with glycosaminoglycans on the surface of cells which give rise to the potential of crosslinking with other cell surface receptors for signalling and cellular activation; disaggregated RANTES appears to lack this ability (Ebisawa *et al.,* 1994; Appay and Rowland-Jones, 2001; Øynebråten *et al.,* 2015). The lack of changes in surface expression on U937 and HL-60 cells may be caused the lack of aggregation of rhRANTES or that concentrations used were too low to generate aggregation. In line with this, it was reported that RANTES exerts its bioactivity at concentrations between 40-8000 ng/mL (Cocchi *et al.,* 1995).

From the results of this study, RANTES appears to have little to no effect on leucocyte activation and that the degree of cell activation is dependent on the bacterial contaminant, with the most virulent strain causing a significant increase in CD54. Whether the bacterial contaminant exist as a planktonic cell or biofilm does not appear to have a considerable effect on the activation status on U937 monocytes and HL-60 neutrophils. The implication of this suggest that the severity of T-AS/T-ABI might be related to the contaminating bacteria species ability to trigger an immune response from platelets and subsequent degranulation. RANTES itself may not cause the upregulation of the activation markers but may work synergistically with other cytokines and chemokines secreted by platelets to modulate expression. In the absence of bacterial contamination, the platelet releasate was able to upregulate surface expression of CD54, CD11b and CD66b implicating that the 273 suspected T-ABI cases negative for bacterial contamination may actually be caused the platelets themselves or other cytokines within the platelet releasate; this warrants further investigation to identify the causative agent (SHOT, 2015; SHOT, 2016; SHOT, 2017; SHOT, 2018).

Overall, the results of this study implicate that virulence of the pathogen affects the strength of the immune response of platelets as multi-resistant *E. coli* contaminated platelet releasate was shown to evoke significant upregulation in activation markers in both cell lines when compared to WT *E. coli* and *S. epidermidis*. However, whether RANTES released from platelet granules were the main driver of platelet immune activity remains to be elucidated. As mentioned previously, it was reported that RANTES concentrations were found to be between 40-8000 ng/mL in addition to specific forms of RANTES has low affinity towards CCR5, this may explain the huge difference in concentrations between other cytokines measured. The lack of activity on HL-60 and U937 cells along with variable activity of rhRANTES on human neutrophils showed that it is probable that RANTES itself may not be responsible for the activation of the immune cells. Rather, TNF-α may be the main cytokine responsible for immune cell activation as it is known that TNF-α and its receptor, type-1 TNF-α receptor, bind with high affinity (Turner *et al.,* 2010). In addition, it was also reported that some cell lines are sensitive to TNF-α at concentrations as low as 8 pg/mL (Khabar *et al.,* 1995). However, this does not rule out the possibility that RANTES may be the main inflammatory signal as concentrations tested were not in the range of its optimal bioactivity.

The myriad of inflammatory molecules stored in intracellular granules of platelets largely contributes to the platelet ability to participate in the immune response. The effector function of platelets can be activated through direct or indirect binding of platelet PRRs such as complement receptors, FcγRIIa, TLRs, GPIIb-IIIa and GPIb, and the pathogen (Ali *et al.,* 2017). The mechanisms behind the interaction between platelets and bacteria has been reviewed extensively by Hamzeh-Cognasse *et al.* The fundamental difference between *S. epidermidis* and *E. coli* was its cell wall components in which LTA was found exclusively in Gram positive bacteria whilst LPS was found in Gram negative bacteria. LTA was reported to be less efficient in inducing an inflammatory response during infection than LPS and may inhibit platelet activation instead, which could explain the lack or minor changes observed in *S. epidermidis* contaminated PRP treated cells (Tunjungputri *et al.,* 2016; Kang *et al.,* 2018). However, it was also reported that administration of LPS to healthy volunteers did not strongly activate platelets however, the results of this study contrasts this as WT *E. coli* was able to upregulate activation markers in its planktonic and biofilm forms (Schrottmaier *et al.,* 2015; Thomas *et al.,* 2015). The discrepancy may be due to different *E. coli* strains used in the studies but majority of literature reported a higher inflammatory response in many infectious models (Yipp *et al.,* 2002; Finney *et al.,* 2012). Multi-resistant *E. coli* had the most significant effect on the activation status of both cell lines than the WT strain and it may be due to the plasmid encoding antimicrobial resistance may also encode for the protease, alpha-toxin. Alpha toxin acts on calpains, a family of proteases involved in signal transduction in the cell cycle, which subsequently leads to the cleavage of Bcl-X (a transmembrane protein involved in platelet survival) and eventual apoptosis of platelets (Kraemer *et al.,* 2012). This event would lead to a substantial release of inflammatory molecules stored in platelet granules and potentially uncontrolled inflammation which may explain the significant upregulation of activation markers of HL-60 and U937 cells compared to WT *E. coli*.

As with the majority of studies, the design of this study is subject to limitations. Current literature on RANTES mainly focus on its role as a potential treatment for HIV and its activity in regulating T cells (Roscic-Mrkic *et al.,* 2003; Crawford *et al.,* 2011; Zhao *et al.,* 2016), as well as its role in neonatal-sepsis (Ng *et al.,* 2006; Manoura *et al.,* 2010; Kasztelewicz *et al.,* 2016) rather than in the context of transfusion. Therefore, the novelty of this study makes it difficult to compare to other studies and whether the results observed is a representative of the

events that may occur within PCs. Another limitation would be the concentration of physiological RANTES does not appear to be established as one study reported that physiological RANTES was found to be at 15 – 150 ng/mL (Trenchevska *et al.,* 2015), while another reported concentration to be at an average of 0.472 ng/mL (Albert *et al.,* 2017). The discrepancies between the studies could be due to the methodology and sensitivity issues with the equipment used for measurements or that the concentration of physiological RANTES is highly variable between individuals. Taking this into account, the concentrations of rhRANTES used in this study would fall into physiological concentrations therefore, minimal to no change in activation marker expression would be observed, in which the study demonstrated with U937 and HL-60 cell lines but not primary human neutrophils. Therefore, it would be important to determine the activity of RANTES at physiological conditions and how increasing or decreasing concentration would affect cell activation.

5.0 Future Work

The focus of this study was on the effects of bacterially-contaminated platelet releasates on monocytes and neutrophils. It was determined that considerable concentrations of RANTES were secreted by platelets after bacterial contamination. However, experiments using rhRANTES showed no effect on U937 and HL-60 cell lines. Yet variable effects were observed on primary human neutrophils. This raises more questions on the exact role of RANTES on the activation status of innate immune cells and its mechanism of action. Further research within this area will deepen the understanding of how bacterial contamination would affect platelet activation and the consequences that would occur after transfusion.

One area to look at in future work would be testing higher concentrations of RANTES than the ones used in this study as the minimum concentration of RANTES for it to exert its chemotatic activity was reported to be 40 ng/mL (Cocchi *et al.,* 1995). Determining the optimal concentration of RANTES to cause adverse transfusion reaction within recipient would be beneficial in the potential development of a detection system that measures the level of cytokines within PCs for bacterial contamination to overcome the sensitivity issues that current bacterial detection technologies face (Jacobs *et al.,* 2008). Another avenue to explore would be the possible synergistic interactions between RANTES and other cytokines present with the platelet releasate for a more accurate depiction of the events that might

occur within PCs in which complex interactions between platelets and cytokines are more likely to be involved than a single cytokine alone (Bartee and McFadden, 2013). Characterisation of the cytokine profiles evoked by different bacteria upon platelet stimulation would also be beneficial for further research as this study and other literature has shown that strain variation within a species can trigger different immune response within a host (Sela *et al.,* 2018). PCR studies looking at gene expression of CD26 and cathepsin G on leucocytes to determine the dominant RANTES form found in the samples could explain the reason as to why zero to variable effects could be seen in the rhRANTES treatment, which may be due to differences in protease expression on immortalized cell lines and primary human cells.

