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Effects of Annexin A5 on Endothelial Inflammation Induced by Lipopolysaccharide-Activated Platelets and Extracellular Vesicles

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Abstract

Sepsis is a dysregulated immune response to infection and the leading cause of mortality globally, accounting for 11 million deaths in 2017. To date, no therapeutics are available to treat the underlying septic response. Previous research from our laboratory has shown that annexin A5 (Anx5) treatment increased survival by 40% in mice with endotoxemia, a model of sepsis. During sepsis, activated platelets release membrane fragments called extracellular vesicles (EVs) with externalization of phosphatidylserine to which annexin A5 binds with a high affinity. We hypothesized that annexin A5 will block the pro-inflammatory response induced by activated platelets and extracellular vesicles in vascular endothelial cells under septic conditions. We showed that treatment with annexin A5 lowered expression of inflammatory cytokines and adhesion molecules induced by lipopolysaccharide (LPS)-activated platelets or EVs in endothelial cells. Furthermore, annexin A5 treatment improved endothelial integrity and reduced monocyte adhesion to endothelial cells in septic conditions. Our study shows that annexin A5 inhibits endothelial inflammation in endotoxic conditions, suggesting its potential as a treatment for sepsis.

Keywords

Sepsis, Inflammation, Annexin A5, Endothelial Cell, Platelet, Extracellular Vesicle, COVID-19

Summary for Lay Audience

Sepsis is a dysregulated immune response to infection and the leading cause of mortality globally, accounting for 11 million deaths in 2017. To date, no therapeutics are available to treat the underlying septic response. Previous research from our laboratory has shown that annexin A5 (Anx5) treatment increased survival by 40% in mice with endotoxemia, a model of sepsis. During sepsis, activated platelets (PLTs) release membrane fragments called extracellular vesicles with externalization of phosphatidylserine to which annexin A5 binds with a high affinity. We hypothesized that annexin A5 will block the pro-inflammatory response induced by activated PLTs and extracellular vesicles in vascular endothelial cells (ECs) under septic conditions. We showed that treatment with annexin A5 lowered expression of inflammatory cytokines and adhesion molecules induced by lipopolysaccharide (LPS)-activated PLTs or extracellular vesicles in ECs. Furthermore, annexin A5 treatment improved EC structural integrity and reduced monocyte adhesion to ECs in septic conditions. Our study shows that annexin A5 inhibits EC inflammation in septic conditions, suggesting its potential as a treatment for sepsis. Severe COVID-19 patients develop sepsis. A phase 2 clinical trial on the effects of annexin A5 in critically ill COVID-19 patients with sepsis is currently underway at London Health Sciences Centre.

Co-Authorship Statement

Dr. Qingping Feng guided and supervised the design of all experiments. Xiangru Lu provided all necessary training and assisted with experimentation, and data collection.

Extracellular vesicles were isolated and purified independently then sent to Janice Gomes in Dr. Stephen Pasternak' laboratory at Robarts research institute for quantification via nanoflow cytometer, or to Dr. Dylan Burger at the University of Ottawa for transmission electron microscopy imaging and nanoparticle tracking analysis.

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Abbreviations

ACE 2	angiotensin converting enzyme 2
ADAM17	A disintegrin and metalloprotease 17
Apoe E	apolipoprotein E
CARS	compensatory anti-inflammatory response syndrome
CD	cluster of differentiation
COVID-19	corona virus disease 2019
DAMP	danger-associated molecular patterns
DIC	disseminated intravascular coagulopathy
DMEM	Dulbecco's modified eagles medium
E. Coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EVs	extracellular vesicles
FBA	fetal bovine albumin
FBS	fetal bovine serum
HMGB-1	high mobility group protein-box 1
HUS	hemolytic uremic syndrome
ICAM-1	intracellular adhesion molecule 1

IL	interleukin
IP	intraperitoneal
IRAK1	interleukin 1 receptor associated kinase 1
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MERS	middle eastern respiratory syndrome
MPs	microparticles
mRNA	messenger ribonucleic acid
miRNA	micro ribonucleic acid
NETs	neutrophil extracellular traps
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptors and pyran domain containing protein
NOD-like	nucleotide-binding oligomerization domain-like
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PKC	protein kinase C
qSOFA	quick sequential organ failure assessment
ROS	reactive oxygen species
RT-qPCR	real time quantitative polymerase chain reaction

SARS CoV-2	severe acute respiratory syndrome corona virus 2
SOFA	sequential organ failure assessment
SIRS	systemic inflammatory response syndrome
TACE	tumour necrosis factor-α converting enzyme
TEER	trans-endothelial electrical resistance
TF	tissue factor
TIR	toll/ interleukin-1 receptor
TLR	toll like receptor
TMEM16	transmembrane protein 16
TMPR2	transmembrane protease serine 2
TNF	tumour necrosis factor
VEGFR-2	vascular endothelial growth factor receptor-2
WHO	world health organization
WT	wild-type

Chapter 1

1. Introduction

1.1 Sepsis

Sepsis is a defined as life-threatening organ dysfunction caused by a dysregulated host response to an infection (Singer *et al*., 2016). Clinically speaking, sepsis is the leading cause of mortality and morbidity among intensive care unit patients globally (Hotchkiss *et al.*, 2016). In 2017, 48.9 million patients developed sepsis (Rudd *et al.,* 2020). 20% of patient with sepsis will die in hospital; however, if sepsis develops into severe sepsis the mortality rate increases to around 40% (Angus *et al.,* 2001), equating to 11 million deaths, or 1 in 5 deaths worldwide (Rudd *et al.,* 2020). With the exception of antibiotics and symptom reducing agents, there are no specific therapeutics indicated for treatment of the widespread septic response (Granja *et al.,* 2019). Sepsis results from a dysregulated immune response, which causes pro-inflammatory cytokine release into the vasculature. Clinically speaking, sepsis is diagnosed when a patient's sequential organ failure assessment (SOFA) score increases by 2 or more and the patient has a suspected or a proven infection (Chousterman *et al.*, 2017). A SOFA score is used to track a patient's tissue and organ health/function during an intensive care unit stay, the higher the score, the greater the organ dysfunction (KaraKike *et al.,* 2019). Severe sepsis is diagnosed when the infection begins to negatively affect patient's organ function and septic shock is characterized by a sharp drop in blood pressure, often leading to respiratory failure or heart failure (Annane *et al*., 2005).

In a large study conducted in the United States in 2000, researchers examined septic patients to determine what microorganisms were leading to a septic response. In the study, 52.1% of sepsis cases were accounted for by a gram-positive bacterial infection, while 32.7% of the septic

patients were infected by gram-negative bacteria (van der Poll & Opal., 2008). While sepsis is often thought of as an infectious disease caused by a bacterial infection; sepsis can also be initiated through a fungal, parasitic, or viral infection (O'Brien *et al.,* 2007). Recently, we have seen hospitalized patients that have been infected with the SARS-CoV-2 virus present with sepsis and more severe cases of SARS-CoV-2 infection, septic shock (Zhou *et al.,* 2020).

Pathogen-associated molecular patterns (PAMP) on gram-positive and gram-negative bacteria are able to bind to toll like receptor 2 (TLR2) and toll like receptor 4 (TLR4), respectively. PAMP-TLR binding leads to a downstream pro-inflammatory response (van der Poll & Opal., 2008). Interestingly, TLRs may be further contributing to the pathogenesis of sepsis by subsequently binding with danger-associated molecular patterns (DAMP). When DAMPs bind to TLR4 an even greater downstream pro-inflammatory response is achieved (van der Poll & Opal., 2008). Although the initial pro-inflammatory response is necessary for our body to fight infection, when the immune response becomes dysregulated, the patient is considered to be experiencing a septic response.

The early stages of a septic response are characterized by a cytokine storm, in which massive increases in inflammatory cytokine release is seen (Chousterman *et al.,* 2017). The cytokine storm leads to what is known as systemic inflammatory response syndrome (SIRS). SIRS experienced during a septic response contributes to tissue and organ damage (Chousterman *et al.,* 2017). Since 1976, researchers have investigated potential treatments to help attenuate the early surge in pro-inflammatory markers seen in sepsis (Schumer, 1976); from high dose steroid treatments used as an immunosuppressant, to neutralization of exogenous endotoxins (Marshall, 2014). Proceeding the inflammatory cytokine-mediated hyper-inflammatory stage of sepsis is the compensatory anti-inflammatory response syndrome (CARS) stage (Ono *et al.,* 2018). In

disagreement with the use of immunosuppressants as described above, some researchers and physicians believe immunostimulatory agents that focus on the CARS stage of sepsis would be most effective in treating sepsis (Ono *et al.*, 2018).

Looking back at the early stages of sepsis, annexin A5 has shown promise with its ability to bind to TLR4, reducing the production of inflammatory cytokines (Arnold *et al.*, 2014) (Figure 1.1). Annexin A5 has also shown anti-inflammatory potential through phosphatidylserine binding on activated platelets and extracellular vesicles (Burger *et al.*, 2013).

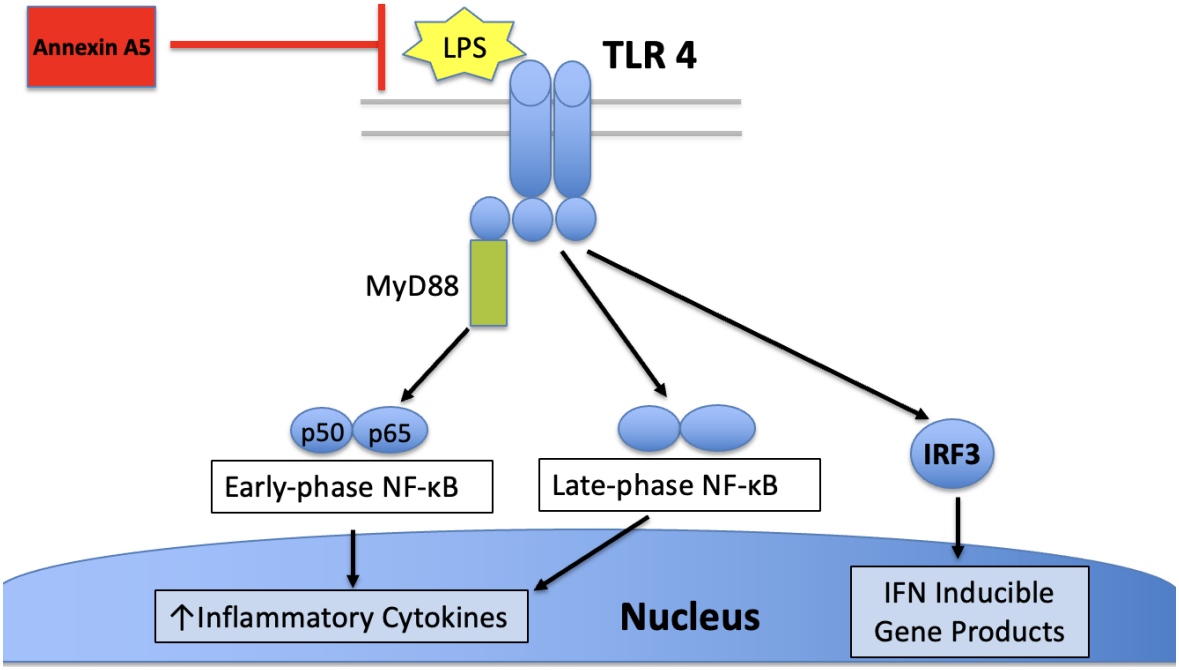


Figure 1.1. Proposed Effects of Annexin A5 on Toll-Like Receptor 4 (TLR4) Signaling. Annexin A5's binding to TLR4 may help explain its anti-inflammatory properties. Binding of annexin A5 to TLR4 may competitively inhibit lipopolysaccharide (LPS) binging leading to downregulation of nuclear factor-κB (NF-κB) activity, ultimately attenuating inflammatory cytokine production and expression.

1.1.1 Toll Like Receptor 4 (TLR4)

The majority of TLR4 expressing cells are myeloid cells (macrophages, dendritic cells, monocytes) (Vaure *et al.*, 2014), however, TLR4 is also found within the membrane of endothelial cells (Dauphinee *et al.*, 2006). TLR4 plays a critical role in an organism's innate immune response (Kawai *et al.*, 2009).

TLR4 does not directly come into contact with LPS, instead LPS binding protein (LBP) binds LPS and cluster of differentiation 14 (CD14) and delivers the bound LPS to the TLR4-MD-2 complex (Monick *et al.*, 2003). MD-2 is bound to TLR4 through ionic and hydrogen bonds to form a TLR4-MD-2 complex (Kawai *et al.*, 2009). Upon LPS stimulation, activated TLR4 forms a homodimer and this dimerization triggers downstream signalling via toll/IL-1R (TIR) complex recruitment, as well as MyD88 recruitment (Kawai *et al.*, 2009). MyD88 forms a complex with interleukin 1 receptor associated kinase 1 (IRAK1) to lead to downstream NF-κB activation, and subsequent production of proinflammatory cytokines. MyD88 can also activate mitogen-activated protein kinases (MAPKs) which lead to further inflammatory cytokine expression (Kawai *et al.*, 2009). This hyperinflammatory state can lead to reduced blood flow and subsequent increased reactive oxygen species (ROS) production and ultimately, organ dysfunction (Pool *et al.*, 2019).

1.1.2 Endotoxemia as a Sepsis Model

Since the observation that the response to LPS simulates severe human sepsis, murine endotoxemia models have been a cornerstone in sepsis research (Deitch, 2005). As mentioned above (Section 1.1), LPS is a coating on gram negative bacteria that elicits a well-defined signalling cascade that begins with the binding of LPS to TLR4 (Poli-de-Figueiredo *et al.*, 2008). LPS can bind to TLR4, ultimately leading to downstream upregulation in inflammatory cytokines such as TNFα. LPS is often used in research to induce endotoxemia, simulating a septic response.

When compared to human sepsis, endotoxemia via intraperitoneal injection of LPS often leads to much shorter-lived inflammatory cytokine expression (Lewis *et al.*, 2016). Through *in vivo* endotoxemia studies our laboratory has shown an LPS dose of 4 mg/kg can lead to increased inflammatory cytokine expression and reduced cardiac function in adult mice (Arnold *et al.*, 2014). However, in human models of endotoxemia, doses of just 1-4 ng/kg can elicit increased temperature and heart rate (Lowry, 2005).

1.2 Annexin Protein Family

The annexin superfamily contains over 1,000 different proteins from various species of plants and animals (Gerke and Moss, 2002; Moss and Morgan, 2004). However, there are 12 annexin genes that are found within humans (Moss and Morgan, 2004). Although annexins are primarily intracellular proteins, they can be found in the extracellular environment where they are able to bind to cell membrane ligands and receptors (Raynal and Pollard, 1994). Physiologically, the annexin family of proteins can affect coagulation and inflammation systemically through binding to negatively charged phospholipids such as phosphatidylserine (Schloer *et al.*, 2018).

All annexins (with the exception of annexin A6), share a conserved core made up of 4 homologous domains (annexin A6 has a core of 8 homologous domains) (Gerke and Moss, 2002) giving annexin proteins similar 3D structure. The domain structures of 12 human annexin proteins are shown in Figure 1.2.