6.0 Conclusions

T-AS and T-ABI still remains as one of the leading causes of morbidity and mortality in transfusion recipients with bacterially contaminated PCs being the most frequently implicated blood product. Even with the advancement of the automated BacT/ALERT® microbial detection system, contaminated PCs can bypass screening tests due to initial low inoculums and consequently cause adverse transfusion reactions. Indeed, in over half of suspected T-ABI/T-AS cases in which the patients are showing obvious clinical symptoms, subsequent microbial follow-up investigations report culture negative or inconclusive. This raises the question on whether these T-AS/T-ABI clinical symptoms are caused by direct interaction of the bacteria themselves, or by bacterially-primed platelets.

CBA analysis demonstrated that the chemokine RANTES was secreted at highly significant concentrations in comparison to IL-1β, IL-6 and TNF-α when PRP was incubated with *S. epidermidis* and *S. marcescens* regardless of incubation times and/or whether the bacteria exist as planktonic cells or biofilms. These results suggest RANTES may play a key role in the initiation of the host immune response if the contaminated PC were to be transfused. Further investigations revealed that bacterially primed platelet releasates primed by either planktonic or biofilm forms of *S. epidermidis* and WT *E. coli* had no significant effect on the expression of CD54, CD11b and CD66b (HL-60 cells only) in U937 monocytes or HL-60 neutrophils. Yet following priming of platelets with planktonic or biofilm forms of multi-resistant *E. coli*, the platelet releasate caused significant upregulation of CD54 expression in both cell types. It is

suspected that this effect can be attributed to its virulence. Indeed, ZoI assays showed that multi-resistant *E. coli* was the least susceptible to antibiotics followed by *S. epidermidis* and WT *E. coli*. However, the precise mechanism behind this upregulation of CD54 activation marker is not fully understood. It must also be noted that plasma, free from bacterial contamination, was able to upregulate surface expression on both cell lines but this was only to a very moderate degree. It could however, suggest that proteins within the bacterially-primed plasma itself could contribute to the immune activation in T-AS.

Incubation of rhRANTES with U937 and HL-60 cells did not induce any significant changes in the expression of CD54, CD11b and CD66b. However, it appeared to have a variable effect on primary human neutrophils. Previous literature has stated that proteases on the surface of leucocytes are responsible for post-translational changes to many cytokines, including RANTES, giving rise to variable forms with different biological activities and efficacies. This may explain the inconsistent expression of CD54 and CD11b on primary neutrophils. Curiously, CD66b expression however, appears to be downregulated as rhRANTES increases.

In summary, despite its significantly upregulated release from bacterially primed platelets, RANTES itself may not be the sole chemokine that stimulates the expression of activation markers on innate immune cells as results from this study demonstrated variable effects. The type of bacterial contaminant does appear to play a key role in inducing the secretion of varying concentrations of specific cytokines from platelets that generate different degrees of activation of leucocytes. However, further investigation is warranted to elucidate the precise mechanism of action that causes these changes.

References

Ajuebor, M. N., Hogaboam, C. M., Kunkel, S. L., Proudfoot, A. E. and Wallace, J. L. (2001) 'The chemokine RANTES is a crucial mediator of the progression from acute to chronic colitis in the rat.' *J Immunol*, 166(1), pp. 552-558.

Akinosoglou, K. and Alexopoulos, D. (2014) 'Use of antiplatelet agents in sepsis: a glimpse into the future.' *Thromb Res*, 133(2), pp. 131-138.

Albert, V., Subramanian, A., Agrawal, D., Bhoi, S. K., Pallavi, P. and Mukhopadhayay, A. K. (2017) 'RANTES levels in peripheral blood, CSF and contused brain tissue as a marker for outcome in traumatic brain injury (TBI) patients.' *BMC Res Notes*, 10(1), p. 139.

Ali, H., Greco-Stewart, V. S., Jacobs, M. R., Yomtovian, R. A., Rood, I. G., de Korte, D. and Ramírez-Arcos, S. M. (2014) 'Characterization of the growth dynamics and biofilm formation of Staphylococcus epidermidis strains isolated from contaminated platelet units.' *J Med Microbiol*, 63(Pt 6), pp. 884-891.

Alves-Filho, J. C., Spiller, F. and Cunha, F. Q. (2010) 'Neutrophil paralysis in sepsis.' *Shock*, 34 Suppl 1, pp. 15-21.

Alves-Filho, J. C., Freitas, A., Souto, F. O., Spiller, F., Paula-Neto, H., Silva, J. S., Gazzinelli, R. T., Teixeira, M. M., Ferreira, S. H. and Cunha, F. Q. (2009) 'Regulation of chemokine receptor by Toll-like receptor 2 is critical to neutrophil migration and resistance to polymicrobial sepsis.' *Proc Natl Acad Sci U S A*, 106(10), pp. 4018-4023.

Appay, V. and Rowland-Jones, S. L. (2001) 'RANTES: a versatile and controversial chemokine.' *Trends Immunol*, 22(2), pp. 83-87.

Appay, V., Brown, A., Cribbes, S., Randle, E. and Czaplewski, L. G. (1999) 'Aggregation of RANTES is responsible for its inflammatory properties. Characterization of nonaggregating, noninflammatory RANTES mutants.' *J Biol Chem*, 274(39), pp. 27505-27512.

Arana, D. M., Rubio, M. and Alós, J. I. (2017) 'Evolution of antibiotic multiresistance in Escherichia coli and Klebsiella pneumoniae isolates from urinary tract infections: A 12-year analysis (2003-2014).' *Enferm Infecc Microbiol Clin*, 35(5), pp. 293-298.

Arslan, U., Erayman, I., Kirdar, S., Yuksekkaya, S., Cimen, O., Tuncer, I. and Bozdogan, B. (2010) 'Serratia marcescens sepsis outbreak in a neonatal intensive care unit.' *Pediatr Int*, 52(2), pp. 208-212.

Bacon, K. B., Premack, B. A., Gardner, P. and Schall, T. J. (1995) 'Activation of dual T cell signaling pathways by the chemokine RANTES.' *Science*, 269(5231), pp. 1727-1730.

Baldwin, J. N., Strickland, R. H. and Cox, M. F. (1969) 'Some properties of the beta-lactamase genes in Staphylococcus epidermidis.' *Appl Microbiol*, 18(4), pp. 628-630.

Bartee, E. and McFadden, G. (2013) 'Cytokine synergy: an underappreciated contributor to innate anti-viral immunity.' *Cytokine*, 63(3), pp. 237-240.

Benjamim, C. F., Ferreira, S. H. and Cunha, F. Q. (2000) 'Role of nitric oxide in the failure of neutrophil migration in sepsis.' *J Infect Dis*, 182(1), pp. 214-223.

Benjamim, C. F., Silva, J. S., Fortes, Z. B., Oliveira, M. A., Ferreira, S. H. and Cunha, F. Q. (2002) 'Inhibition of leukocyte rolling by nitric oxide during sepsis leads to reduced migration of active microbicidal neutrophils.' *Infect Immun*, 70(7), pp. 3602-3610.

Benjamin, R. J. and Wagner, S. J. (2007) 'The residual risk of sepsis: modeling the effect of concentration on bacterial detection in two-bottle culture systems and an estimation of false-negative culture rates.' *Transfusion*, 47(8), pp. 1381-1389.

Bester, J. and Pretorius, E. (2016) 'Effects of IL-1β, IL-6 and IL-8 on erythrocytes, platelets and clot viscoelasticity.' *Sci Rep*, 6, p. 32188.

Blajchman, M. A. and Goldman, M. (2001) 'Bacterial contamination of platelet concentrates: incidence, significance, and prevention.' *Semin Hematol*, 38(4 Suppl 11), pp. 20-26.

Blajchman, M. A., Beckers, E. A., Dickmeiss, E., Lin, L., Moore, G. and Muylle, L. (2005) 'Bacterial detection of platelets: current problems and possible resolutions.' *Transfus Med Rev*, 19(4), pp. 259-272.

Bleibtreu, A., Gros, P. A., Laouénan, C., Clermont, O., Le Nagard, H., Picard, B., Tenaillon, O. and Denamur, E. (2013) 'Fitness, stress resistance, and extraintestinal virulence in Escherichia coli.' *Infect Immun*, 81(8), pp. 2733-2742.