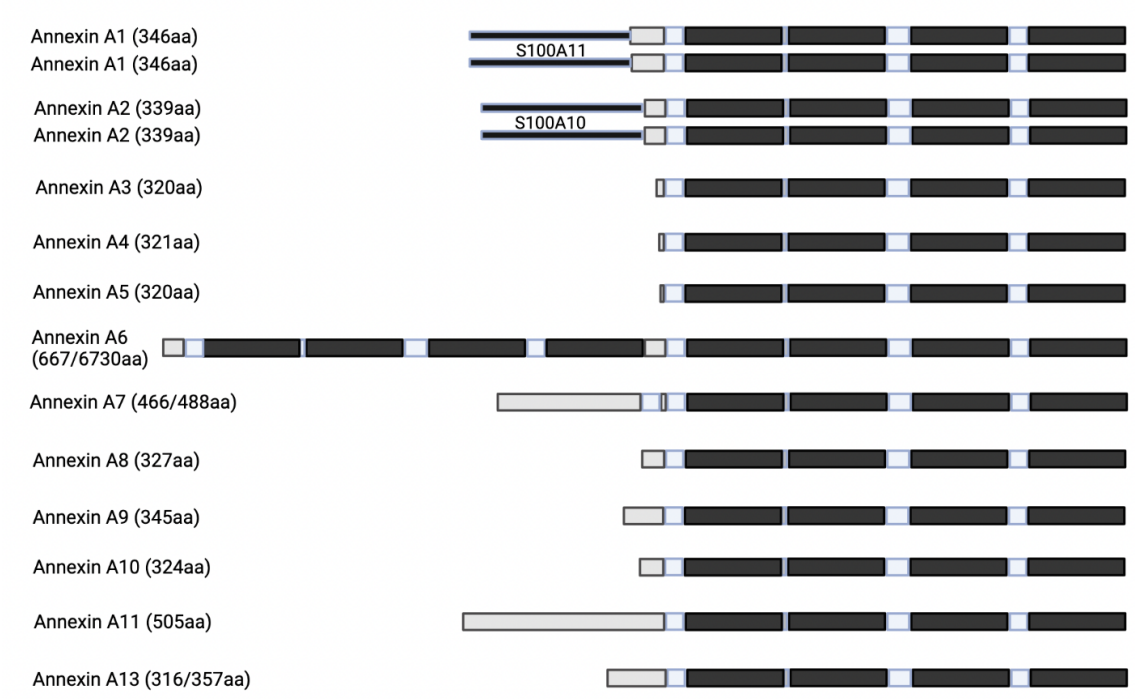


Figure 1.2 Annexin Protein Family. Domain structures for the 12 human annexin proteins. Conserved core regions of annexin domains are shown in black and variable end terminal shown in shaded grey. The number of amino acid (aa) is indicated in brackets. Annexin A1 and A2 present as dimers through interactions with S100 protein family members. Image is modified from Moss and Morgan, 2004 and created in BioRender.com.

1.2.1 Annexin A5

Annexin A5 is an endogenous calcium-binding protein that is highly expressed by endothelial cells and under conditions of stress, annexin A5 is secreted into the extracellular space and the bloodstream (Maloberti *et al.*, 2017). Annexin A5 is translocated from the inner leaflet of the cell membrane into the extracellular space through a TMEM16F scramblase specific reaction (Stewart *et al.*, 2018). TMEM16F is a scramblase protein that externalizes phospholipid such as phosphatidylserine in a calcium specific, energy independent manner (Pomorski and Menon, 2016; Suzuki *et al.*, 2010). Individuals with TMEM16F dysfunction have reduced coagulation ability

due to their reduced ability to externalize phosphatidylserine (Zwall *et al.,* 2004). Through the process of phospholipid externalization annexin A5 that is bound to the phospholipids on the inner leaflet are also externalized (Stewart *et al.,* 2018). Circulating plasma levels of annexin A5 range from ~6-7 ng/ml in humans (Rand *et al.,* 2006). Annexin A5 is able to act as an anticoagulant by forming two-dimensional crystals on externalized phospholipids of cells to reduce phospholipid-dependent coagulation (Rand *et al.,* 2006*).* Binding of annexin A5 to phosphatidylserine also provides an anti-apoptotic function (Gidon-Jeangirard *et al.,* 1999; Krauling *et al.,* 1999).

Functional applications of annexin A5's ability to bind to phosphatidylserine go beyond anticoagulation. Annexin A5's binding of phosphatidylserine also leads to inhibition of phagocytosis by shielding ligands located near phosphatidylserine that contribute to the process of phosphatidylserine expressing cells being engulfed (Kenis *et al.,* 2006). Annexin A5's binding on live cells also acts as a physical restraint to inhibit extracellular vesicle blebbing from host cells (Thiagarajan *et al.,* 1991) (Figure 1.4).

Annexin A5 may act as a signalling protein for vascular endothelial growth factor receptor-2 (VEGFR-2). By interacting with VEGFR-2, annexin A5 may act as a mediator for vascular endothelial cells proliferation (Wen *et al.,* 1999). Annexin A5 has also been shown to improve endothelial function and reduce vascular inflammation in apolipoprotein E (ApoE) knockout mice with atherosclerosis (Ewing *et al.,* 2011).

Lipopolysaccharide (LPS) is a lipid-polysaccharide molecule that coats gram-negative bacteria and can induce inflammation via the TLR4 pathway (Akira & Takeda, 2004). Previous research from our lab shows that binding of recombinant annexin A5 to leucine rich repeats on TLR4 can attenuate inflammatory cytokine release induced by LPS (Arnold *et al.,* 2014). With the knowledge that annexin A5 is also able to selectively bind to phosphatidylserine (Burger *et al.,* 2013), a

proinflammatory and procoagulant phospholipid present on the membrane of activated platelets and EVs, annexin A5 shows potential as a therapeutic.

For many years, it has been well established that annexin A5 is also able to bind to endothelial cells (van Heerde *et al.,* 1994), in doing so, annexin A5 protects the endothelial monolayer from coagulation and inflammatory induced damage (Mak and Koo, 2014). By repairing the luminal membrane of endothelial cells, annexin A5 may reduce vascular permeability and organ damage during sepsis. Annexin A5 binds to heparan sulfate, a prominent glycosaminoglycan on the endothelial glycocalyx (Capila *et al.,* 2001).

1.2.2 Therapeutic Potential of Other Annexin Proteins

Annexin A1

Annexin A1, also known as lipocortin, is present in various tissues such as the lungs, kidneys, bone marrow, intestines, spleen, thymus, brain, and seminal fluid (Fava *et al.,* 1989). Annexin A1 is often present intracellularly, when it is externalized, it possesses anti-inflammatory properties (Gavins and Hickey, 2012). Annexin A1's anti-inflammatory affects are achieved through disruption and modification of adhesion and migratory leukocytes (Gavins and Hickey, 2012). Trans-endothelial migration is inhibited by annexin A1's ability to cause L-selectin shedding from leukocytes (De Coupade *et al.,* 2003). Annexin A1 can also bind and activate cell messengers which activate apoptosis, ultimately leading to a reduction in necrotic cell death, and reducing inflammation (Minghetti *et al.,* 1999). Lastly, extracellular annexin A1 increases T cell proliferation to enhance the immune response to a pathogen (D'Acquisto *et al.,* 2007).

Annexin A2

Annexin A2 has been shown to activate human macrophages through TLR4 activation leading to macrophage internalization and subsequent anti-inflammatory cytokine release (Zhang *et al.,*

2015). Annexin A2 has not been fully exposed as a therapeutic due to the less potent anti-inflammatory and anti-coagulant properties when compared to that of annexin A1 and A5 (Bharadwaj *et al.,* 2013).

Annexin A3

Like annexin A5, Annexin A3 binds to negatively charged phospholipids in a calcium specific manner (Toufiq *et al.,* 2020). Annexin A3 is almost exclusively expressed on neutrophils (Toufiq *et al.,* 2020). It is well established that annexin A3 levels are significantly elevated during sepsis (Khaenam *et al.,* 2014), highlighting a potential link between annexin A3 and the pathogenesis of sepsis. Annexin A3 promotes granule-granule and granule-phagosome aggregation (Le Cabec *et al.,* 1994), a role that is important in the process of degranulation. Although degranulation is necessary to fight off an infectious agent, excessive degranulation can lead to tissue damage (Eichelberger and Goldman, 2020). Annexin A3's ability to enhance degranulation is hypothesized to play a role in mediating microbial function and pathogen clearance through neutrophils (Toufiq *et al.,* 2020). Conversely, elevated levels of annexin A3 in sepsis may play a detrimental role, annexin A3 also promotes tumorigenesis (Ma *et al.,* 2018) via caspase 3 inhibition (Wang L. *et al.,* 2019) which could potentially increase neutrophil longevity and contribute to organ damage (Perl *et al.,* 2007). Like annexin A5, annexin A3 has also been shown to have anti-apoptotic properties (Wang Y. *et al.,* 2019).

Annexin A4 and A6

Both annexin A4 and A6 are involved in cell membrane repair and both annexin A4 and A6 are upregulated in cancer. Boye and colleagues found that annexin A4 and A6 are needed for cell repair to occur and that both annexins are recruited to cell injury sites (Boye *et al.,* 2017). Little research was found on the relevance of annexin A4 and A6 to sepsis, perhaps annexin A4 and A6

are recruited to sites of endothelial cell injury and damaged tissue sites during severe sepsis and septic shock. The role of these annexins in the repair of endothelial cells and tissue would be an interesting avenue of research for future inquiry.

Annexin A7 and A9

Annexin A7 is expressed in a broad range of tissues throughout the body (Moss and Morgan, 2004). Annexin A7 has been shown to have a tumour-suppression role in a wide range of cancers including glioblastoma, glioblastoma multiforme, melanoma and prostate cancer (Guo *et al.,* 2013). Conversely, annexin A7 seems to promote tumour growth in cancers such as liver cancer, gastric cancer, nasopharyngeal carcinoma, colorectal cancer, and breast cancer (Guo *et al.,* 2013). Similarly, annexin A9 expression is increased in colorectal cancer and silencing of the annexin A9 protein expression led to a decrease in metastasis, indicating the role of annexin A9 in promotion of cell growth (Yu *et al.,* 2018).

Annexin A8

Annexin A8 is largely expressed in the skin and within the placenta during pregnancy (Moss and Morgan, 2004). Annexin A8 plays a major role in P-selectin and CD63 presentation on human umbilical vein endothelial cells (Poeter *et al.,* 2014). P-selectin and CD63 exposure on endothelial cells allows for leukocyte adhesion onto the endothelium, so the presence of annexin A8 at the site of tissue injury is critical for leukocyte recruitment and host immune response. With that in mind, as discussed earlier, annexin A8 is predominantly present in skin cells and the placenta during pregnancy so its role in a septic response has not been researched in detail.

Annexin A10

Annexin A10 is predominantly expressed in the stomach (Moss and Morgan, 2004). Annexin A10 has been shown to be downregulated in gastric cancer and it has been shown that upregulation of

annexin A10 in cancer cell lines decreased cell growth and the overexpression exacerbated apoptosis (Kim *et al.,* 2010). This pro-apoptotic role is antagonistic to the anti-apoptotic function of annexin A5 (van Genderen *et al.,* 2018). Apoptosis in organ tissue during the septic response contributes to the progression of multi-organ dysfunction (Sharron *et al*., 2012), thus increasing apoptosis as we would expect with annexin A10 could have a deleterious effect on patients with sepsis.

Annexin A11

Annexin A11 is expressed relatively ubiquitously throughout the body (Moss and Morgan, 2004). Interestingly, annexin A11 plays a synergistic role in facilitating staphylokinase (a blood dissolving agent) in attenuating blood clot formation. Staphylokinase alone is an effective blood clot dissolving molecule, when annexin A11 is added as an adjunctive treatment and a staphylokinase-annexin A11 complex is formed the efficacy of clot retardation is increased (Wang *et al.,* 2014). With blood clotting being a major contributor to the pathogenesis of sepsis, annexin A11 shows some promise as a potential adjunctive treatment in septic patients.

Annexin A13

Annexin A13 is predominantly expressed in the small intestine (Moss and Morgan, 2004) and it is hypothesized to be the original progenitor to the annexin superfamily of proteins (Turnay *et al.,* 2005). Like other annexins, annexin A13 plays a role in tumorigenesis, annexin A13 is associated with colorectal cancer cell invasion *in vitro* as well as lymph node metastasis in colorectal cancer (Jiang *et al.,* 2017). Annexin A13 has been proposed as a biomarker for diagnosis and prognosis of colorectal cancer (Jiang *et al.,* 2017).

1.3 Platelets in Sepsis

Platelets are anuclear, disk-shaped cells that are present within blood of humans and animals. Platelets play a key role in maintaining hemostasis, their role in hemostasis has been studied in detail (Jenne *et al.,* 2013). Platelets are able to contribute to coagulation by converting inactive prothrombin into its active form, thrombin, which converts fibrinogen into fibrin, leading to the formation of a thrombus, or blood clot (Monroe *et al.,* 2002). However, platelets are also able to coordinate neutrophils, endothelial cells, and lymphocytes to elicit an immune response against tissue damage or an infection (Jenne *et al.,* 2013). When platelets are bound to neutrophils, neutrophils are stimulated to release granule proteins and chromatin to form an extracellular fibril matrix known as neutrophil extracellular traps (NETs) to fight either viral or bacterial infections. Platelets are able to fulfil their pro-coagulant role in hemostasis by externalizing phosphatidylserine (Presseizen *et al.,* 2002).

Phosphatidylserine is a negatively charged membrane bound phospholipid that, at rest, is contained on the inner leaflet of the platelet membrane (Kay & Grinstein, 2011). Calcium-dependent phospholipid scramblase is able to translocate phosphatidylserine, so it is exposed to the extracellular environment (Bevers and Williamson, 2010), a process observed during a septic response (Ma *et al.,* 2017). As phosphatidylserine is externalized, platelet dependent thrombin generation appears to increase (Presseizen *et al.,* 2002), through increased binding sites for factors Va and Xa to form prothrombinase (Zhang *et al.,* 2016). Ultimately leading to greater coagulation in the vasculature, in some instances leading to sepsis-associated disseminated intravascular coagulation (DIC). Markers of platelet activation were significantly higher in patients with DIC vs healthy individuals Wegryzyn *et al* (2021), showing the importance of platelet activation in the process of sepsis-associated coagulopathy. Thrombin is an enzyme that can cleave inactive

fibrinogen into fibrin. Active fibrin can bind to activated platelets to form blood clots within the vasculature (Levi, 2017). P-selectin released from activated platelets (P-selectin levels are increased during inflammation) can bind to monocytes to further increases expression of tissue factor VIIa which can: increase inflammation (which positively feeds back to further increase coagulation), supress anticoagulant pathways and fibrinolysis and increase thrombin generation (Levi, 2017). Exacerbated coagulation during sepsis can contribute to the organ ischemia and organ dysfunction seen in patients with severe sepsis and septic shock (Levi, 2017). During the process of coagulation, platelet counts can drop; clinically, this drop can lead to thrombocytopenia (platelet count $<150x10^9$/ dL blood). Severe drops in platelet counts can partly be attributed to increased adhesion of activated platelets to adhesion molecules on endothelial cells causing hemophagocytosis (phagocytosis of platelet progenitor cells by mononuclear cells) to occur (Levi, 2017). As platelet count decreases in a septic patient the severity of prognosis increases (Levi, 2017), this highlights the importance of controlling coagulation during sepsis.

1.4 Extracellular Vesicles

Activated platelets are also able to 'pinch' off portions of their membrane to form small extracellular vesicles (EVs) (Burger *et al.*, 2013). The role of EVs on the vasculature is not fully understood. EVs range in size from 100 nm to 1000 nm in diameter (Burger *et al.*, 2013), and they are known to contain RNA, protein, and lipid components from their parent cells (Kao *et al.*, 2019). In septic conditions, the quantity of phosphatidylserine-positive-platelet derived EVs is increased by 1.49-fold (Zhang *et al.*, 2016).

In the past, it was believed that EVs were simply biomarkers of inflammation; however, Burger (2013) has shown that platelet derived EVs can contribute to inflammation. As EVs are shed from

parent cells, phosphatidylserine is externalized on the parent cell (Argañaraz *et al.,* 2020) leading to concentrated phosphatidylserine exposure on the outer membrane of EVs. In fact, the surface of platelet derived EVs were found to be 50-100 times more pro-coagulant than the surface of activated platelets (Burger *et al.,* 2013), suggesting that platelet derived EVs may have a crucial role in the pro-inflammatory and pro-coagulant response seen in septic patients. Some of the potent pro-inflammatory and pro-coagulant activity of EVs can be attributed to their ability to produce reactive oxygen species (ROS) (Cat *et al.,* 2013), ROS has been shown to increase NF-κB activity (Blaser *et al.,* 2016). Once activated, NF-κB can carry out its role as transcriptional activator of inflammatory cytokines (Nennig *et al.,* 2017). The ability of EVs to produce ROS may be contributing to their increased pro-inflammatory and pro-coagulant potential.