Boehlen, F. and Clemetson, K. J. (2001) 'Platelet chemokines and their receptors: what is their relevance to platelet storage and transfusion practice?' *Transfus Med*, 11(6), pp. 403-417.

Boomer, J. S., Green, J. M. and Hotchkiss, R. S. (2014) 'The changing immune system in sepsis: is individualized immuno-modulatory therapy the answer?' *Virulence*, 5(1), pp. 45-56.

Braunersreuther, V., Pellieux, C., Pelli, G., Burger, F., Steffens, S., Montessuit, C., Weber, C., Proudfoot, A., Mach, F. and Arnaud, C. (2010) 'Chemokine CCL5/RANTES inhibition reduces myocardial reperfusion injury in atherosclerotic mice.' *J Mol Cell Cardiol*, 48(4), pp. 789-798.

Brecher, M. E. (2018) 'Do older platelets increase the risk of transfusion-associated sepsis?' *Transfusion*, 58(1), pp. 1-2.

Brecher, M. E., Holland, P. V., Pineda, A. A., Tegtmeier, G. E. and Yomtovian, R. (2000) 'Growth of bacteria in inoculated platelets: implications for bacteria detection and the extension of platelet storage.' *Transfusion*, 40(11), pp. 1308-1312.

Capoulade-Métay, C., Ayouba, A., Kfutwah, A., Lole, K., Pêtres, S., Dudoit, Y., Deterre, P., Menu, E., Barré-Sinoussi, F., Debré, P. and Theodorou, I. (2006) 'A natural CCL5/RANTES variant antagonist for CCR1 and CCR3.' *Immunogenetics*, 58(7), pp. 533-541.

Cavaillon, J. M. and Adib-Conquy, M. (2005) 'Monocytes/macrophages and sepsis.' *Crit Care Med*, 33(12 Suppl), pp. S506-509.

Cavaillon, J. M., Adib-Conquy, M., Fitting, C., Adrie, C. and Payen, D. (2003) 'Cytokine cascade in sepsis.' *Scand J Infect Dis*, 35(9) pp. 535-544.

Chousterman, B. G., Swirski, F. K. and Weber, G. F. (2017) 'Cytokine storm and sepsis disease pathogenesis.' *Semin Immunopathol*, 39(5), pp. 517-528.

Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C. and Lusso, P. (1995) 'Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells.' *Science*, 270(5243), pp. 1811-1815.

Cognasse, F., Hamzeh-Cognasse, H., Lafarge, S., Delezay, O., Pozzetto, B., McNicol, A. and Garraud, O. (2008) 'Toll-like receptor 4 ligand can differentially modulate the release of cytokines by human platelets.' *Br J Haematol*, 141(1), pp. 84-91.

Conceição, R. A., Ludovico, M. S., Andrade, C. G. and Yano, T. (2012) 'Human sepsis-associated Escherichia coli (SEPEC) is able to adhere to and invade kidney epithelial cells in culture.' *Braz J Med Biol Res*, 45(5), pp. 417-424.

Conklyn, M. J., Neote, K. and Showell, H. J. (1996) 'Chemokine-dependent upregulation of CD11b on specific leukocyte subpopulations in human whole blood: effect of anticoagulant on rantes and MIP-1 beta stimulation.' *Cytokine*, 8(10), pp. 762-766.

Crawford, A., Angelosanto, J. M., Nadwodny, K. L., Blackburn, S. D. and Wherry, E. J. (2011) 'A role for the chemokine RANTES in regulating CD8 T cell responses during chronic viral infection.' *PLoS Pathog*, 7(7), p. e1002098.

Cristina, M. L., Sartini, M. and Spagnolo, A. M. (2019) 'Infections in Neonatal Intensive Care Units (NICUs).' *Int J Environ Res Public Health*, 16(4), p. 610

Czaplewski, L. G., McKeating, J., Craven, C. J., Higgins, L. D., Appay, V., Brown, A., Dudgeon, T., Howard, L. A., Meyers, T., Owen, J., Palan, S. R., Tan, P., Wilson, G., Woods, N. R., Heyworth, C. M., Lord, B. I., Brotherton, D., Christison, R., Craig, S., Cribbes, S., Edwards, R. M., Evans, S. J., Gilbert, R., Morgan, P., Randle, E., Schofield, N., Varley, P. G., Fisher, J., Waltho, J. P. and Hunter, M. G. (1999) 'Identification of amino acid residues critical for aggregation of human CC chemokines macrophage inflammatory protein (MIP)-1alpha, MIP-1beta, and RANTES. Characterization of active disaggregated chemokine variants.' *J Biol Chem*, 274(23), pp. 16077-16084.

Delano, M. J. and Ward, P. A. (2016) 'The immune system's role in sepsis progression, resolution, and long-term outcome.' *Immunol Rev*, 274(1), pp. 330-353.

Desroches, M., Clermont, O., Lafeuillade, B., Rodriguez, C., Darty, M., Royer, G., Bouvet, O., Ounnoughene, N., Noizat-Pirenne, F., Denamur, E. and Decousser, J. W. (2018) 'Genotypic and phenotypic characteristics of Escherichia coli involved in transfusion-transmitted bacterial infections: implications for preventive strategies.' *Transfusion*, 58(8), pp. 1940-1950.

Dessì, A., Puddu, M., Testa, M., Marcialis, M. A., Pintus, M. C. and Fanos, V. (2009) 'Serratia marcescens infections and outbreaks in neonatal intensive care units.' *J Chemother*, 21(5), pp. 493-499.

Di Domenico, E. G., Cavallo, I., Bordignon, V., Prignano, G., Sperduti, I., Gurtner, A., Trento, E., Toma, L., Pimpinelli, F., Capitanio, B. and Ensoli, F. (2018) 'Inflammatory cytokines and biofilm production sustain Staphylococcus aureus outgrowth and persistence: a pivotal interplay in the pathogenesis of Atopic Dermatitis.' *Sci Rep*, 8(1), p. 9573.

Doi, Y., Yokoyama, K., Yamane, K., Wachino, J., Shibata, N., Yagi, T., Shibayama, K., Kato, H. and Arakawa, Y. (2004) 'Plasmid-mediated 16S rRNA methylase in Serratia marcescens conferring high-level resistance to aminoglycosides.' *Antimicrob Agents Chemother*, 48(2), pp. 491-496.

Dong, Y., Speer, C. P. and Glaser, K. (2018) 'Beyond sepsis: Staphylococcus epidermidis is an underestimated but significant contributor to neonatal morbidity.' *Virulence*, 9(1), pp. 621-633.

Drayson, M. T., Michell, R. H., Durham, J. and Brown, G. (2001) 'Cell proliferation and CD11b expression are controlled independently during HL60 cell differentiation initiated by 1,25 alpha-dihydroxyvitamin D(3) or all-trans-retinoic acid.' *Exp Cell Res*, 266(1), pp. 126-134.

Drewry, A. M., Ablordeppey, E. A., Murray, E. T., Dalton, C. M., Fuller, B. M., Kollef, M. H. and Hotchkiss, R. S. (2018) 'Monocyte Function and Clinical Outcomes in Febrile and Afebrile Patients With Severe Sepsis.' *Shock*, 50(4), pp. 381-387.

Eash, K. J., Greenbaum, A. M., Gopalan, P. K. and Link, D. C. (2010) 'CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow.' *J Clin Invest*, 120(7), pp. 2423-2431.

Ebisawa, M., Yamada, T., Bickel, C., Klunk, D. and Schleimer, R. P. (1994) 'Eosinophil transendothelial migration induced by cytokines. III. Effect of the chemokine RANTES.' *J Immunol*, 153(5), pp. 2153-2160.

El-Sharkawy, H., Kantarci, A., Deady, J., Hasturk, H., Liu, H., Alshahat, M. and Van Dyke, T. E. (2007) 'Platelet-rich plasma: growth factors and pro- and anti-inflammatory properties.' *J Periodontol*, 78(4), pp. 661-669.

Eladli, M., Alharbi, N., Khaled, J., Kadaikunnan, S., Alobaidi, A. and Alyahya, S. (2019). 'Antibiotic-resistant Staphylococcus epidermidis isolated from patients and healthy

students comparing with antibiotic-resistant bacteria isolated from pasteurized milk.' *Saudi Journal of Biological Sciences*.

Ellis, M., al-Ramadi, B., Hedström, U., Frampton, C., Alizadeh, H. and Kristensen, J. (2005) 'Significance of the CC chemokine RANTES in patients with haematological malignancy: results from a prospective observational study.' *Br J Haematol*, 128(4), pp. 482-489.

Ertel, W., Kremer, J. P., Kenney, J., Steckholzer, U., Jarrar, D., Trentz, O. and Schildberg, F. W. (1995) 'Downregulation of proinflammatory cytokine release in whole blood from septic patients.' *Blood*, 85(5), pp. 1341-1347.

Faix, J. D. (2013) 'Biomarkers of sepsis.' *Crit Rev Clin Lab Sci*, 50(1), pp. 23-36.

Farrell, D. J., Mendes, R. E., Ross, J. E., Sader, H. S. and Jones, R. N. (2011) 'LEADER Program results for 2009: an activity and spectrum analysis of linezolid using 6,414 clinical isolates from 56 medical centers in the United States.' *Antimicrobial Agents Chemotherapy*, 55(8), pp. 3684-3690.

Ferri, L. E., Chia, S., Benay, C., Giannias, B. and Christou, N. V. (2009) 'L-selectin shedding in sepsis limits leukocyte mediated microvascular injury at remote sites.' *Surgery*, 145(4), pp. 384-391.

Finney, S. J., Leaver, S. K., Evans, T. W. and Burke-Gaffney, A. (2012) 'Differences in lipopolysaccharide- and lipoteichoic acid-induced cytokine/chemokine expression.' *Intensive Care Med*, 38(2), pp. 324-332.

Fleischmann, C., Scherag, A., Adhikari, N. K., Hartog, C. S., Tsaganos, T., Schlattmann, P., Angus, D. C., Reinhart, K. and Trialists, I. F. o. A. C. (2016) 'Assessment of Global Incidence and Mortality of Hospital-treated Sepsis. Current Estimates and Limitations.' *Am J Respir Crit Care Med*, 193(3), pp. 259-272.

França, A., Pérez-Cabezas, B., Correia, A., Pier, G. B., Cerca, N. and Vilanova, M. (2016) 'Biofilm-Released Cells Induce a Prompt and More Marked.' *Front Microbiol*, p. 1530.

Fujihara, M., Ikebuchi, K., Wakamoto, S. and Sekiguchi, S. (1999) 'Effects of filtration and gamma radiation on the accumulation of RANTES and transforming growth factor-beta1 in apheresis platelet concentrates during storage.' *Transfusion*, 39(5), pp. 498-505.

Gravemann, U., Handke, W., Müller, T. H. and Seltsam, A. (2019) 'Bacterial inactivation of platelet concentrates with the THERAFLEX UV-Platelets pathogen inactivation system.' *Transfusion*, 59(4), pp. 1324-1332.

Greco, C., Martincic, I., Gusinjac, A., Kalab, M., Yang, A. F. and Ramírez-Arcos, S. (2007) 'Staphylococcus epidermidis forms biofilms under simulated platelet storage conditions.' *Transfusion*, 47(7), pp. 1143-1153.

Greco-Stewart, V. S., Brown, E. E., Parr, C., Kalab, M., Jacobs, M. R., Yomtovian, R. A. and Ramírez-Arcos, S. M. (2012) 'Serratia marcescens strains implicated in adverse transfusion reactions form biofilms in platelet concentrates and demonstrate reduced detection by automated culture.' *Vox Sang*, 102(3), pp. 212-220.

Greenwood, D. and O'Grady, F. (1978) 'The responses of Escherichia coli to nalidixic acid.' *Chemotherapy*, 24(4) pp. 249-258.

Guo, H., Callaway, J. B. and Ting, J. P. (2015) 'Inflammasomes: mechanism of action, role in disease, and therapeutics.' *Nat Med*, 21(7), pp. 677-687.

Haddix, P. L. and Shanks, R. M. Q. (2018) 'Prodigiosin pigment of Serratia marcescens is associated with increased biomass production.' *Arch Microbiol*, 200(7), pp. 989-999.

Hafezi-Moghadam, A., Thomas, K. L., Prorock, A. J., Huo, Y. and Ley, K. (2001) 'L-selectin shedding regulates leukocyte recruitment.' *J Exp Med*, 193(7), pp. 863-872.

Hamzeh-Cognasse, H., Damien, P., Chabert, A., Pozzetto, B., Cognasse, F. and Garraud, O. (2015) 'Platelets and infections - complex interactions with bacteria.' *Frontiers in Immunology*, 6, p. 18.

Han, S. H., Kim, J. H., Martin, M., Michalek, S. M. and Nahm, M. H. (2003) 'Pneumococcal lipoteichoic acid (LTA) is not as potent as staphylococcal LTA in stimulating Toll-like receptor 2.' *Infect Immun*, 71(10), pp. 5541-5548.

Hanke, M. L., Heim, C. E., Angle, A., Sanderson, S. D. and Kielian, T. (2013) 'Targeting macrophage activation for the prevention and treatment of Staphylococcus aureus biofilm infections.' *J Immunol*, 190(5), pp. 2159-2168.

Heltberg, O., Skov, F., Gerner-Smidt, P., Kolmos, H. J., Dybkjaer, E., Gutschik, E., Jerne, D., Jepsen, O. B., Weischer, M. and Frederiksen, W. (1993) 'Nosocomial epidemic of Serratia marcescens septicemia ascribed to contaminated blood transfusion bags.' *Transfusion*, 33(3), pp. 221-227.

Hodgson, S. D., Greco-Stewart, V., Jimenez, C. S., Sifri, C. D., Brassinga, A. K. and Ramirez-Arcos, S. (2014) 'Enhanced pathogenicity of biofilm-negative Staphylococcus epidermidis isolated from platelet preparations.' *Transfusion*, 54(2), pp. 461-470.

Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A. and Miller, S. I. (2005) 'Aminoglycoside antibiotics induce bacterial biofilm formation.' *Nature*, 436(7054), pp. 1171-1175.

Hong, H., Xiao, W., Lazarus, H. M., Good, C. E., Maitta, R. W. and Jacobs, M. R. (2016) 'Detection of septic transfusion reactions to platelet transfusions by active and passive surveillance.' *Blood*, 127(4), pp. 496-502.

Hoogerwerf, J. J., de Vos, A. F., Bresser, P., van der Zee, J. S., Pater, J. M., de Boer, A., Tanck, M., Lundell, D. L., Her-Jenh, C., Draing, C., von Aulock, S. and van der Poll, T. (2008) 'Lung

inflammation induced by lipoteichoic acid or lipopolysaccharide in humans.' *Am J Respir Crit Care Med*, 178(1), pp. 34-41.

Hotchkiss, R. S., Monneret, G. and Payen, D. (2013) 'Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy.' *Nat Rev Immunol*, 13(12), pp. 862-874.

Houard, X., Touat, Z., Ollivier, V., Louedec, L., Philippe, M., Sebbag, U., Meilhac, O., Rossignol, P. and Michel, J. B. (2009) 'Mediators of neutrophil recruitment in human abdominal aortic aneurysms.' *Cardiovasc Res*, 82(3), pp. 532-541.

Huang, H., Wang, S., Jiang, T., Fan, R., Zhang, Z., Mu, J., Li, K., Wang, Y., Jin, L., Lin, F., Xia, J., Sun, L., Xu, B., Ji, C., Chen, J., Chang, J., Tu, B., Song, B., Zhang, C., Wang, F. and Xu, R. (2018). 'High levels of circulating GM-CSF+CD4+ T cells are predictive of poor outcomes in sepsis patients: a prospective cohort study. *Cellular & Molecular Immunology*, 16(6), pp.602-610.

Högman, C. F., Fritz, H. and Sandberg, L. (1993) 'Posttransfusion Serratia marcescens septicemia.' *Transfusion*, 33(3), pp. 189-191.

Idriss, H. T. and Naismith, J. H. (2000) 'TNF alpha and the TNF receptor superfamily: structure-function relationship(s).' *Microsc Res Tech*, 50(3), pp. 184-195.