EVs have also been shown to affect a range of physiological processes such as intracellular exchange of proteins and RNA, and the rate of angiogenesis within an organism (Mulcahy *et al.,* 2014).

Annexin A5 treatment has been shown to reduce EV formation *in vitro* through a process of phosphatidylserine binding (Gidon-Jeangirard *et al.,* 1999). As annexin A5 binds to externalized phosphatidylserine on an EV producing cell, it causes two-dimensional-crystallization at the cell surface, acting as a physical restraint to EV "blebbing" (shown in figure 1.4). Figure 1.4 shows annexin A5's ability to selectively bind externalized phosphatidylserine on activated host cells as well as on EVs

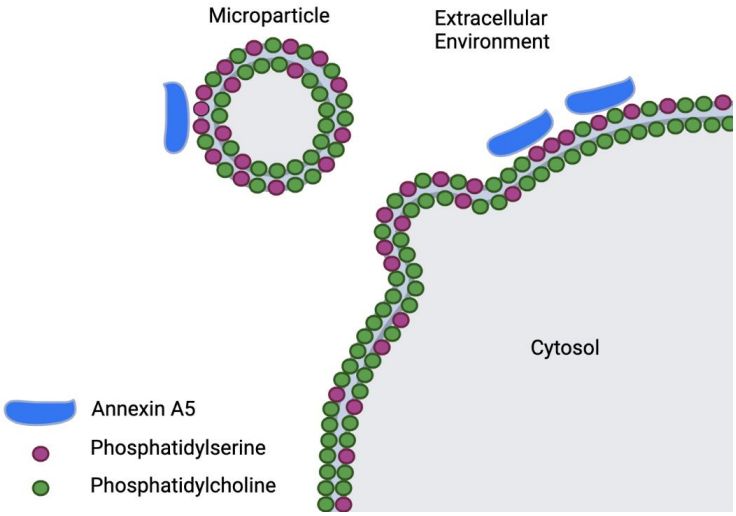


Figure 1.4. Annexin A5 Binds Phosphatidylserine on Host Cells and EVs. Image depicting annexin A5's ability to cause two-dimensional-crystallization through phosphatidylserine binding. Image was created in BioRender.com.

Interestingly, annexin A5 treatment has also been shown to reduce the circulating levels of platelet derived EVs through increasing EV uptake by dendritic immune cells (Tontanahal, *et al.*, 2021). Potentially further contributing to the anti-inflammatory properties exhibited by annexin A5.

Within the last decade, it has become clear that EVs play a critical role in cell-to-cell communications (Mulcahy *et al.*, 2014). Valadi and colleagues (2007) demonstrated the ability of EVs to transfer genetic information in an experiment in which human mast cells were incubated with mouse EVs. After the incubation period, mouse proteins were found within the human mast cells. For EVs to release their nucleic acid and protein components into target cells to alter the cells phenotype, they must fuse with the membrane of the target cells or fuse with the endocytic vesicle during or after the process of endocytosis (Mulcahy *et al.*, 2014). mRNA and miRNA that are transported through the extracellular fluid within EVs can be deposited within target cells and

effect gene expression of target cells, ultimately altering the cell's phenotype. Many EVs are internalized by target cells in a process which is dependent on the extracellular proteins and glycoprotein of the EVs as well as the host cell (Mulcahy *et al.,* 2014). Potential routes of internalization include clathrin-dependent endocytosis and clathrin-independent endocytosis such as caveolin-mediated uptake, micropinocytosis, phagocytosis, and lipid raft–mediated internalization (Mulcahy *et al.,* 2014).

EV clearance can be viewed as a mechanism by which phagocytic immune cells mitigate the effects of EVs. In the case of sepsis, EV clearance via immune cells contributes to our bodies' anti-inflammatory and anti-coagulation efforts. An example of the deleterious effects of EVs is shown in hemolytic uremic syndrome (HUS), HUS is characterized by an E. Coli infection in the colon in which Shiga toxin is produced. The toxin within the colon can lead to diarrhea or hemorrhagic colitis. In severe cases, the Shiga toxin can be transported to the kidneys via EVs leading to HUS. Tontanahal (2021), has shown that in HUS mice, circulating levels of EVs were significantly lower in mice treated with annexin A5 for 6 days. This corresponded with delayed onset of clinical disease in a dose-dependent manner. The findings suggest that the delayed onset of disease in the annexin A5 treatment groups was a result of increase EV uptake from phagocytic immune cells, ultimately reducing the transfer of HUS inducing Shiga toxin to the kidneys (Tontanahal *et al.,* 2021). Notably, they showed that EVs induce phosphatidylserine exposure on phagocytic membranes, and that EV clearance was not affected when phagocytic cells alone were treated with annexin A5, indicating that annexin A5 coated EVs can be readily cleared from phagocytosis by macrophages and monocytes (Tontanahal *et al.,* 2021). These findings were contradictory to previous beliefs that annexin A5 treated EVs are phagocytosed at a lower rate by macrophages and natural killer cells compared to EVs without annexin A5 coating (Yuyama *et al.,*

2012; Nolte-'t Hoen *et al.*, 2009). The reduced uptake of annexin A5 coated exosomes by dendritic cells highlighted the importance of phosphatidylserine in triggering EV uptake by immune cells.

1.5 Proinflammatory and Procoagulant Effects of Phosphatidylserine

Under normal physiological conditions, phosphatidylserine is asymmetrically distributed between the inner and outer leaflets of the cell membrane, with essentially all phosphatidylserines contained within the inner leaflet of a cell at rest (Leventis *et al.*, 2010). The asymmetry between the inner and outer leaflets of the cell membrane is maintained by action of 10-transmembrane domain aminophospholipid translocases known as "flippases", which relocates phosphatidylserine back into the inner leaflet (Lemke 2017). A subset of flippases known as ATP11C are inactivated by caspase cleavage during apoptosis, allowing phosphatidylserine to carry out its pro-apoptotic properties (Segawa *et al.*, 2014). During processes such as platelet activation, EV shedding, and cell activation/death, cells externalize phosphatidylserine via TMEM16 scramblase activity (Pomorski & Menon, 2016). Externalized phosphatidylserine has been shown to modulate tissue factor (TF) activation and initiate the coagulation cascade (Bach *et al.*, 1989). Phosphatidylserine provides a platform for aggregation of various coagulation factors, increasing prothrombin production by creating binding sites with factors Va and Xa. (Zhang *et al.*, 2016). The importance of externalized phosphatidylserine is in highlighted in Scott's syndrome, in which a mutation in the TMEM16 scramblase protein leads to inability to externalize phosphatidylserine leading to increased bleeding (Brooks *et al.*, 2015).

Phosphatidylserine has also been shown to provide docking sites for protein kinase C (PKC) (Argañaraz *et al.*, 2020). Various isoforms of PKC have shown potential to mediate the

assembly of NOD-Like receptor family members such as NOD-Like receptor C4 (NDRC4), leading to the production of the inflammatory cytokine IL-1β (Qu *et al.,* 2012). A disintegrin and metalloprotease 17 (ADAM17), also known as tumour necrosis factor-α converting enzyme (TACE) is a membrane-bound enzyme that cleaves various cell surface proteins such as cytokines (TNFα), cytokine receptors (IL-6R and TNF-R) and adhesion proteins (L-Selectin and ICAM-1) (Scheller *et al.,* 2011). When membrane bound, some inflammatory cytokines such as IL-6 and TNFα not only do not possess their normal pro-inflammatory properties but may possess anti-inflammatory properties (Scheller *et al.,* 2011). However, once these cytokines are cleaved from their host cell membrane by ADAM17, they possess their pro-inflammatory properties (Scheller *et al.,* 2011). Sommer *et al.,* (2016) has shown that phosphatidylserine is an important activation site for ADAM17 sheddase activity providing a possible link between phosphatidylserine and the underlying mechanism of cytokine release (Argañaraz *et al.,* 2020).

Cauvi *et al.,* (2019) has shown that injecting liposomes expressing externalized phosphatidylserine into the peritoneal cavity of mice via IP injection led to higher expression of the inflammatory cytokines TNFα and IL-6 in their peritoneal cells when compared to the peritoneal cells that were treated with liposomes expressing externalized phosphatidylcholine. They also found that the liposomes with externalized phosphatidylserine were predominantly taken up by macrophages (~93% of labelled phosphatidylserine) via endocytosis, and they have proposed that changes in transcriptome (increased inflammatory cytokine expression) is most likely due to peritoneal macrophage activity (Cauvi *et al.,* 2019).

Lastly, phosphatidylserine positive liposomes within the peritoneal cavity led to massive increases in chemokine and cytokine expression ultimately causing significant increases in neutrophil counts when compared with mice treated with phosphatidylcholine liposomes (Cauvi

et al., 2019). When recruited, neutrophils can engulf foreign particles, produce NETs and release inflammatory cytokines (Rosales, 2018). In this study phosphatidylserine exposure appears to elicit its pro-inflammatory response through cytokine -cytokine receptor binding, as the cytokine-cytokine receptor interaction pathways was significantly upregulated in the phosphatidylserine liposome treatment when compared with mice treated with phosphatidylcholine liposome treatment (Cauvi *et al.,* 2019).

1.6 Endothelial Cells During Sepsis

Vascular endothelial cells line blood vessels within our body and they act as a median between the blood and vascular wall (Yilmaz *et al.,* 2019). In advanced cases of sepsis, the endothelial cells that line the vasculature can degrade, leading to leakage of blood components out of the vasculature, known as extravasation (Granja *et al.,* 2019). Extravasation can lead to ischemic and inflammatory organ damage, a characteristic of severe sepsis and septic shock (Granja *et al.,* 2019).

Endothelial Inflammation and Adhesion in Sepsis

Vascular endothelial cells are known to play a critical role in the crosstalk between vascular inflammation and coagulation during a septic response (Aird, 2001). Endothelial cells can become activated by inflammatory cytokines released from leukocytes and they can, in turn release inflammatory cytokines themselves as well as express adhesion molecules on their surface to increase blood cell and immune cell adhesion as well as release growth factors (Levi and Poll, 2013). In normal conditions, endothelial cells produce and externalize von Willbrand factor (vWF) multimers from vWF monomers (Dong *et al.* 2002). In a healthy state, an enzyme called ADAMTS13 will cleave a large portion of the externalized monomer, however in conditions like

sepsis, ADAMTS13 is downregulated leading to less vWF cleavage and more platelet adhesion to the injured endothelial cells (Zheng, 2013). The ensuing thrombotic microangiopathy has been connected to multi organ dysfunction seen in sepsis (Booth *et al.,* 2011). Interestingly, during a septic response endothelial cell also have increased externalized phosphatidylserine after a 24-hour incubation in septic serum (Zhang *et al.*, 2016), which could be another major contributor to the pro-inflammatory and procoagulant response.

During inflammation, adhesion molecules on endothelial cells become exposed for circulating leukocytes to bind (Ince *et al.*, 2016). Exposed adhesion molecules on the endothelium initiate leukocyte adhesion which can ultimately lead to leukocyte infiltration into the tissue. Presence of exposed selectins (E, P and L) allow leukocytes to bind to and roll along the endothelial monolayer, while exposed intracellular adhesion molecule-1 (ICAM-1) allows for firm adhesion and transmigration of leukocytes through the endothelium (Ince *et al.,* 2016). Once white blood cells transmigrate into the local tissue, they release inflammatory cytokines and reactive molecules that aid in killing the pathogen, while at the same time raise the risk for tissue death (Ince *et al.*, 2016). This process is highly inflammatory and needs to be tightly controlled while occurring during the inflammatory stages of the septic response.

Endothelial Glycocalyx in Sepsis

The endothelial monolayer contains a gel-like layer on the luminal surface known as the glycocalyx (Ince *et al.*, 2016), the glycocalyx is composed of negatively charged proteoglycans, glycoproteins, glycolipids and glycosaminoglycans (Yilmaz *et al.*, 2019). The glycocalyx is involved in mediating many key endothelial functions. The main functions of focus for this project are controlling vascular permeability, and controlling blood cell adhesion (Yilmaz *et al.*, 2019). An intact glycocalyx protects against hypercoagulation and facilitates fluid homeostasis (Iba &

Levy, 2019). In patients suffering from severe sepsis or septic shock, the glycocalyx can shed, contributing to the compromised structural integrity of the endothelial monolayer (Ince *et al.,* 2016). Detecting glycocalyx components in the circulation is thought of as a marker for sepsis (Iba & Levy, 2019). Many factors can lead to shedding of the glycocalyx, including elevated levels of the inflammatory cytokine tumour necrosis factor alpha TNFα (Ince *et al.,* 2016). With that in mind, it appears that degeneration of the endothelial monolayer is critical for the pathological progression from sepsis to severe sepsis and septic shock, as the glycocalyx sheds, we see an increase in coagulation and an increase in vascular permeability (Ince *et al.,* 2016).

With the importance of the endothelial glycocalyx clear, we know that the most prominent glycosaminoglycans in the glycocalyx is heparan sulfate, making up 60-90% of the glycocalyx (Iba & Levy, 2019). In inflammatory diseases such as sepsis, heparan sulfate is cleaved from the endothelial glycocalyx by heparanase 1. Heparan sulfate cleavage can lead to increased vascular permeability, contributing to hypovolemia in shock patients, and neutrophil adhesion (Garsen *et al.,* 2015; Kiyan *et al.,* 2019). Caren and colleagues (2015), have shown that heparanase deficient mice showed less heparan sulfate cleavage, leading to reduced vascular permeability and neutrophil adhesion in LPS treated mice. Along with preserving vascular permeability, intact heparan sulfate on the endothelial monolayer has been shown to interfere with LPS-TLR4 interaction, helping to reduce inflammatory cytokine expression in LPS treated mice (Kiyan *et al.,* 2019).

Intracellular and extracellular annexin A5 can form a scaffolding in the membrane to help with membrane dynamics, cytoskeleton stability, induce membrane repair and link adjacent membranes (Tontanahal, *et al.,* 2021). It has been shown that annexin A5 is able to bind to heparan

sulfate within the endothelial glycocalyx (Capila *et al.,* 2001). This may represent another beneficial effect of annexin A5 in attenuating endothelial dysfunction in sepsis.

1.7 Annexin A5 as a Potential Therapeutic in COVID-19

The COVID-19 pandemic is caused by SARS-CoV-2 viral infection in the airway and the lungs. The SARS CoV-2 virus utilizes its surface spike protein to bind with angiotensin converting enzyme 2 (ACE2) on host cells to cause infection. This process begins with the respiratory tract epithelial cells and then infects various cells throughout the body, including endothelial cells (Figure 4.8) (Mui *et al.,* 2021).

The imbalance of inflammation and coagulation seen in COVID-19 can lead to septic shock and multi-organ failure (Blanco-Melo *et al.,* 2020). In fact, 100% (54) of hospitalized COVID-19 patients who deceased during the study were septic, opposed to 42% (58) of hospitalized survivors. Additionally, 70% (38) of hospitalized COVID-19 patients who passed away had sepsis progress to septic shock (Zhou *et al.,* 2020). Thus, development of sepsis and septic shock has a major negative impact on COVID-19 patients.

1.8 Summary and Rationale

The reservoir of evidence exhibiting annexin A5's ability to attenuate inflammation (Arnold *et al.,* 2014; Park *et al.,* 2016), reduce coagulation (Rand *et al.,* 2006*),* and potentially maintain endothelial monolayer permeability and hemostasis (Capila *et al.,* 2001; Ince *et al.,* 2016; Iba & Levy, 2019) in sepsis or endotoxemia is ever-growing. Our research is looking to account for a significant gap in patient care and sepsis treatment, as there are no pharmacological agents available to treat the underlying septic response (Granja *et al.,* 2019). With the knowledge that

sepsis is the leading cause of death in the world, accounting for 11 million deaths per year (Rudd *et al.,* 2020) it is paramount that a pharmacological agent be formulated to account for this issue.