Jacobi, J. (2002) 'Pathophysiology of sepsis.' *Am J Health Syst Pharm*, 59 Suppl 1, pp. S3-8.

Jacobs, M. R., Good, C. E., Lazarus, H. M. and Yomtovian, R. A. (2008) 'Relationship between bacterial load, species virulence, and transfusion reaction with transfusion of bacterially contaminated platelets.' *Clin Infect Dis*, 46(8), pp. 1214-1220.

Jauréguy, F., Carbonnelle, E., Bonacorsi, S., Clec'h, C., Casassus, P., Bingen, E., Picard, B., Nassif, X. and Lortholary, O. (2007) 'Host and bacterial determinants of initial severity and outcome of Escherichia coli sepsis.' *Clin Microbiol Infect*, 13(9), pp. 854-862.

Jefferson, K. (2004). 'What drives bacteria to produce a biofilm?' *FEMS Microbiology Letters*, 236(2), pp.163-173.

Jiang, D., Liang, J. and Noble, P. W. (2007) 'Hyaluronan in tissue injury and repair.' *Annu Rev Cell Dev Biol*, 23 pp. 435-461.

Jones, A. E., Trzeciak, S. and Kline, J. A. (2009) 'The Sequential Organ Failure Assessment score for predicting outcome in patients with severe sepsis and evidence of hypoperfusion at the time of emergency department presentation.' *Crit Care Med*, 37(5), pp. 1649-1654.

Juda, M., Chudzik-Rzad, B. and Malm, A. (2016) 'The prevalence of genotypes that determine resistance to macrolides, lincosamides, and streptogramins B compared with spiramycin susceptibility among erythromycin-resistant Staphylococcus epidermidis.' *Mem Inst Oswaldo Cruz*, 111(3), pp. 155-160.

Kameyoshi, Y., Dörschner, A., Mallet, A. I., Christophers, E. and Schröder, J. M. (1992) 'Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils.' *J Exp Med*, 176(2), pp. 587-592.

Kang, S., Kim, S., Baik, J., Ko, E., Ahn, K., Yun, C. and Han, S., 2018. Staphylococcal LTA antagonizes the B cell-mitogenic potential of LPS. *Scientific Reports*, 8(1).

Kasztelewicz, B., Piotrowska, E., Tołłoczko, J., Borszewska-Kornacka, M. K., Gregorek, H. and Dzierżanowska-Fangrat, K. (2016) 'Assessment of interleukin-17A, C5a and RANTES for early diagnosis of neonatal sepsis - a preliminary study.' *Cent Eur J Immunol*, 41(4), pp. 376-382.

Kaufmann, I., Hoelzl, A., Schliephake, F., Hummel, T., Chouker, A., Peter, K. and Thiel, M. (2006) 'Polymorphonuclear leukocyte dysfunction syndrome in patients with increasing sepsis severity.' *Shock*, 26(3), pp. 254-261.

Khabar, K., Siddiqui, S. and Armstrong, J., 1995. WEHI-13VAR: a stable and sensitive variant of WEHI 164 clone 13 fibrosarcoma for tumor necrosis factor bioassay. *Immunology Letters*, 46(1-2), pp.107-110.

Klinger, M. H., Wilhelm, D., Bubel, S., Sticherling, M., Schröder, J. M. and Kühnel, W. (1995) 'Immunocytochemical localization of the chemokines RANTES and MIP-1 alpha within human platelets and their release during storage.' *Int Arch Allergy Immunol*, 107(4), pp. 541-546.

Koupenova, M., Mick, E., Mikhalev, E., Benjamin, E. J., Tanriverdi, K. and Freedman, J. E. (2015) 'Sex differences in platelet toll-like receptors and their association with cardiovascular risk factors.' *Arterioscler Thromb Vasc Biol*, 35(4), pp. 1030-1037.

Kraemer, B., Campbell, R., Schwertz, H., Franks, Z., Vieira de Abreu, A., Grundler, K., Kile, B., Dhakal, B., Rondina, M., Kahr, W., Mulvey, M., Blaylock, R., Zimmerman, G. and Weyrich, A., 2012. Bacteria differentially induce degradation of Bcl-xL, a survival protein, by human platelets. *Blood*, 120(25), pp.5014-5020.

Kuehnert, M. J., Roth, V. R., Haley, N. R., Gregory, K. R., Elder, K. V., Schreiber, G. B., Arduino, M. J., Holt, S. C., Carson, L. A., Banerjee, S. N. and Jarvis, W. R. (2001) 'Transfusion-transmitted bacterial infection in the United States, 1998 through 2000.' *Transfusion*, 41(12), pp. 1493-1499.

Kumar, V. (2018) 'Inflammasomes: Pandora's box for sepsis.' *J Inflamm Res*, 11, pp. 477-502.

Lakschevitz, F. S., Hassanpour, S., Rubin, A., Fine, N., Sun, C. and Glogauer, M. (2016) 'Identification of neutrophil surface marker changes in health and inflammation using high-throughput screening flow cytometry.' *Exp Cell Res*, 342(2), pp. 200-209.

Latger-Cannard, V., Besson, I., Doco-Lecompte, T. and Lecompte, T. (2004) 'A standardized procedure for quantitation of CD11b on polymorphonuclear neutrophil by flow cytometry: potential application in infectious diseases.' *Clin Lab Haematol*, 26(3), pp. 177-186.

Le, K. Y., Park, M. D. and Otto, M. (2018) 'Immune Evasion Mechanisms of.' *Front Microbiol*, p. 359.

Lerman, Y. V. and Kim, M. (2015) 'Neutrophil migration under normal and sepsis conditions.' *Cardiovasc Hematol Disord Drug Targets*, 15(1) pp. 19-28.

Levy, M. M., Fink, M. P., Marshall, J. C., Abraham, E., Angus, D., Cook, D., Cohen, J., Opal, S. M., Vincent, J. L., Ramsay, G. and SCCM/ESICM/ACCP/ATS/SIS. (2003) '2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference.' *Crit Care Med*, 31(4), pp. 1250-1256.

Lim, J. K., Burns, J. M., Lu, W. and DeVico, A. L. (2005) 'Multiple pathways of amino terminal processing produce two truncated variants of RANTES/CCL5.' *J Leukoc Biol*, 78(2), pp. 442-452.

Lim, J. K., Lu, W., Hartley, O. and DeVico, A. L. (2006) 'N-terminal proteolytic processing by cathepsin G converts RANTES/CCL5 and related analogs into a truncated 4-68 variant.' *J Leukoc Biol*, 80(6), pp. 1395-1404.

Limb, G. A., Webster, L., Soomro, H., Janikoun, S. and Shilling, J. (1999) 'Platelet expression of tumour necrosis factor-alpha (TNF-alpha), TNF receptors and intercellular adhesion molecule-1 (ICAM-1) in patients with proliferative diabetic retinopathy.' *Clin Exp Immunol*, 118(2), pp. 213-218.

Lintomen, L., Franchi, G., Nowill, A., Condino-Neto, A., de Nucci, G., Zanesco, A. and Antunes, E. (2008) 'Human eosinophil adhesion and degranulation stimulated with eotaxin and RANTES in vitro: lack of interaction with nitric oxide.' *BMC Pulm Med*, 8, p. 13.

Loza-Correa, M., Ayala, J. A., Perelman, I., Hubbard, K., Kalab, M., Yi, Q. L., Taha, M., de Pedro, M. A. and Ramirez-Arcos, S. (2019) 'The peptidoglycan and biofilm matrix of Staphylococcus epidermidis undergo structural changes when exposed to human platelets.' *PLoS One*, 14(1), p. e0211132.

Manoura, A., Gourgiotis, D., Galanakis, E., Matalliotakis, E., Hatzidaki, E., Korakaki, E., Saitakis, E., Marmarinos, A. S. and Giannakopoulou, C. (2010) 'Circulating concentrations of α- and β-chemokines in neonatal sepsis.' *Int J Infect Dis*, 14(9), pp. e806-809.