Past research from our laboratory has shown that annexin A5 reduces inflammation and improves survival by 40% in endotoxic mice (Arnold *et al.*, 2014) but more work is required to understand annexin A5's mechanisms of action as a potential treatment option for sepsis.

In a septic response, phosphatidylserine is externalized on platelets, EVs and endothelial cells, leading to increased inflammation and coagulation in the vasculature. Exaggerated inflammatory cytokine release and coagulation seen in a septic response can lead to endothelial dysfunction and contribute to multi-organ failure (Chousterman *et al.,* 2017). Of particular interest, annexin A5 has the innate ability to bind to phosphatidylserine in a calcium-dependent manner. With the knowledge that annexin A5 selectively binds to the pro-coagulant, pro-inflammatory phosphatidylserine, we believe that treatment with annexin A5 can attenuate the pro-inflammatory and pro-coagulant activity induced by LPS-activated platelets and EVs. We also believe that annexin A5 may reduce E-selectin and ICAM-1 expression during the inflammatory stages of sepsis, ultimately reducing the risk of organ dysfunction during sepsis and platelet and leukocyte adhesion to the endothelium.

1.9 Hypothesis and Specific Objectives

We hypothesize that annexin A5 will block the pro-inflammatory response induced by LPS activated platelets and extracellular vesicles in vascular endothelial cells.

Specific Objectives:

1) Examine the effects of recombinant human annexin A5 on endothelial inflammation induced by LPS activated platelets and EVs
2) Determine if LPS activated platelets and EVs affect the integrity of the endothelial monolayer by measuring trans-endothelial electrical resistance (TEER).
3) Investigate platelet and monocyte adhesion to endothelial cells in septic conditions with and without recombinant human annexin A5.

Chapter 2

2. Methods

2.1 Animal Ethics and Approval

This study utilized mice in accordance with the Guidelines for the Care and Use of Animals by the Canadian Council of Animal Care. The study protocol was approved by the Animal Care Committee at Western University, Canada. Wild-type (WT) C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice were given water and chow *ad libitum* and held on a 12h-12h light-dark cycle. Male and female mice were used in this project.

2.2 Mouse Skeletal Muscle Endothelial Cell Culture and Passage

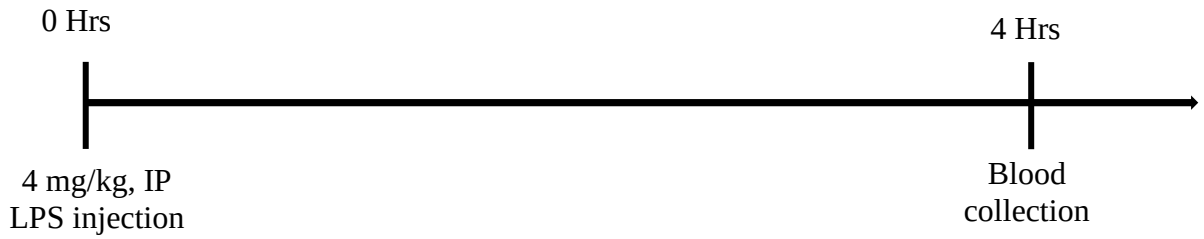
Primary skeletal muscle microvascular endothelial cells were isolated from the hind limb of C57BL/6 mice. Hind limb skeletal muscle was chosen because the hind limb of C57BL/6 mice contains a rich microvascular bed, with a vessel density of 656 $\pm$ 22 vessels /mm^2 in the gastrocnemius (Scholz *et al.,* 2002). Hind limb skeletal muscle was excised from 1-2 sacrificed C57BL/6 mice, ranging in age from 2 weeks to 2 months. Male and female mice were used for this project. Mice were sedated using intraperitoneal injection of 10 μL/gram body weight of a ketamine solution (264 μg/g body mass of ketamine, 13.2 ug/g body mass acepromazine, 13.2 μg/g body mass xylazine, and 660 ng/g body mass atropine). Following ketamine injection, mice were euthanized via cervical dislocation. Isolated muscle was digested in a 15 ml solution containing DMEM medium, 12.8 mg of collagenase II, 2 mg of dispase II and 24 mg of fetal bovine albumin (FBA) at 40 ^{o}C for ~40 minutes or until skeletal muscle was sufficiently digested. Digested skeletal muscle tissue was further broken down by aspiration with an electronic pipette

using a 10 ml tip. The solution was spun down at 300 x g for 5 minutes and the supernatant was removed and strained using a 70 µm filter. The filtered solution was once again spun at 300 x g for 5 minutes and the resulting pellet contained endothelial cells. The cell pellet was resuspended in ~4 ml of DMEM medium containing 20% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine, microvascular growth supplement (Thermo Scientific, Waltham, United States), and 5 U/ml heparin and then added to a 25 cm^2 flask. Cells were incubated in 37 ^oC with 5% CO_2 until the cells reached 80-90% confluence, at the time when the cells reached this confluence, cells were passaged from one 25 cm^2 flask into two. Endothelial cell cultures were labelled with isolectin B4 (Vector, Burlington, Ontario) to visualize cell purity. Culture purity was consistently ~98% (supplementary data Figure 5.1).

To passage the endothelial cells, media was removed from the flask and added to a 15 ml tube. PBS was added to the flask for around 1 minute to wash the endothelial cells. PBS was then removed and 1x trypsin-EDTA was added for 1-2 minutes with periodic shaking to allow cells to detach from the flask. Flasks were then aspirated to remove the semi-detached cells from the flask and the resuspended cells were added to a 15 ml tube and spun at 300 x g. Pelleted cells were resuspended in fresh culture media containing 20% FBS, 1% penicillin/streptomycin, 1% L-glutamine, microvascular growth supplement (Thermo Scientific, Waltham, United States), and 5 U/ml heparin in DMEM media. After 2-3 passages, the cells were passaged (at least 80% confluence) onto a 24 well plate, coverslips, or inserts for experimentation.

2.3 *In Vivo* LPS Induced Endotoxemia

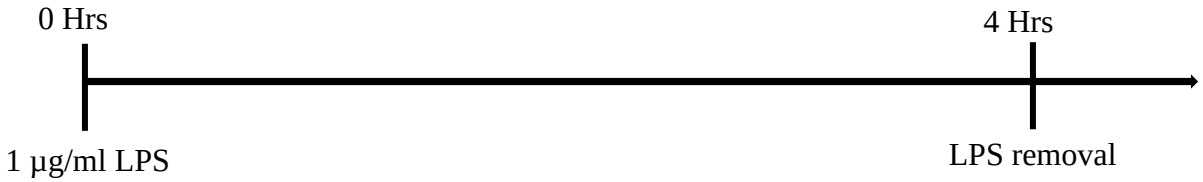
LPS (from Salmonella typhosa, Cat. L7136, Sigma Aldrich, St. Louis, Missouri) induced endotoxemia on C57BL/6 mice was carried out as shown below.



In order to investigate the role of *in vivo* activated platelets and EVs, 4 mg/kg bodyweight of LPS was injected into C57BL/6 mice via intraperitoneal (IP) injection. Blood was collected for platelet and EV isolation, 4 hours after the LPS injection.

2.4 *In Vitro* LPS Induced Platelet and Extracellular Vesicle Activation

LPS treatment on isolated platelets and EVs was carried out as shown below:



Once platelets and EVs are isolated from C57BL/6 mouse blood and suspended in PBS, LPS was added at a concentration of 1 µg/ml PBS for 4 hours. Similarly, LPS was added to endothelial cells on a 24 well plate at a concentration of 1 µg/ml culture medium in an LPS treatment group. After the 4-hour treatment, the platelets, EVs, and endothelial cells were washed thoroughly to remove all traces of LPS. In order to wash platelets, the solution containing platelets suspended in PBS and 1 µg/ml LPS was spun at 800 x g for 10 minutes at 4 ^{o}C twice, first to remove the 1 µg/ml LPS supernatant and then again to remove a PBS wash. Similarly, EVs were

washed with two spins at 19,500 x g for 20 minutes each. Once platelets and EVs are washed their pellets were aspirated onto a 24 well plate containing endothelial cells using the culture medium covering the monolayer. Wells containing endothelial cells were assigned to 1 of 6 groups; control (containing only endothelial cells in medium), LPS treatment (1 µg/ml LPS added to medium), *in vitro* activated platelets, *in vitro* activated EVs, *in vivo* activated platelets, or *in vivo* activated EVs. The aspirated platelets and EVs were incubated in the endothelial culture at 37 ^{o}C and 5% CO_2 for 4 hours The endothelial cells were washed by removing the culture medium containing LPS and then washed with PBS three times. The treated endothelial cells were stored at -80 ^{o}C for analysis of the inflammatory cytokine and adhesion molecule mRNA levels via RT-qPCR.

2.5 Annexin A5 Treatment

Once LPS was added to the solutions, platelets and EVs remained in the solution for 4 hours to allow for *in vitro* platelet and EV activation. After the 4-hour treatment, the platelets and EVs were washed twice, and then aspirated onto a 24 well plate containing an endothelial monolayer with or without 1 µg/ml of recombinant human annexin A5 treatment in the endothelial monolayer media. Both platelet and EV experiments contained 4 groups:

1. Control
2. Recombinant human annexin A5 (1 µg/ml)
3. Activated platelets or EVs
4. Activated platelet or EVs+ recombinant human annexin A5 (1 µg/ml)

Once again, the platelets and EVs were incubated in the endothelial culture at 37 ^{o}C and 5% CO_2 for 4 hours, following the 4-hour treatment endothelial cells were washed 3-5 times. The treated

endothelial cells were stored at -80 ^{o}C for analysis of the inflammatory cytokine and adhesion molecule mRNA levels via RT-qPCR.

2.6 Blood Sample Collection

In this procedure mice were sedated using intraperitoneal injection of a ketamine containing solution at a dose of 10 µl/gram body mass (264 µg/g body mass of ketamine, 13.2 µg/g body mass acepromazine, 13.2 µg/g body mass xylazine, and 660 ng/g body mass atropine). Once the mouse was sedated, the ribcage was exposed and a 1 ml heparin coated syringe with a 23 gauge, 1-inch needle was used to draw as much blood as possible from the heart. An average of around 800 µl of blood per mouse was collected into a 1.5 ml heparin coated Eppendorf tube for platelet and EV isolation.

2.6.1 Platelet and Extracellular Vesicle Isolation

Platelets and EVs were isolated from the blood of C57BL/6 mice that were either LPS challenged or control mice. Whole blood was obtained from 1-2 adult mice per experiment via cardiac puncture. Once blood was collected, the mice were sacrificed, and platelets and EVs were isolated following a slightly modified protocol from Burger *et al.* (2013). In this procedure, whole blood was spun at 150 x g at 4 ^{o}C for 8 minutes to separate the blood plasma from the red blood cells. The blood plasma was then spun at 800 x g at 4 ^{o}C for 10 minutes in order to obtain a platelet pellet. Lastly, the supernatant was removed and spun down at 19,500 x g at 4 ^{o}C for 20 minutes to obtain an EV pellet. Both pellets were resuspended in 1000 µl of PBS for further treatment. EVs isolated from a genetically modified mouse line expressing td-Tomato-labelled mTmG is depicted in Figure 5.3, validating our EV isolation obtained cell membrane encapsulated fragments of the correct size (<1µm).

The isolated platelets were quantified using a 0.1 mm deep hemocytometer (Bright-Line). 10 µl of the diluted platelet solution was added to the hemocytometer for quantification under a light microscope. On average, one mouse platelet isolation yields 20-30 million platelets. Thus, 10-15 million activated platelets were added to each experimental treatment group.

2.6.2 Extracellular Vesicle Quantification

Extracellular vesicles were quantified using a nanoflow cytometer, quantification of cellular fragments that were within the expected size range of extracellular vesicles (180-880 nm) were obtained. To quantify EVs specifically, isolated extracellular vesicles were diluted into 100 µl of PBS. Samples were labelled with FITC-conjugated annexin A5 (Biolegend, San Diego California) to label externalized phosphatidylserine, which is characteristically found on the external membranes of EVs. The count of annexin A5 labelled cell fragments that ranged in size from (180nm-880nm) were deemed to be EVs. Alexa fluor 647-conjugated CD41 (Biolegend, San Diego California) which is a platelet specific marker was also added to the EV solution to determine the proportion of EVs that were from platelet origin. An fluor 647-conjugated rat IgG,κ isotype control (Biolegend, San Diego California), was used to ensure CD41 binding specificity.

2.6.3 Nanoparticle Tracking Analysis of Extracellular Vesicles

Blood was collected via cardiac puncture from control C57BL/6 mice and mice treated with LPS (4 mg/kg) for 4 hours. EVs were isolated via differential centrifugation and EV samples were suspended in 100 µL of PBS and frozen at -80 ^{o}C until analysis. Samples were further diluted 1:200 to a final volume of 20ml. 1ml of diluted EV sample was loaded into a nanoparticle tracking analysis machine (Zetaview PMX110, Particle Metrix, Meerbusch, Germany) after calibration using 105 nm and 500 nm polystyrene beads. ZetaView software (version 8.02.28) was used for analysis of light scattering at 11 camera positions with 2 second video lengths, a camera frame rate

of 15 frames per second, and system temperature of ~ 21 ^{o}C to obtain the size profiling of isolated extracellular vesicles.

2.6.4 Transmission Electron Microscopic Analysis of Extracellular Vesicles

Blood was collected via cardiac puncture from control C57BL/6 mice. EVs were isolated via differential centrifugation and EV samples were suspended in 100 µL of PBS and frozen at -80 ^{o}C until analysis. Samples were loaded onto Formvar/carbon coated copper grinds, samples were visualized, and images were captured using a JEOL JEM-1400 plus transmission electron microscope (Akishima, Tokyo, Japan).

2.7 Monocyte Isolation

Monocytes were obtained from femoral bone marrow of C57BL/6 mice. In this procedure mice were sedated using intraperitoneal injection of 10 µl/gram body mass of ketamine (264 µg/g body mass of ketamine, 13.2 µg/g body mass acepromazine, 13.2 µg/g body mass xylazine, and dislocation. Once euthanized, both hind limbs were surgically removed from one, C57BL/6 mouse and the femur was cut at both ends, exposing the bone marrow. A 1 ml syringe with a 26 gauge, 1-inch needle was inserted into the bone marrow cavity of the femur in order to flush out as much bone marrow as possible using PBS. For both femurs, around 10 ml of PBS was used. After flushing, the PBS containing bone marrow was filtered in order to remove any traces of mouse hair or skeletal muscle before being centrifuged at 250 x g for 10 minutes. After the first spin, a pellet was obtained containing monocytes as well as red blood cells; in order to remove the red blood cells a red blood cell lysis buffer was used. After a 10-minute suspension in the red blood cell lysis buffer the solution was once again spun at 250 x g for 10 minutes to obtain isolated monocytes. On average, a monocyte isolation from one mouse yields ~12 million monocytes.

2.8 Endothelial Cell RNA Isolation

Treated and untreated endothelial cells were removed from the -80 ^{o}C freezer and rested on ice while TRIzol reagent (Life Technologies, London Ontario) (500 µl/well) was added to each well of cells. A 1000 µl pipette tip was used to scratch off as many adhered endothelial cells as possible and then the cells together with TRIzol solution were transferred to a 1.5 ml Eppendorf tube. Reagent grade chloroform (100 µl) was then added to the Eppendorf tubes containing endothelial cells suspended in TRIzol. Samples were vigorously vortexed for 20 seconds and then incubated at room temperature for 5 minutes. Following incubation, samples were centrifuged at 12,000 x g at 4 ^{o}C for 15 minutes. The top layer of the solution was removed and added to a clean Eppendorf tube and 250 µl of 100% isopropanol was added, vortexed then incubated with the solution for 10 minutes. Following incubation, the solutions were centrifuged at 14,000 x g at 4 ^{o}C for 15 minutes to obtain an RNA pellet. The supernatant was discarded, and the pellet was washed by adding 500 µl of 70% ethanol in 0.1% DEPC water to the pellet and vertexing before centrifuging the solutions at 12,000 x g at 4 ^{o}C for 10 minutes to obtain a washed RNA pellet. The supernatant was removed and discarded, and the Eppendorf tubes containing the RNA pellets were left open and placed in a fume hood for ~30 minutes or until the pellet was dry. The dried RNA pellet was resuspended in 30-60 µl of 0.1% DEPC water and heated at 70 ^{o}C for 5 minutes before RNA quantification using a nanodrop lite spectrophotometer (Thermo Scientific, Waltham, United States).