Mase, K., Hasegawa, T., Horii, T., Hatakeyama, K., Kawano, Y., Yamashino, T. and Ohta, M. (2000) 'Firm adherence of Staphylococcus aureus and Staphylococcus epidermidis to human hair and effect of detergent treatment.' *Microbiol Immunol*, 44(8) pp. 653-656.

Martinon, F., Burns, K. and Tschopp, J. (2002). The Inflammasome: A Molecular Platform Triggering Activation of Inflammatory Caspases and Processing of proIL-β. *Molecular Cell*, 10(2), pp.417-426.

McDonald, C., Allen, J., Brailsford, S., Roy, A., Ball, J., Moule, R., Vasconcelos, M., Morrison, R. and Pitt, T. (2017) 'Bacterial screening of platelet components by National Health Service Blood and Transplant, an effective risk reduction measure.' *Transfusion*, 57(5), pp. 1122-1131.

Mohr, H., Lambrecht, B., Bayer, A., Spengler, H., Nicol, S., Montag, T. and Muller, T. (2006). Sterility testing of platelet concentrates prepared from deliberately infected blood donations. *Transfusion*, 46(3), pp.486-491

Mohs, A., Kuttkat, N., Reißing, J., Zimmermann, H. W., Sonntag, R., Proudfoot, A., Youssef, S. A., de Bruin, A., Cubero, F. J. and Trautwein, C. (2017) 'Functional role of CCL5/RANTES for HCC progression during chronic liver disease.' *J Hepatol*, 66(4), pp. 743-753.

Monneret, G., Finck, M. E., Venet, F., Debard, A. L., Bohé, J., Bienvenu, J. and Lepape, A. (2004) 'The anti-inflammatory response dominates after septic shock: association of low

monocyte HLA-DR expression and high interleukin-10 concentration.' *Immunol Lett*, 95(2), pp. 193-198.

Mosavie, M., Blandy, O., Jauneikaite, E., Caldas, I., Ellington, M. J., Woodford, N. and Sriskandan, S. (2019) 'Sampling and diversity of Escherichia coli from the enteric microbiota in patients with Escherichia coli bacteraemia.' *BMC Res Notes*, 12(1), p. 335.

Murphy, W. G., Foley, M., Doherty, C., Tierney, G., Kinsella, A., Salami, A., Cadden, E. and Coakley, P. (2008) 'Screening platelet concentrates for bacterial contamination: low numbers of bacteria and slow growth in contaminated units mandate an alternative approach to product safety.' *Vox Sang*, 95(1), pp. 13-19.

Nazareth, H., Genagon, S. A. and Russo, T. A. (2007) 'Extraintestinal pathogenic Escherichia coli survives within neutrophils.' *Infect Immun*, 75(6), pp. 2776-2785.

Ng, P. C., Li, K., Leung, T. F., Wong, R. P., Li, G., Chui, K. M., Wong, E., Cheng, F. W. and Fok, T. F. (2006) 'Early prediction of sepsis-induced disseminated intravascular coagulation with interleukin-10, interleukin-6, and RANTES in preterm infants.' *Clin Chem*, 52(6), pp. 1181-1189.

Niemira, B. (2005). Nalidixic Acid Resistance Increases Sensitivity ofEscherichia coliO157:H7 to Ionizing Radiation in Solution and on Green Leaf Lettuce. *Journal of Food Science*, 70(2), pp.M121-M124.

Ono, S. J., Nakamura, T., Miyazaki, D., Ohbayashi, M., Dawson, M. and Toda, M. (2003) 'Chemokines: roles in leukocyte development, trafficking, and effector function.' *J Allergy Clin Immunol*, 111(6), pp. 1185-1199; quiz 1200.

Pan, Z. Z., Parkyn, L., Ray, A. and Ray, P. (2000) 'Inducible lung-specific expression of RANTES: preferential recruitment of neutrophils.' *Am J Physiol Lung Cell Mol Physiol*, 279(4), pp. L658-666.

Petros, A., Lamb, G., Leone, A., Moncada, S., Bennett, D. and Vallance, P. (1994) 'Effects of a nitric oxide synthase inhibitor in humans with septic shock.' *Cardiovasc Res*, 28(1), pp. 34-39.

Proost, P., De Meester, I., Schols, D., Struyf, S., Lambeir, A. M., Wuyts, A., Opdenakker, G., De Clercq, E., Scharpé, S. and Van Damme, J. (1998) 'Amino-terminal truncation of chemokines by CD26/dipeptidyl-peptidase IV. Conversion of RANTES into a potent inhibitor of monocyte chemotaxis and HIV-1-infection.' *J Biol Chem*, 273(13), pp. 7222-7227.

Proudfoot, A. E., Power, C. A., Hoogewerf, A. J., Montjovent, M. O., Borlat, F., Offord, R. E. and Wells, T. N. (1996) 'Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist.' *J Biol Chem*, 271(5), pp. 2599-2603.

Ramakrishna, C. and Cantin, E. M. (2018) 'IFNγ inhibits G-CSF induced neutrophil expansion and invasion of the CNS to prevent viral encephalitis.' *PLoS Pathog*, 14(1), p. e1006822.

Ramos, C. D., Canetti, C., Souto, J. T., Silva, J. S., Hogaboam, C. M., Ferreira, S. H. and Cunha, F. Q. (2005) 'MIP-1alpha[CCL3] acting on the CCR1 receptor mediates neutrophil migration in immune inflammation via sequential release of TNF-alpha and LTB4.' *J Leukoc Biol*, 78(1), pp. 167-177.

Ramírez-Arcos, S., Chin-Yee, I., Hume, H., Fearon, M., Goldman, M., Eckert, K., Martincic, I., Peters, G., Kovach, D. and Richardson, S. E. (2006) 'Fatal septic shock associated with transfusion-transmitted Serratia marcescens.' *Transfusion*, 46(4), pp. 679-681.

Reichel, C. A., Khandoga, A., Anders, H. J., Schlöndorff, D., Luckow, B. and Krombach, F. (2006) 'Chemokine receptors Ccr1, Ccr2, and Ccr5 mediate neutrophil migration to postischemic tissue.' *J Leukoc Biol*, 79(1), pp. 114-122.

Reinhart, K., Daniels, R., Kissoon, N., Machado, F. R., Schachter, R. D. and Finfer, S. (2017) 'Recognizing Sepsis as a Global Health Priority - A WHO Resolution.' *N Engl J Med*, 377(5), pp. 414-417.

Rigato, O., Ujvari, S., Castelo, A. and Salomão, R. (1996) 'Tumor necrosis factor alpha (TNF-alpha) and sepsis: evidence for a role in host defense.' *Infection*, 24(4), pp. 314-318.

Rincón, E., Rocha-Gregg, B. L. and Collins, S. R. (2018) 'A map of gene expression in neutrophil-like cell lines.' *BMC Genomics*, 19(1), p. 573.

Roscic-Mrkic, B., Fischer, M., Leemann, C., Manrique, A., Gordon, C. J., Moore, J. P., Proudfoot, A. E. and Trkola, A. (2003) 'RANTES (CCL5) uses the proteoglycan CD44 as an auxiliary receptor to mediate cellular activation signals and HIV-1 enhancement.' *Blood*, 102(4), pp. 1169-1177.

Rot, A., Krieger, M., Brunner, T., Bischoff, S. C., Schall, T. J. and Dahinden, C. A. (1992) 'RANTES and macrophage inflammatory protein 1 alpha induce the migration and activation of normal human eosinophil granulocytes.' *J Exp Med*, 176(6), pp. 1489-1495.

Roth, V. R., Arduino, M. J., Nobiletti, J., Holt, S. C., Carson, L. A., Wolf, C. F., Lenes, B. A., Allison, P. M. and Jarvis, W. R. (2000) 'Transfusion-related sepsis due to Serratia liquefaciens in the United States.' *Transfusion*, 40(8), pp. 931-935.

Salomao, R., Brunialti, M. K., Kallás, E. G., Martins, P. S., Rigato, O. and Freudenberg, M. (2002) 'Lipopolysaccharide-cell interaction and induced cellular activation in whole blood of septic patients.' *J Endotoxin Res*, 8(5) pp. 371-379.

Schall, T. J., Bacon, K., Toy, K. J. and Goeddel, D. V. (1990) 'Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES.' *Nature*, 347(6294), pp. 669-671.

Schnoor, M. and Parkos, C. A. (2008) 'Disassembly of endothelial and epithelial junctions during leukocyte transmigration.' *Front Biosci*, 13, pp. 6638-6652.

Schrottmaier, W., Kral, J., Zeitlinger, M., Salzmann, M., Jilma, B. and Assinger, A., 2016. Platelet activation at the onset of human endotoxemia is undetectablein vivo. *Platelets*, 27(5), pp.479-483.

Sela, U., Euler, C. W., Correa da Rosa, J. and Fischetti, V. A. (2018) 'Strains of bacterial species induce a greatly varied acute adaptive immune response: The contribution of the accessory genome.' *PLoS Pathog*, 14(1), p. e1006726.

Sepsis Trust. (2019) *References and Sources*. [Online] [Accessed on 9th August 2019] https://sepsistrust.org/about/about-sepsis/references-and-sources/

Serious Hazards of Transfusion. (2016) *Annual SHOT Report 2015.* [Online] [Accessed on 31st July 2019] https://www.shotuk.org/wp-content/uploads/myimages/SHOT-2015-Annual-Report-Web-Edition-Final-bookmarked-1.pdf

Serious Hazards of Transfusion. (2017) *Annual SHOT Report 2016.* [Online] [Accessed on 31st July 2019] https://www.shotuk.org/wp-content/uploads/myimages/SHOT-Report-2016_web_11th-July.pdf

Serious Hazards of Transfusion. (2018) *Annual SHOT Report 2017.* [Online] [Accessed on 31st July 2019] https://www.shotuk.org/wp-content/uploads/myimages/SHOT-Report-2017-WEB-Final-v4-25-9-18.pdf

Serious Hazards of Transfusion. (2019) *Annual SHOT Report 2018.* [Online] [Accessed on 31st July 2019] https://www.shotuk.org/wp-content/uploads/myimages/SHOT-Report-2018_Web_Version.pdf

Shalova, I. N., Lim, J. Y., Chittezhath, M., Zinkernagel, A. S., Beasley, F., Hernández-Jiménez, E., Toledano, V., Cubillos-Zapata, C., Rapisarda, A., Chen, J., Duan, K., Yang, H., Poidinger,

M., Melillo, G., Nizet, V., Arnalich, F., López-Collazo, E. and Biswas, S. K. (2015) 'Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1α.' *Immunity*, 42(3), pp. 484-498.

Shankar-Hari, M., Harrison, D. A., Ferrando-Vivas, P., Rubenfeld, G. D. and Rowan, K. (2019) 'Risk Factors at Index Hospitalization Associated With Longer-term Mortality in Adult Sepsis Survivors.' *JAMA Netw Open*, 2(5), p. e194900.

Shapiro, N., Howell, M. D., Bates, D. W., Angus, D. C., Ngo, L. and Talmor, D. (2006) 'The association of sepsis syndrome and organ dysfunction with mortality in emergency department patients with suspected infection.' *Ann Emerg Med*, 48(5), pp. 583-590, 590.e581.

Sheikh, N. A. and Jones, L. A. (2008) 'CD54 is a surrogate marker of antigen presenting cell activation.' *Cancer Immunol Immunother*, 57(9), pp. 1381-1390.

Shen, X. F., Cao, K., Jiang, J. P., Guan, W. X. and Du, J. F. (2017) 'Neutrophil dysregulation during sepsis: an overview and update.' *J Cell Mol Med*, 21(9), pp. 1687-1697.

Shin, W. G., Park, B. J., Lee, S. J. and Kim, J. G. (2018) 'Infection of human intestinal epithelial cells by invasive bacteria activates NF-κB and increases ICAM-1 expression through NOD1.' *Korean J Intern Med*, 33(1), pp. 81-90.

Singer, M., Deutschman, C. S., Seymour, C. W., Shankar-Hari, M., Annane, D., Bauer, M., Bellomo, R., Bernard, G. R., Chiche, J. D., Coopersmith, C. M., Hotchkiss, R. S., Levy, M. M., Marshall, J. C., Martin, G. S., Opal, S. M., Rubenfeld, G. D., van der Poll, T., Vincent, J. L. and Angus, D. C. (2016) 'The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).' *JAMA*, 315(8), pp. 801-810.

Sinistro, A., Almerighi, C., Ciaprini, C., Natoli, S., Sussarello, E., Di Fino, S., Calò-Carducci, F., Rocchi, G. and Bergamini, A. (2008) 'Downregulation of CD40 ligand response in monocytes from sepsis patients.' *Clin Vaccine Immunol*, 15(12), pp. 1851-1858.

Sjölund, M., Tano, E., Blaser, M. J., Andersson, D. I. and Engstrand, L. (2005) 'Persistence of resistant Staphylococcus epidermidis after single course of clarithromycin.' *Emerg Infect Dis*, 11(9), pp. 1389-1393.

Souto, F. O., Alves-Filho, J. C., Turato, W. M., Auxiliadora-Martins, M., Basile-Filho, A. and Cunha, F. Q. (2011) 'Essential role of CCR2 in neutrophil tissue infiltration and multiple organ dysfunction in sepsis.' *Am J Respir Crit Care Med*, 183(2), pp. 234-242.

Spiliopoulou, A. I., Kolonitsiou, F., Krevvata, M. I., Leontsinidis, M., Wilkinson, T. S., Mack, D. and Anastassiou, E. D. (2012) 'Bacterial adhesion, intracellular survival and cytokine induction upon stimulation of mononuclear cells with planktonic or biofilm phase Staphylococcus epidermidis.' *FEMS Microbiol Lett*, 330(1), pp. 56-65.

Struyf, S., De Meester, I., Scharpé, S., Lenaerts, J. P., Menten, P., Wang, J. M., Proost, P. and Van Damme, J. (1998) 'Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor.' *Eur J Immunol*, 28(4), pp. 1262-1271.

Szewzyk, U., Szewzyk, R. and Stenström, T. A. (1993) 'Growth and survival of Serratia marcescens under aerobic and anaerobic conditions in the presence of materials from blood bags.' *J Clin Microbiol*, 31(7), pp. 1826-1830.

Sáenz, Y., Zarazaga, M., Briñas, L., Ruiz-Larrea, F. and Torres, C. (2003) 'Mutations in gyrA and parC genes in nalidixic acid-resistant Escherichia coli strains from food products, humans and animals.' *J Antimicrob Chemother*, 51(4), pp. 1001-1005.

Sáenz, Y., Briñas, L., Domínguez, E., Ruiz, J., Zarazaga, M., Vila, J. and Torres, C. (2004) 'Mechanisms of resistance in multiple-antibiotic-resistant Escherichia coli strains of human, animal, and food origins.' *Antimicrob Agents Chemother*, 48(10), pp. 3996-4001.

Sônego, F., Castanheira, F. V., Ferreira, R. G., Kanashiro, A., Leite, C. A., Nascimento, D. C., Colón, D. F., Borges, V. e. F., Alves-Filho, J. C. and Cunha, F. Q. (2016) 'Paradoxical Roles of the Neutrophil in Sepsis: Protective and Deleterious.' *Front Immunol*, 7, p. 155.

Stock, I., Grueger, T. and Wiedemann, B. (2003). Natural antibiotic susceptibility of strains of Serratia marcescens and the S. liquefaciens complex: S. liquefaciens sensu stricto, S. proteamaculans and S. grimesii. *International Journal of Antimicrobial Agents*, 22(1), pp.35-47.

Taha, M., Kalab, M., Yi, Q. L., Maurer, E., Jenkins, C., Schubert, P. and Ramirez-Arcos, S. (2016) 'Bacterial survival and distribution during buffy coat platelet production.' *Vox Sang*, 111(4), pp. 333-340.

Tang, B. M., Huang, S. J. and McLean, A. S. (2010) 'Genome-wide transcription profiling of human sepsis: a systematic review.' *Crit Care*, 14(6), p. R237.