2.9 Reverse Transcription and Real Time PCR (RT-qPCR)

2.9.1 Reverse Transcription

The isolated RNA (250-500 ng) was diluted in 0.1% DEPC water for a total volume of 11.5 µl and then 1 µl of random primer (20 µM) was added and the solution was heated to 95 ^{o}C for 5 minutes. Then 7.5 µl of mater mix (4µl of 5x FirstStrand buffer, 2µl of 0.1M DTT, 1µl of 10 mM dNTP, and 0.5 µl of 200 U/µl MMLV reverse transcriptase) was added to the diluted RNA and random primer, to make a final volume of 20 µl. The solution was then placed in a 37 ^{o}C incubator for four hours to allow reverse transcription to occur to produce cDNA. The solution was then heated to 70 ^{o}C for 5 minutes to inactivate the reverse transcription process. Finally, the cDNA was stored at -20 ^{o}C for qPCR.

2.9.2 RT-qPCR

In order to perform qPCR, the following solutions were added to an opaque 96 well PCR plate; 2.5 µL ddH2O, 5µL 2x qPCR Buffer (EvaGreen), 0.5 µL of gene specific primer mixture (Forward + Reverse; 20 µM each), and 2µL of cDNA. 35 cycles were run at temperatures Tm ranging from 62 – 64 ^{o}C. C_T values were obtained from the qPCR and expression ratios were obtained by dividing the gene of interest expression by the 28S expression. 28S samples were diluted 1:500. Ratios were expressed as fold change by dividing the ratios by the average ratio in the control group and then normalized to reduce variance among treatment groups.

2.9.3 PCR Primers

TNF-α (Sigma): Forward= 5’CGGCATCCATCTCAAAGACA

Reverse= 5’CTTGACGGCAGAGAGGAGGT

IL-6 (Sigma): Forward= 5’CAAAGCCAGAGTCCTTCAGAG

Reverse= 5’ATGGTCTTGGTCCTTAGCCAC

ICAM-1 (Sigma): Forward= 5’GTGATGCTCAGGTATCCATCCA

Reverse= 5’CACAGTTCTCAAAGCACAGCG

E-Selectin (Sigma): Forward= 5’ATGCCTCGCGCTTTCTCTC

Reverse= 5’GTAGTCCCGCTGACAGTATGC

2.10 Trans-Endothelial Electrical Resistance (TEER)

The endothelial cells were cultured onto transwell inserts for our 24 well plates (1 µm pore, Greiner Bio-One). TEER were determined using an epithelial volt/ohm meter 3 (EVOM3, WPI, Florida, USA) with STX2-plus electrodes that were fixed at 2-3 mm apart. We measured the initial resistance (ohms) in the presence of transwell inserts in the cell culture media. We then passaged cultured endothelial cells onto the transwell inserts and allowed the cells to grow to full confluence (100%). TEER measurements were taken twice a day. The greater the resistance, the more intact the monolayer (Chen *et al.*, 2015). When TEER peaked, the confluent endothelial cells were challenged with LPS, LPS-activated platelets or LPS-activated EVs in the absence or presence of annexin A5, and TEER was assessed every 30 minutes for 4 hours and then at 8 and 24 hours. In each experiment, a control replicate was included to assess changes of TEER in confluent endothelial cells throughout the 24-hour period without any septic challenges or annexin A5 treatment. Each timepoint represents the average of three replicated resistance measurements at that timepoint divided by the average 0-hour measurement from all samples.

2.10.1 Heparanase Expression via RT-qPCR

Endothelial cells were grown to 100% confluence in a 24-well plate, treatment groups included control, recombinant annexin A5 (1µg/ml) treatment alone, LPS (1µg/ml) activated platelets alone and LPS activated platelets supplemented with recombinant annexin A5. Cells were treated for 4

hours and then washed before RNA was isolated and reverse transcribed (as described in 2.8 and 2.9). cDNA was then quantified using RT-qPCR with the following primer.

Heparanse Primer Sequence (Sigma):
Froward: 5' CCTCGTTCCTGTCCATCACC
Reverse: 5' CCGGCTCAGACCTGCAAATA

2.11 Monocyte Adhesion Assay

Cultured endothelial cells were passaged into 8 wells of a black-walled 96 well plate and cells were grown to 100% confluence. LPS was added to culture media containing cells at a concentration of 1µg/ml in two of the four wells for 3-hours. After 3 hours of LPS treatment, monocytes were isolated from a wildtype mouse and added to cell culture media containing endothelial cells. Monocytes were added to the culture media for 1 hour and then media was removed, cells were gently washed with PBS to remove debris and un-adhered monocytes. Wells were then fixed with 4% paraformaldehyde (4% PFA) before visualization under the light microscope. Treatment groups included a control, recombinant annexin A5 (1µg/ml) treatment alone, LPS alone (1µg/ml) and LPS+ recombinant annexin A5. Monocytes were added to all groups. Random image fields were obtained, and adhered monocytes were quantified from a 100 µm x 100 µm area. Values were expressed as adhered monocytes per 10,000 μm^2.

2.12 Platelet Adhesion Assay

Cultured endothelial cells were passaged onto 4 laminin coated coverslips and cells were grown to 100% confluence. Coverslips were sued to allow for high magnification viewing and laminin coating was used to ensure endothelial cells could adhere to the glass coverslips. LPS was added to culture media containing cells at a concentration of 1 µg/ml in two of the four coverslips for 3

hours. After 3 hours of LPS treatment, platelets were isolated from whole blood obtain via cardiac puncture (described in section 2.2) and labelled with calcein AM (Thermo Scientific, Waltham, United States). Labelled platelets were added to cell culture media containing endothelial cells for 1 hour and then media was removed, cells were gently washed with PBS to remove debris and un-adhered platelets. An image depicting Calcein AM labelled platelets is shown in Figure 5.2. Slides were then fixed with 4% paraformaldehyde (4% PFA), stained with Hoechst stain, and mounted onto 1 x 3x 1.0 mm. slides (Leica, Wetzler, Germany) before visualization via florescence microscope. Treatment groups included a control, recombinant annexin A5 (1 µg/ml) treatment alone, LPS alone (1 µg/ml) and LPS+ recombinant annexin A5. Platelets were added to all groups. Random image fields were obtained, and adhered platelets were counted per random field. Results were normalized to the average platelet count of each replicate's control and expressed as fold change vs the control.

2.13. Statistical Analysis

Data were presented as mean ± SEM. EV data was analyzed using an unpaired, two-way t-test. All mRNA expression data was analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's comparison. All TEER data were analyzed using two-way ANOVA followed by Fishers LSD test. Monocyte and platelet adhesion data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. A Grubb's test (alpha= 0.05) was used to remove any outlier replicates (GraphPad Prism 6 for Mac).

Chapter 3

3. Results

3.1 Analysis of Extracellular Vesicles (EVs)

Prior to experimental treatment with EVs, analysis was needed to ensure that EV isolation procedures described in Section 2.2.1 yielded appropriate quantities of cellular fragments within the appropriate size range.

EV samples were labelled with FITC conjugated annexin A5 to label externalized phosphatidylserine, which is characteristically found on the external membranes of EVs. Cell fragments that were labelled with annexin A5 labelled and ranged in size from 180-880nm were deemed to be EVs. Alexa fluor 647-conjugated CD41 which is a platelet specific marker was also added to the EV solution to determine the proportion of EVs that were from platelet origin. The EVs from platelet origin in this study were obtained from LPS stimulated and unstimulated WT mice. Low $CD41^+$ values (~10-12%) were obtained in our samples compared to what has been shown in the literature (38.5%) (Flaumenhaft *et al.*, 2009). (Table 3.1). EVs from LPS stimulated mice had a significantly higher proportion of annexin $A5^+$ EVs when compared to non-stimulated mice (80% and 69%, respectively) (Table 3.1).

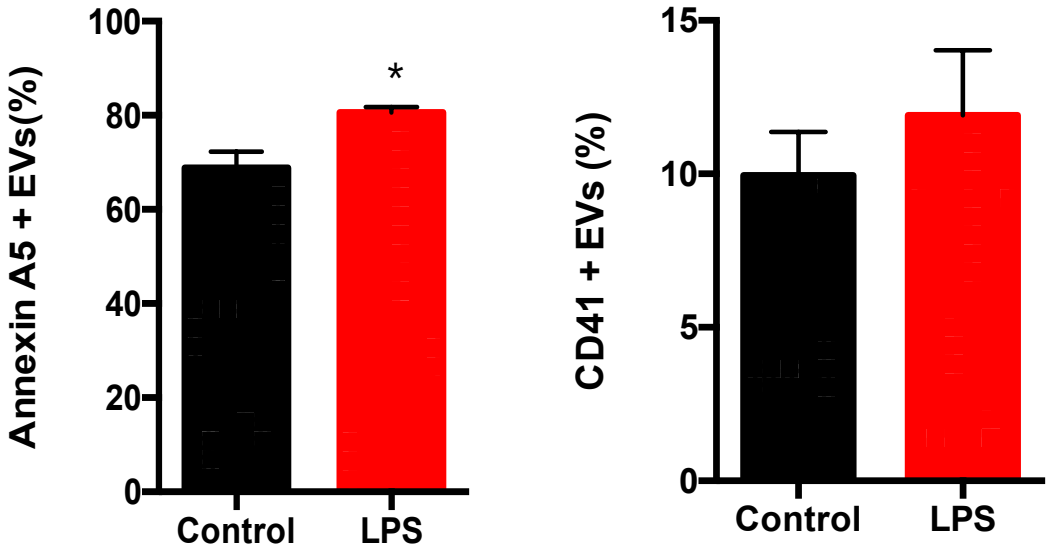


Figure 3.1 Analysis of Extracellular Vesicles. EVs were isolated from WT mice and labelled with Alexa fluor 647 labelled CD41 and FITC labelled annexin A5 (Biolegend, San Diego, California). Samples were run in a nanoflow cytometer. Values presented as mean $\pm$ SEM. Two-tailed, unequal variance T-test was used for statistical analysis. n=5. * $p<0.05$.

3.1.1 Extracellular Vesicles Transmission Electron Microscopy Images

In order to assess the morphology of the isolated EVs, transmission electron microscopic analysis was performed. In these electron microscopic images, cellular components can be visualized by the dark shading visualized prominently within the EV shown with the red arrow. EVs visualized with less shading within the cytoplasm most likely have small perforation in the membrane leading to leakage of cytoplasmic material during the freeze thaw cycle of the samples or during loading onto carbon coated copper grinds. EVs visualized were well within the expected size range of 100-1000 nm.

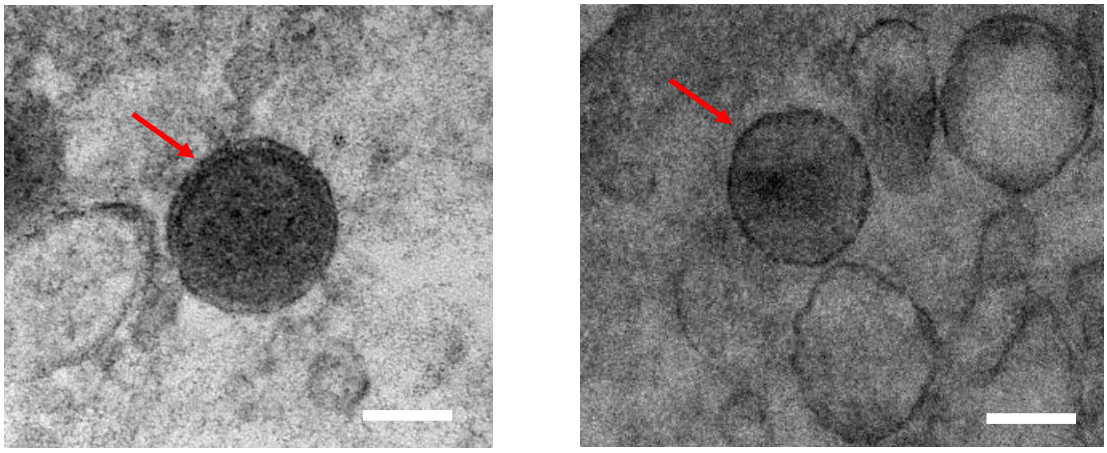


Figure 3.1.1. Transmission Electron Microscope Image. Scale bar= 100nm. Images were obtained from Dr. Dylan Burger at the University of Ottawa. Red arrow pointing to structurally intact EVs.

3.1.2 Nanoparticle Tracking Analysis

Our nanoparticle tracking analysis has revealed that the majority (~65%) of our control and LPS treated EVs ranged in size from 120 nm to 210 nm with negligible quantities of EVs measured over 500 nm in diameter for both the EVs isolated from control mice and the *in vivo* LPS stimulated mice (Figure 3.1.2).

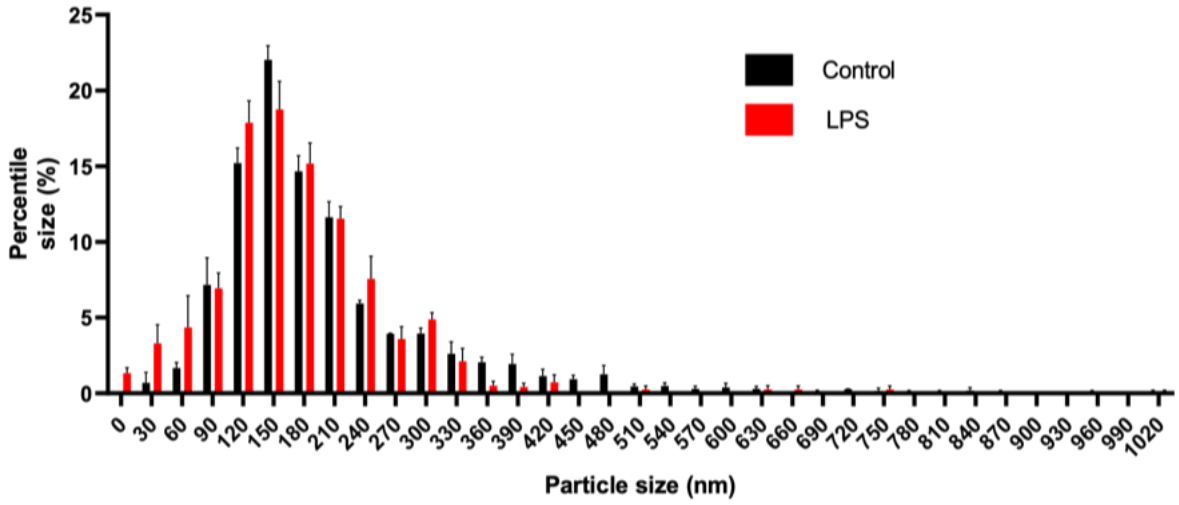


Figure 3.1.2. Nanoparticle Tracking Analysis. Data are mean ± SEM from n=3 (control) and n=4 (LPS) independent EV isolations per group.

3.1.3 LPS Stimulation Increases Plasma EV Levels

As seen in the literature (Burgelman *et al.,* 2021), the quantity of EVs from LPS stimulated mice was significantly higher than the quantity of EVs isolated from control mice. EV plasma levels were around 50% higher in the LPS stimulated mice vs. control mice (Figure 3.1.3).

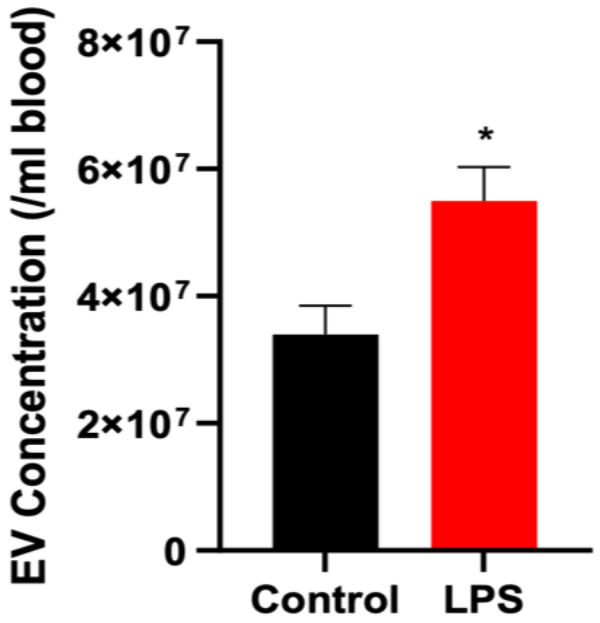


Figure 3.1.3. EV Concentration. EVs isolated from control and LPS (4 mg/kg) mice. EV concentrations were obtained from extrapolating NTA data. Data are mean ± SD from n=3 (control) n=4 (LPS) independent EV isolations per group. T-test was used for statistical analysis ***p<0.001, vs control.

3.2 Activated Platelets and EVs Increase Endothelial Expression of Inflammatory Cytokines and Adhesion Molecules

In order to validate that both *in vitro* and *in vivo* LPS-activated platelets and EVs could increase inflammatory cytokine and adhesion molecule expression within endothelial cells both techniques of stimulation were used. In this experiment, mice were treated with LPS (4 mg/kg, IP) for 4 hours and the activated platelets and EVs were isolated. Additionally, platelets and EVs were isolated from control mice and treated with LPS (1 μg/ml) for 4 hours *in vitro*. Through RT-qPCR analysis, it was determined that the addition of either *in vivo* or *in vitro* LPS-activated platelets or EVs onto cultured endothelial cells for 4 hours led to significantly higher mRNA levels of the inflammatory cytokines TNF-α and IL-6 and the adhesion molecules ICAM-1 and E-Selectin compared to the control endothelial cells. These findings show that the activated platelets and EVs induce endothelial cell inflammatory response.

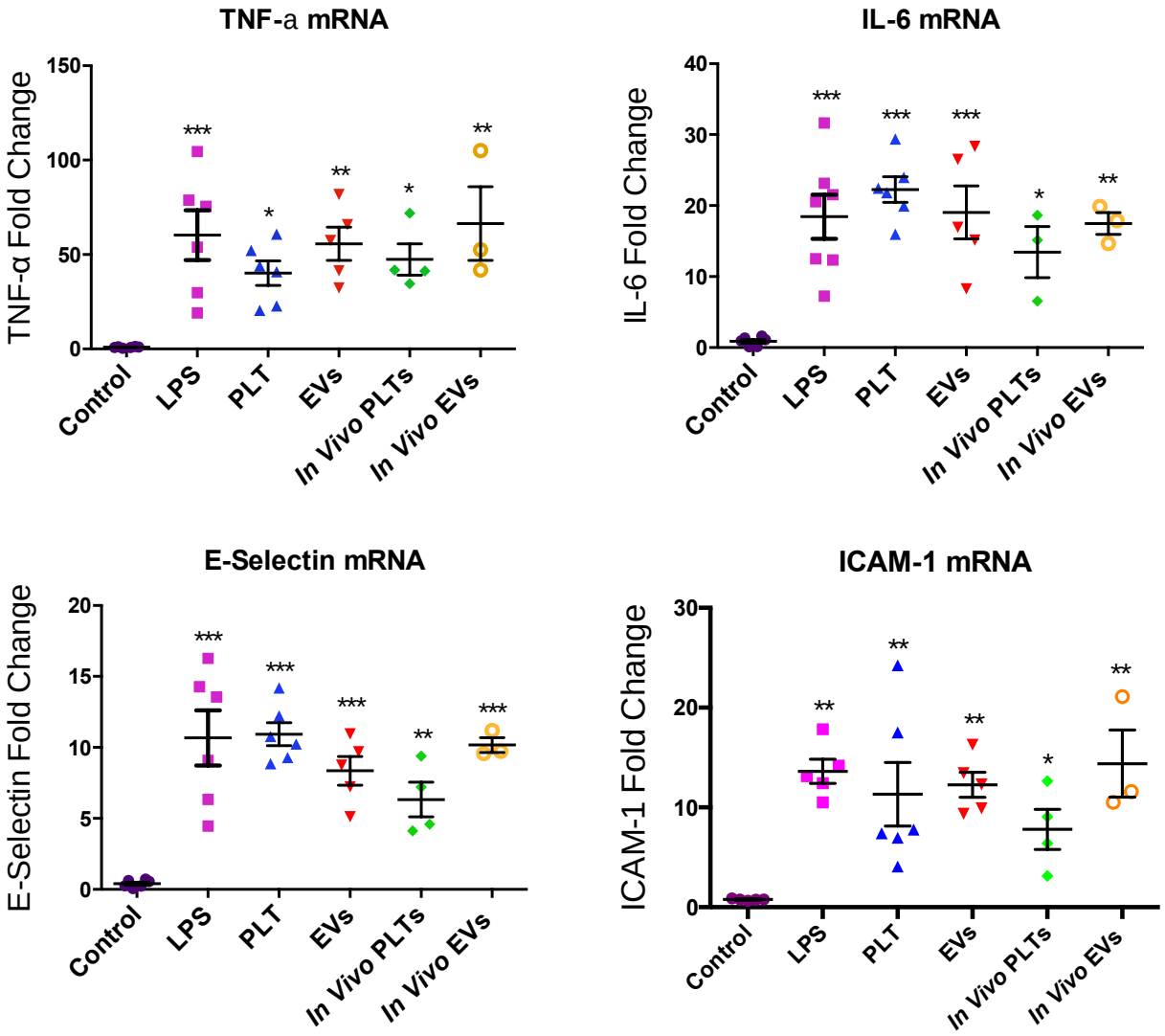


Figure 3.2. Effects of LPS-activated platelets (PLTs) or EVs on cytokine and adhesion molecule mRNA levels in cultured microvascular endothelial cells. *In vitro* LPS (1 µg/ml) treatment was used as a positive control. Adult mice were treated with LPS (4 mg/kg, IP) for 4 hours and the *in vivo* activated platelets and EVs were isolated. Additionally, platelets and EVs were isolated from control mice and treated with LPS (1 µg/ml) for 4 hours *in vitro* before they were incubated with cultured endothelial cells. The mRNA levels of TNF-α, IL-6, E-Selectin and ICAM-1 in cultured microvascular endothelial cells were analyzed by RT-qPCR. One-way ANOVA followed by Dunnett's test was used for statistical analysis. Data are mean ± SEM from n=3-7 independent cell cultures per group. *p<0.05, ** p<0.01, ***p<0.001, vs control.

3.3 Effects of Annexin A5 on Endothelial Inflammatory Response Induced by Activated Platelets

In order to test the anti-inflammatory and anti-adhesive effects of annexin A5, recombinant human annexin A5 was used as a treatment in cell media containing an endothelial monolayer being treated with activated platelets or EVs. Our data showed that the activated platelets alone induced significantly higher inflammatory cytokine and adhesion molecule expression in endothelial cells when compared to the control endothelial cells (Figure 3.2). Recombinant human annexin A5 alone had no effect on cytokine levels. Notably, annexin A5 treatment (1 μg/ml) significantly lowered inflammatory cytokine and adhesion molecule expression induced by activated platelets (Figure 3.3).

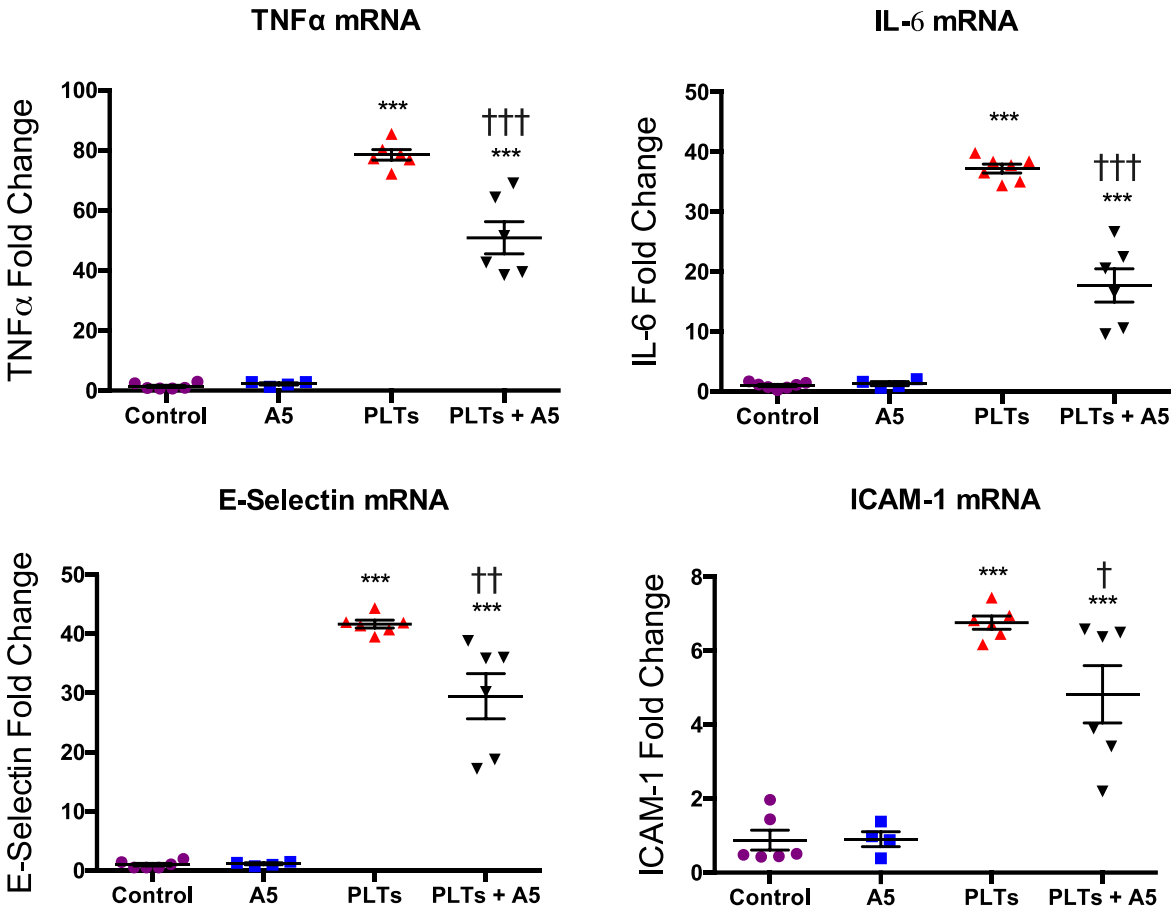


Figure 3.3 Effects of annexin A5 on mRNA levels of cytokines and adhesion molecules in cultured microvascular endothelial cells induced by LPS-activated platelets (PLTs). PLTs were isolated from mouse blood and incubated with LPS (1 µg/ml) *in vitro* for 4 hours and washed. Cultured microvascular endothelial cells were challenged with LPS-activated PLTs in the absence or presence of recombinant human annexin A5 (1 µg/ml). The mRNA levels of TNF-α, IL-6, E-Selectin and ICAM-1 in cultured microvascular endothelial cells were analyzed by RT-qPCR. One-way ANOVA followed by Tukey's multiple comparison test was used for analysis. Data are mean ± SEM from n=4-7 independent cell cultures per group. ***p<0.001 vs control. † p<0.05, †† p<0.01, ††† p<0.001 vs. PLTs.

3.4 Effects of Annexin A5 on Endothelial Inflammatory Response Induced by Activated EVs

Similarly, activated EVs alone induced significantly higher inflammatory cytokine and adhesion molecule expression in endothelial cells when compared to the control endothelial cells (Figure 3.2). Annexin A5 alone had no effect on cytokine levels. Notably, annexin A5 treatment (1 µg/ml) significantly lowered inflammatory cytokine and adhesion molecule expression induced by activated EVs (Figure 3.4).

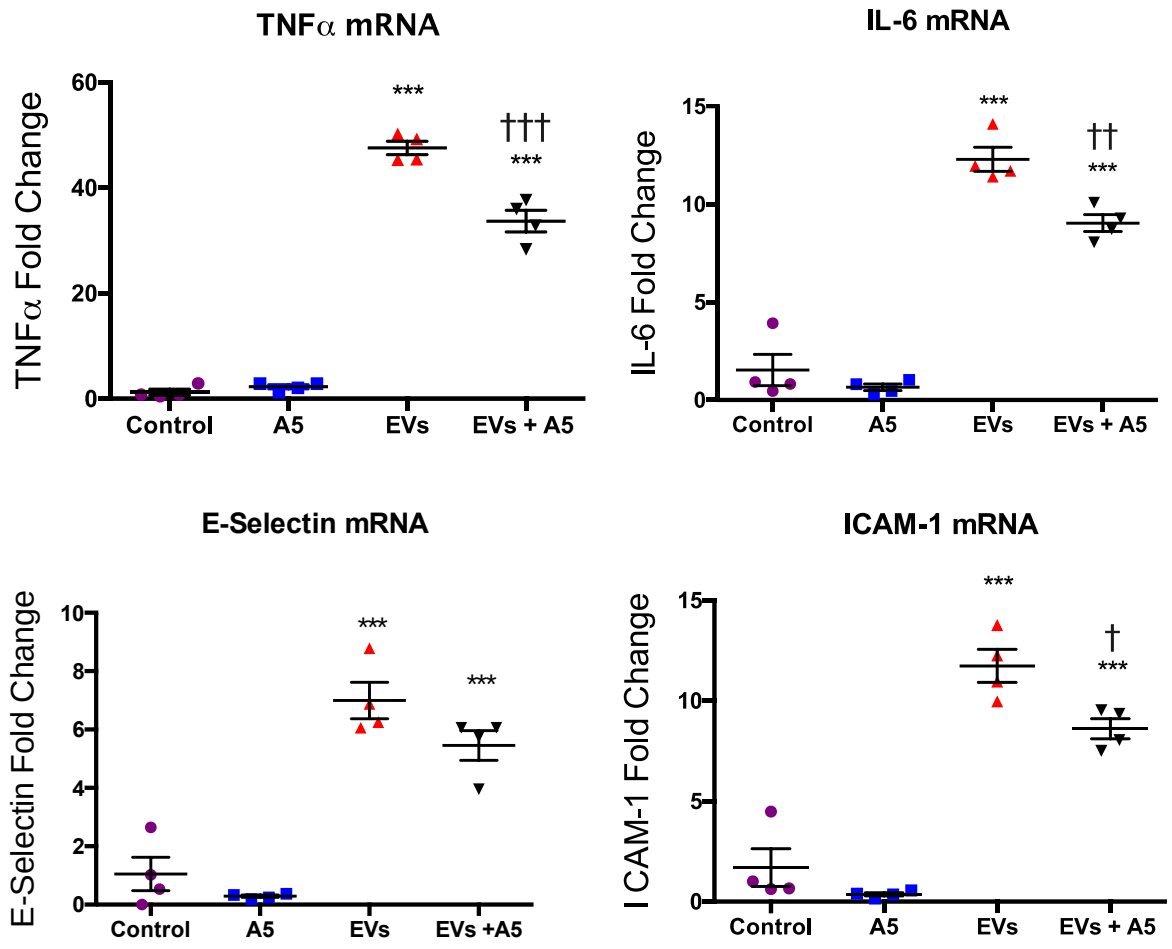


Figure 3.4 Effects of annexin A5 on mRNA levels of cytokines and adhesion molecules in cultured microvascular endothelial cells induced by LPS-activated extracellular vesicles (EVs). EVs were isolated from mouse blood and incubated with LPS (1 µg/ml) *in vitro* for 4 hours and washed. Cultured microvascular endothelial cells were challenged with LPS-activated EVs in the absence or presence of recombinant human annexin A5 (1 µg/ml). The mRNA levels of TNF-a, IL-6, E-Selectin and ICAM-1 in cultured microvascular endothelial cells were analyzed by RT-qPCR. One-way ANOVA followed by Tukey's multiple comparison test was used for analysis. Data are mean ± SEM from n=4 independent cell cultures per group. ***p<0.001 vs control. †p<0.05, †† p<0.01, ††† p<0.001 vs. EVs.