Tavares-Murta, B. M., Cunha, F. Q. and Ferreira, S. H. (1998) 'The intravenous administration of tumor necrosis factor alpha, interleukin 8 and macrophage-derived neutrophil chemotactic factor inhibits neutrophil migration by stimulating nitric oxide production.' *Br J Pharmacol*, 124(7), pp. 1369-1374.

Thomas, M. R., Outteridge, S. N., Ajjan, R. A., Phoenix, F., Sangha, G. K., Faulkner, R. E., Ecob, R., Judge, H. M., Khan, H., West, L. E., Dockrell, D. H., Sabroe, I. and Storey, R. F. (2015) 'Platelet P2Y12 Inhibitors Reduce Systemic Inflammation and Its Prothrombotic Effects in an Experimental Human Model.' *Arterioscler Thromb Vasc Biol*, 35(12), pp. 2562-2570.

Thurlow, L. R., Hanke, M. L., Fritz, T., Angle, A., Aldrich, A., Williams, S. H., Engebretsen, I. L., Bayles, K. W., Horswill, A. R. and Kielian, T. (2011) 'Staphylococcus aureus biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo.' *J Immunol*, 186(11), pp. 6585-6596.

Tiru, B., DiNino, E. K., Orenstein, A., Mailloux, P. T., Pesaturo, A., Gupta, A. and McGee, W. T. (2015) 'The Economic and Humanistic Burden of Severe Sepsis.' *Pharmacoeconomics*, 33(9), pp. 925-937.

Tisoncik, J. R., Korth, M. J., Simmons, C. P., Farrar, J., Martin, T. R. and Katze, M. G. (2012) 'Into the eye of the cytokine storm.' *Microbiol Mol Biol Rev*, 76(1), pp. 16-32.

Trenchevska, O., Sherma, N. D., Oran, P. E., Reaven, P. D., Nelson, R. W. and Nedelkov, D. (2015) 'Quantitative mass spectrometric immunoassay for the chemokine RANTES and its variants.' *J Proteomics*, 116, pp. 15-23.

Tunjungputri, R., van de Heijden, W., Urbanus, R., de Groot, P., van der Ven, A. and de Mast, Q., 2016. Higher platelet reactivity and platelet-monocyte complex formation in Gram-positive sepsis compared to Gram-negative sepsis. *Platelets*, 28(6), pp.595-601.

Turner, D., Paszek, P., Woodcock, D., Nelson, D., Horton, C., Wang, Y., Spiller, D., Rand, D., White, M. and Harper, C., 2010. Physiological levels of TNF stimulation induce stochastic dynamics of NF- B responses in single living cells. *Journal of Cell Science*, 123(16), pp.2834-2843.

van den Bogaard, A. E., London, N., Driessen, C. and Stobberingh, E. E. (2001) 'Antibiotic resistance of faecal Escherichia coli in poultry, poultry farmers and poultry slaughterers.' *J Antimicrob Chemother*, 47(6), pp. 763-771.

Van Der Meeren, A., Squiban, C., Gourmelon, P., Lafont, H. and Gaugler, M. H. (1999) 'Differential regulation by IL-4 and IL-10 of radiation-induced IL-6 and IL-8 production and ICAM-1 expression by human endothelial cells.' *Cytokine*, 11(11), pp. 831-838.

van der Poll, T., van de Veerdonk, F. L., Scicluna, B. P. and Netea, M. G. (2017) 'The immunopathology of sepsis and potential therapeutic targets.' *Nat Rev Immunol*, 17(7), pp. 407-420.

Venkata, C., Kashyap, R., Farmer, J. C. and Afessa, B. (2013) 'Thrombocytopenia in adult patients with sepsis: incidence, risk factors, and its association with clinical outcome.' *J Intensive Care*, 1(1), p. 9.

Vincent, J. L., Moreno, R., Takala, J., Willatts, S., De Mendonça, A., Bruining, H., Reinhart, C. K., Suter, P. M. and Thijs, L. G. (1996) 'The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine.' *Intensive Care Med*, 22(7), pp. 707-710.

Vincent, J. L., Sakr, Y., Sprung, C. L., Ranieri, V. M., Reinhart, K., Gerlach, H., Moreno, R., Carlet, J., Le Gall, J. R., Payen, D. and Investigators, S. O. i. A. I. P. (2006) 'Sepsis in European intensive care units: results of the SOAP study.' *Crit Care Med*, 34(2), pp. 344-353.

Voelz, A., Müller, A., Gillen, J., Le, C., Dresbach, T., Engelhart, S., Exner, M., Bates, C. J. and Simon, A. (2010) 'Outbreaks of Serratia marcescens in neonatal and pediatric intensive care units: clinical aspects, risk factors and management.' *Int J Hyg Environ Health*, 213(2), pp. 79-87.

von Hundelshausen, P. and Weber, C. (2007) 'Platelets as immune cells: bridging inflammation and cardiovascular disease.' *Circ Res*, 100(1), pp. 27-40.

Watson, C. N., Kerrigan, S. W., Cox, D., Henderson, I. R., Watson, S. P. and Arman, M. (2016) 'Human platelet activation by Escherichia coli: roles for FcγRIIA and integrin αIIbβ3.' *Platelets*, 27(6), pp. 535-540.

Wilson-Nieuwenhuis, J. S. T., Dempsey-Hibbert, N., Liauw, C. M. and Whitehead, K. A. (2017) 'Surface modification of platelet concentrate bags to reduce biofilm formation and transfusion sepsis.' *Colloids Surf B Biointerfaces*, 160, pp. 126-135.

Wilson-Nieuwenhuis, J., Whitehead, K. A., Dempsey-Hibbert, N. C. (2018) ‘Bacterial contamination of platelet transfusion units affects platelet haemostatic function and immunological activity.’ *EMJ Hematology*, 6(1), pp. 48-49.

Wolkow, P. P. (1998) 'Involvement and dual effects of nitric oxide in septic shock.' *Inflamm Res*, 47(4), pp. 152-166.

Yao, Y., Sturdevant, D. E., Villaruz, A., Xu, L., Gao, Q. and Otto, M. (2005) 'Factors characterizing Staphylococcus epidermidis invasiveness determined by comparative genomics.' *Infect Immun*, 73(3), pp. 1856-1860.

Yeaman, M. R. (2010) 'Platelets in defense against bacterial pathogens.' *Cell Mol Life Sci*, 67(4), pp. 525-544.

Yipp, B. G., Andonegui, G., Howlett, C. J., Robbins, S. M., Hartung, T., Ho, M. and Kubes, P. (2002) 'Profound differences in leukocyte-endothelial cell responses to lipopolysaccharide versus lipoteichoic acid.' *J Immunol*, 168(9), pp. 4650-4658.

Youssefian, T., Drouin, A., Massé, J. M., Guichard, J. and Cramer, E. M. (2002) 'Host defense role of platelets: engulfment of HIV and Staphylococcus aureus occurs in a specific subcellular compartment and is enhanced by platelet activation.' *Blood*, 99(11), pp. 4021-4029.

Zarbock, A., Polanowska-Grabowska, R. K. and Ley, K. (2007) 'Platelet-neutrophil-interactions: linking hemostasis and inflammation.' *Blood Rev*, 21(2), pp. 99-111.

Zhao, J., She, S., Xie, L., Chen, X., Mo, C., Huang, L. and Tang, W. (2016) 'The Effects of RANTES Polymorphisms on Susceptibility to HIV-1 Infection and Disease Progression: Evidence from an Updated Meta-Analysis.' *AIDS Res Hum Retroviruses*, 32(6), pp. 517-528.

Zhou, H., Deng, M., Liu, Y., Yang, C., Hoffman, R., Zhou, J., Loughran, P. A., Scott, M. J., Neal, M. D. and Billiar, T. R. (2018) 'Platelet HMGB1 is required for efficient bacterial clearance in intra-abdominal bacterial sepsis in mice.' *Blood Adv*, 2(6), pp. 638-648.

Øynebråten, I., Barois, N., Bergeland, T., Küchler, A. M., Bakke, O. and Haraldsen, G. (2015) 'Oligomerized, filamentous surface presentation of RANTES/CCL5 on vascular endothelial cells.' *Sci Rep*, 5, p. 9261.