3.5 Trans-Endothelial Electrical Resistance in Endotoxic Conditions

Endothelial cells were cultured in trans-well inserts and septic challenges with or without annexin A5 treatment were started when cells were confluent and resistance readings around 200 Ω were reached (average baseline reading was 200.5 Ω $\pm$2.8 Ω). As the resistance reading drops below 100% it signifies the endothelial monolayer structural integrity is being compromised. Our data showed that all three septic conditions reduced endothelial monolayer resistance below 50% of their baseline value (resistance before treatment when cells were 100% confluent) with the largest reduction occurring between the 0-minute and the 30-minute timepoint (Figure 3.5.1-3.5.3). In cells challenged by LPS, annexin A5 treatment significantly reserved endothelial monolayer structure at various timepoint throughout the experiment (Figure 3.5.1). Treatment with annexin A5 led to significantly higher resistance values by the 24-hour timepoints, starting at 3.5 hours in cells challenged by LPS-activated platelets (Figure 3.5.2) and at 4 hours in cells challenged by LPS-activated EVs (Figure 3.5.3).

To ensure the membrane resistance maintained its baseline value throughout the 24-hour measurement period, a control well was measured, and it was found that resentence after 24 hours of measurement was the same as values at baseline (no treatment results not shown).

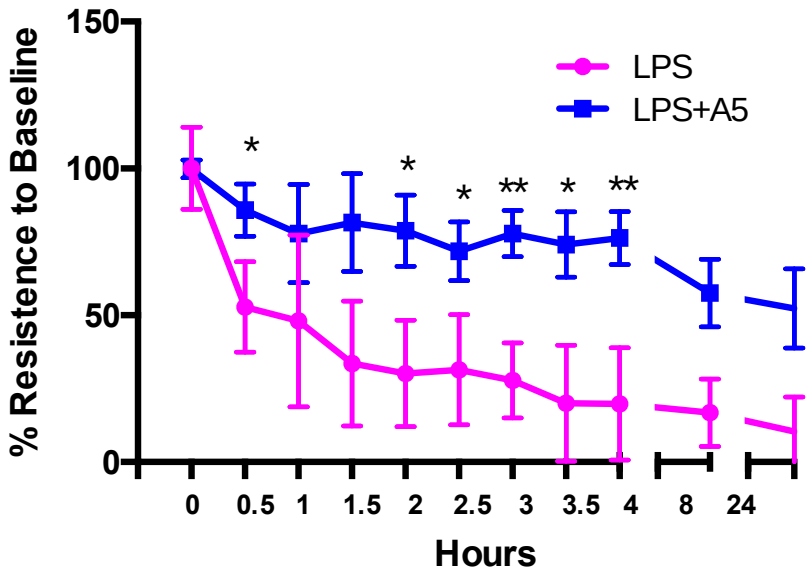


Figure 3.5.1. Effects of annexin A5 on trans-endothelial electrical resistance (TEER) induced by LPS. The confluent endothelial cells were challenged with LPS (4 µg/ml) in the absence or presence of annexin A5 (1 µg/ml). TEER was determined using an EVOM3 meter. Data are mean ± SEM, and were analyzed by 2-way ANOVA followed by Fisher's LSD test. n=4 individual cultures. *$p<0.05$, **$p<0.01$ vs. LPS.

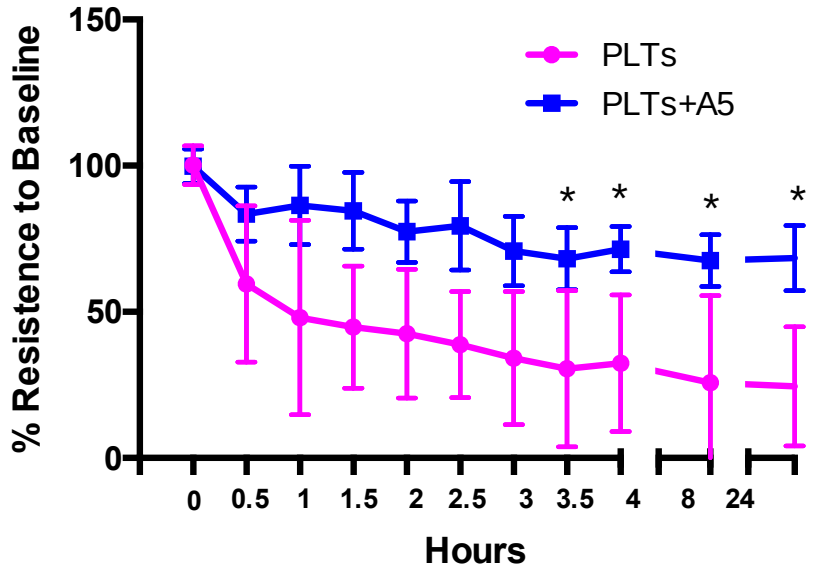


Figure 3.5.2 Effects of annexin A5 on trans-endothelial electrical resistance (TEER) induced by LPS-activated platelets (PLTs). The isolated PLTs from control mice were incubated with LPS (1 µg/ml) for 4 hours *in vitro* and washed. Confluent endothelial cells on transwell inserts were challenged with LPS-activated PLTs in the absence or presence of annexin A5 (1 µg/ml). TEER was determined using an EVOM3 meter. Data are mean ± SEM, and were analyzed by 2-way ANOVA followed by Fisher's LSD test. n=5 individual cultures, *$p<0.05$, vs. PLTs.

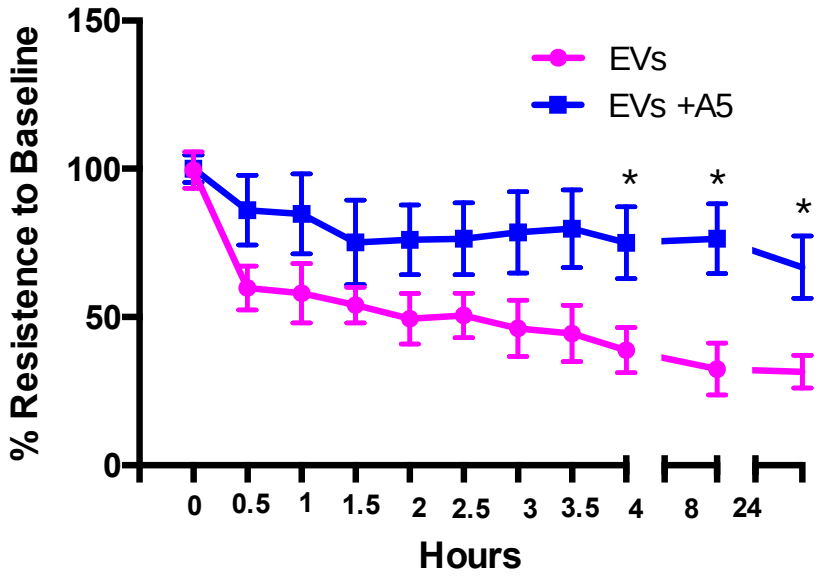


Figure 3.5.3 Effects of annexin A5 on trans-endothelial electrical resistance (TEER) induced by LPS-activated extracellular vesicles (**EVs).** The isolated EVs from control mice were incubated with LPS (1 µg/ml) for 4 hours *in vitro* and washed. Confluent endothelial cells on transwell inserts were challenged with LPS-activated PLTs in the absence or presence of annexin A5 (1 µg/ml). TEER was determined using an EVOM3 meter. Data are mean ± SEM, and were analyzed by 2-way ANOVA followed by Fisher's LSD test. n=5 individual cultures, *$p<0.05$, vs. EVs.

3.6 Heparanase mRNA Expression

In order to understand annexin A5's ability to maintain the structural integrity of the endothelial monolayer during septic conditions, heparanse expression in septic conditions with or without annexin A5 treatment was measured. Heparanase is an enzyme that cleaves heparan sulfate in inflammatory conditions, leading to exposed surface selectins on endothelial cells as well as increased extravasation (Iba *et al.,* 2018).

Using RT-qPCR analysis, we showed that heparanase mRNA levels were significantly higher in endothelial cells challenged with LPS-activated platelets compared to control endothelial cells. Annexin A5 (1 µg/ml) treatment significantly lowered heparanase expression to levels similar to those seen within the control groups.

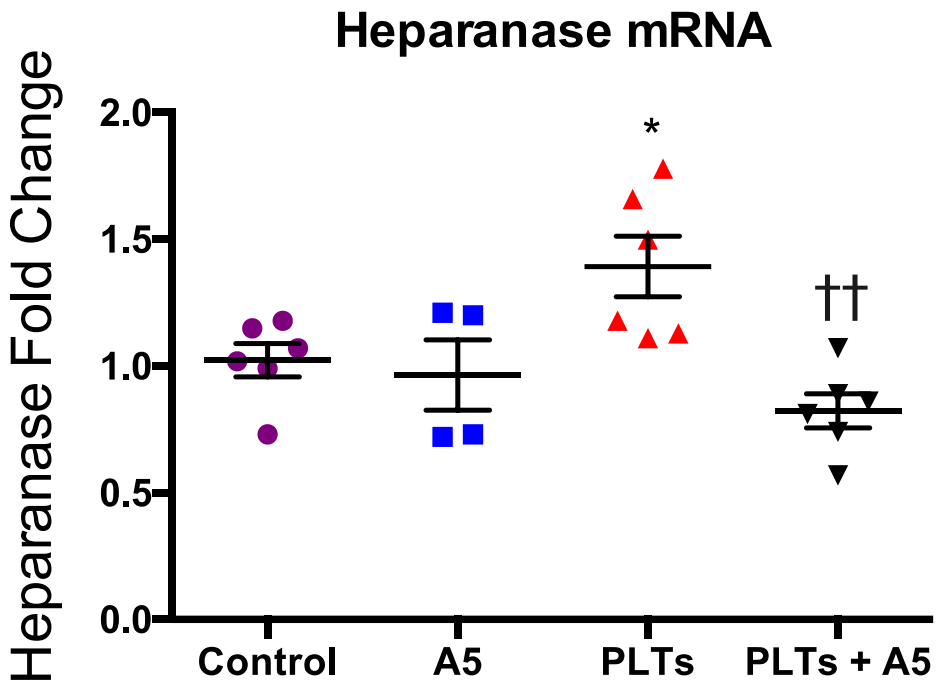


Figure 3.6 Effects of annexin A5 on heparanase mRNA expression induced by LPS-activated platelets (PLTs) in cultured microvascular endothelial cells. PLTs were isolated from mouse blood and incubated with LPS (1 µg/ml) *in vitro* for 4 hours and washed. The cultured microvascular endothelial cells were challenged with LPS-activated PLTs in the absence or presence of annexin A5 (1 µg/ml). Heparanase mRNA levels were assessed by RT-qPCR analysis. Data are mean± SEM, and were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. n=4-6 of independent cultures per group. *$p<0.05$ vs control. †† $p<0.01$ vs. PLTs.

3.7 Effects of Annexin A5 on LPS-induced Monocyte Adhesion to Endothelial Cells

To complement previous findings in this study showing that annexin A5 can reduce adhesion molecule expression (Figures 3.3 and 3.4), a monocyte adhesion assay was completed. Leukocyte adhesion is increased in septic patients when compared to non-septic patients and those with higher leukocyte adhesion to the endothelial cells show a higher death rate (Fabian-Jessing *et al.,* 2018).

In the present study, LPS-induced monocyte adhesion to cultured microvascular endothelial cells was assessed with and without recombinant human annexin A5. The representative images were shown in Figure 3.7.1. Treatment with annexin A5 (1 µg/ml) did not significantly alter monocyte adhesion vs controls. As expected, LPS (1 µg/ml) challenge significantly increased monocyte adhesion to the endothelial monolayer, which was significantly inhibited by annexin A5 (1 µg/ml) treatment, bringing adhesion levels back down to levels comparable to those seen in the control group (Figure 3.7.2).

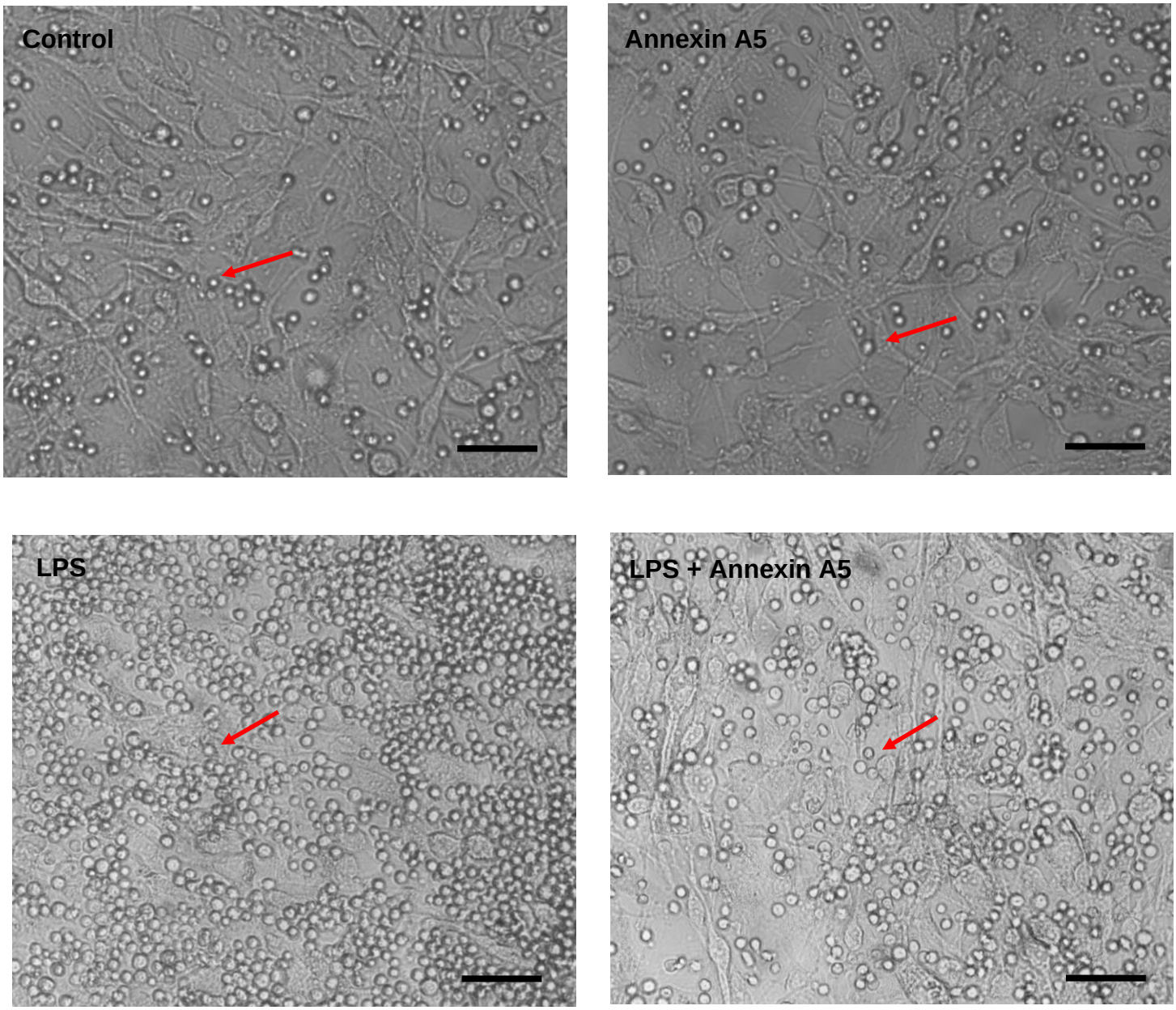


Figure 3.7.1. Representative Images of Monocyte Adhesion on Endothelial Cells. Monocytes were isolated from bone marrow of C57BL/6 mice. LPS/Annexin A5, 1µg/ml treatment for 3 hours prior to 1-hour monocyte treatments. Images were obtained from light microscope. Red arrows pointing to monocytes. Scale bar= 50 µm.

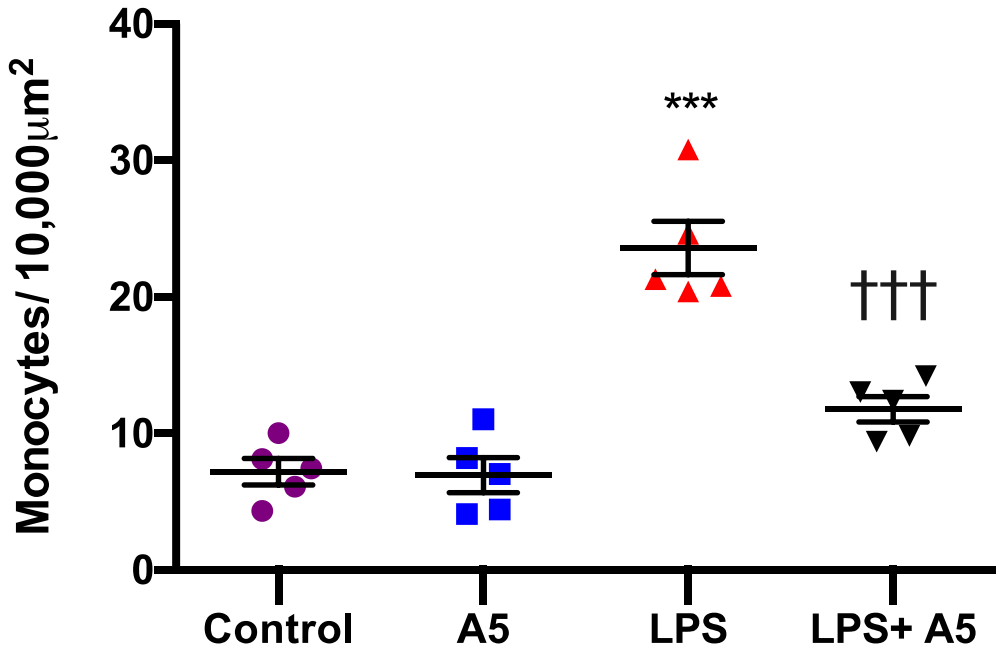


Figure 3.7.2. Quantification of Adhered Monocytes on Endothelial Cells. Quantification was achieved by counting adhered monocytes in randomly chosen 100µm x 100µm area of endothelium. One-way ANOVA followed by Tukey's Post Hoc test was used for analysis. Data are mean± SEM, and were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. n=5 of independent cultures per group. ***p<0.001 vs control. ††† p<0.001 vs. LPS.

3.8 Effects of Annexin A5 on LPS-induced Platelet Adhesion to Endothelial Cells

To provide a more complete picture of the adhesive properties of endothelial cells in a septic response, platelet adhesion was also investigated. In the present study, LPS-induced platelet adhesion to cultured microvascular endothelial cells was assessed with and without recombinant human annexin A5. Representative images are shown in Figure 3.8.1. Treatment with annexin A5 (1 μg/ml) did not significantly alter platelet adhesion in controls. As expected, LPS (1 μg/ml) challenge significantly increased platelet adhesion to the endothelial monolayer, which was significantly inhibited by annexin A5 (1 μg/ml) treatment (Figure 3.8.2).

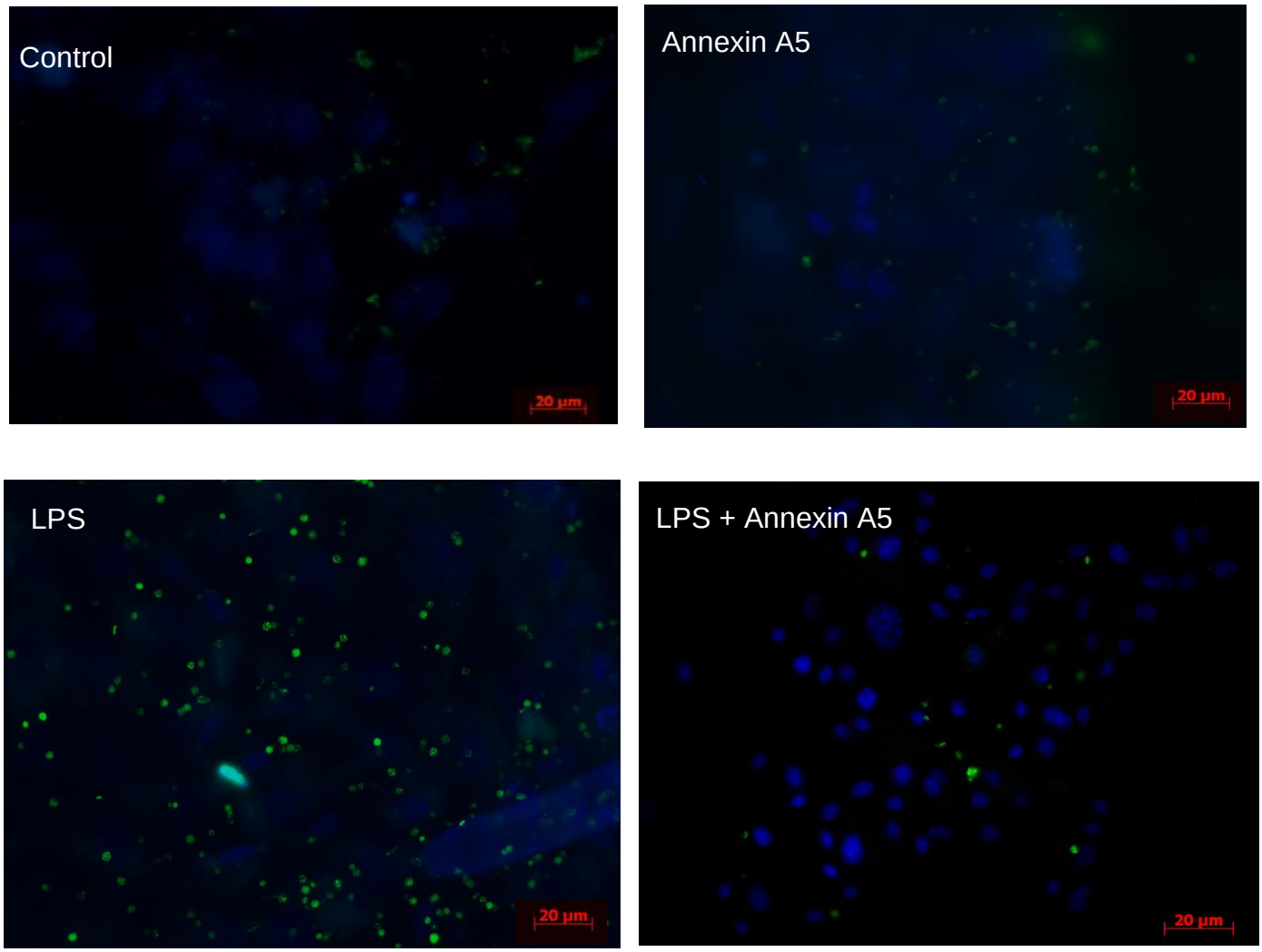


Figure 3.8.1. Representative Images of Platelet Adhesion on Endothelial Cells. Images obtained via florescent microscope. 1µg/ml LPS/Annexin A5 treatment for 3 hours prior to 1-hour platelet treatments. Platelets isolated from WT mice and labelled with calcein AM (labelled in green) and nuclei were stained using Hoechst stain (blue).

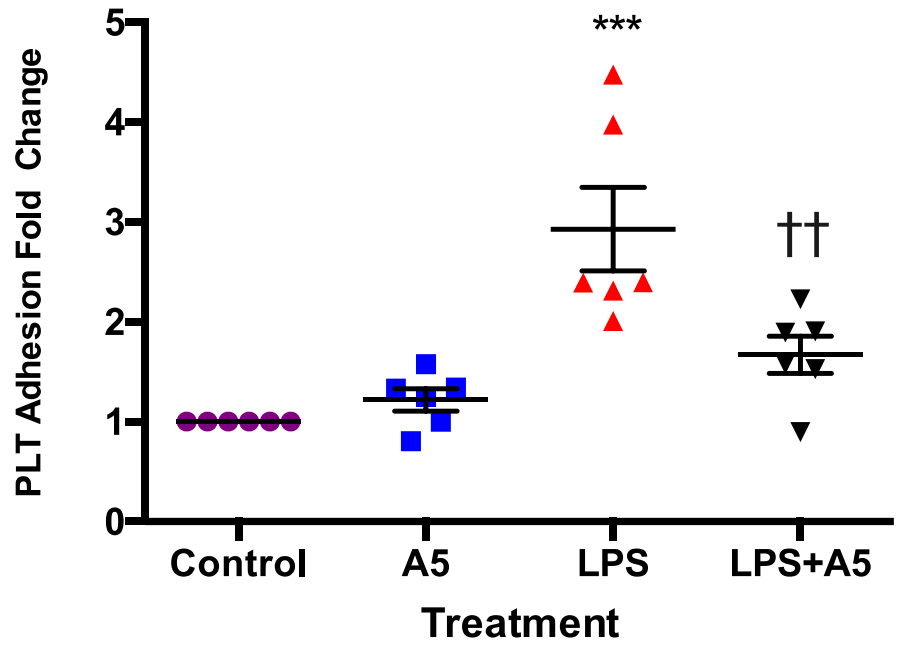


Figure 3.8.2. Effects of Annexin A5 on LPS-induced Platelet Adhesion to Endothelial Cells. Platelets were isolated from WT mice and labelled with calcein AM. Results were normalized to the average platelet count to its replicate's control. Data are mean± SEM, and were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. n=6 of independent cultures per group. *** $p<0.001$ vs control. †† $p<0.01$ vs LPS.

Chapter 4

4. Discussion

4.1 Summary

At the time of the present study, no approved therapeutic is available to treat the underlying septic response (Papafilippou *et al.*, 2021). With that in mind, this study has been conducted at an opportune time in hopes that the results will contribute to the growing literature showing that annexin A5 has the potential to act as a pharmaceutical treatment for the leading cause of death in the world, potentially saving millions of lives. As discussed above, we used LPS-induced endotoxemia as our model of sepsis. Due to the absence of gram-positive bacteria and lack of microbial source, endotoxemia models do not fully recapitulate the human septic response, but it is a useful method to investigate specific pathways involved in the septic response (Lewis *et al.*, 2016).

Our study showed that LPS-activated platelets or EVs significantly increased inflammatory cytokine and adhesion molecule expression of endothelial cells after 4-hour incubation with LPS-activated platelets or EVs. Annexin A5 treatment significantly lowered the expression of inflammatory cytokines and adhesion molecules in the cultured endothelial cells challenged by LPS-activated platelets or EVs.

We next measured the integrity of the endothelial monolayer during the septic response by assessing trans-endothelial electrical resistance. As expected, the septic conditions induced by LPS, LPS-activated platelets, or LPS-activated EVs added onto the endothelial monolayer, all led to marked reductions in the endothelial monolayer resistance, signifying a disruption of the

endothelial monolayer integrity. Annexin A5 treatment significantly increased electrical resistance readings in all three septic conditions.

During a septic response, platelets and monocytes adhere to the endothelial monolayer leading to a wide range of pathological changes in sepsis. To study platelet and monocyte adhesion to endothelial cells, the endothelial monolayer was incubated with LPS for 3 hours prior to addition of platelets or monocytes. Our data has shown that LPS induced significant increases in platelet and monocyte adhesion which was prevented by annexin A5 treatment.

4.2 Analysis of Extracellular Vesicles (EVs)

It is well established that the proportion of platelet derived EVs increases dramatically in conditions of injury or stress (Said *et al.*, 2018). One study has shown that under physiological conditions, 38.5% of EVs were from platelets origins and 43.5% of EVs were endothelial derived, whereas in stimulated states, platelet derived EVs account for ~80% of total EVs (Flaumenhaft *et al.*, 2009). In human samples, 74.7% of EVs were phosphatidylserine positive (annexin $A5^+$) (Suades *et al.*, 2012). Although we saw similar annexin $A5^+$ EVs in our mice samples, our $CD41^+$ levels indicating the EVs are from platelet origin were very low when compared to the literature (Table 3.1). Low $CD41^+$ signalling (~10-12% of EVs in both control and LPS groups) may be in part due to inadequate antibody labelling in which not enough CD41 antibody was loaded into the EV solution. Our nanoflow cytometry data is also limited by the fact that we were unable to quantify EVs less than 180 nm in diameter. Using our data from nanoparticle tracking analysis (Figure 3.1.2) as reference, we see that we are excluding a large portion of our EVs from analysis by excluding the cohort of sample below 180 nm in diameter. The nanoflow analysis has shown that a significantly higher proportion of EVs from LPS stimulated mice were annexin $A5^+$ meaning

that a larger proportion of EVs from LPS stimulated mice possess externalized phosphatidylserine (Figure 3.1). During the process of cell activation phosphatidylserine is externalized (Argañaraz *et al.,* 2020), this phenomenon helps explain the results highlighting that externalized phosphatidylserine levels are higher on EVs isolated from LPS stimulated mice

Transmission electron microscopic images show that our EV samples contained EVs with typical morphologies in which the cell membrane is still intact and the EVs still contain cytoplasmic material (Figure 3.1.1). For electron microscopic analysis the samples were froze, the freeze/thaw process can lyse the membrane of EVs. For all the bellow experiments, freshly isolated EVs were used, further protecting against cell membrane rupture.

Nanoparticle tracking analysis has shown that EVs isolated from control mice and LPS stimulated mice are similar in size (Figure 3.1.2). As discussed earlier, EVs can range in size from 100 -1000 nm (Burger *et al.,* 2013), both our control and LPS stimulated mice had high concentrations of EVs in the 100-210 nm range. This is within the lower limits of potential EV size range. More recent literature has shown that control mice produced EVs that were 197 nm on average and LPS stimulated mice produced EVs that were 157 nm in diameter, on average (Xu *et al.,* 2018).

Previous research has established that septic conditions cause increased plasma EV levels, Xu *et al.* (2018) has shown that cecal ligation and puncture induced sepsis led to a 1.7-fold increase in plasma EVs of mice when compared to sham mice. Similarly, LPS (4 mg/kg) stimulated mice showed ~1.65-fold increase in plasma EV levels through our nanoparticle tracking analysis (Figure 3.1.3).

4.3 Activated Platelets and EVs Enhance Inflammatory Cytokine and Adhesion Molecule Expression

Under normal physiological conditions, negatively charged phosphatidylserine is contained within the inner leaflet of the cell membrane (Kay & Grinstein, 2011). An enzyme known as scramblase is involved in the process of externalizing phosphatidylserine in a calcium-dependent manner (Bevers and Williamson, 2010), and this externalization process occurs during a septic or endotoxic response (Ma *et al.*, 2017). As phosphatidylserine is externalized it creates a pro-inflammatory and pro-coagulant environment on the surface of the cell (Presseizen *et al.*, 2002). In the present study, LPS-activated platelets or EVs significantly increases the expression of inflammatory cytokines (TNFα and IL-6) and adhesion molecules (ICAM-1 and E-Selectin). A common feature of the activated platelets and EVs is the externalization of phosphatidylserine. The witnessed increase in inflammatory cytokines and adhesion molecules is possibly due to the externalization of phosphatidylserine since phosphatidylserine is known to contribute to the hyperinflammatory environment observed in a septic response by activating the "sheddase" activity of a disintegrin and metalloprotease 17 (ADAM17) which is able to cleave pro-inflammatory molecules such as TNFα and IL-6R (Argañaraz *et al.*, 2020). Externalized phosphatidylserine has been shown to modulate tissue factor (TF) activation and initiate the coagulation cascade (Bach *et al.*, 1989). Phosphatidylserine can also increase prothrombin production by creating binding sites with factors Va and Xa. (Zhang *et al.*, 2016).

4.4 Annexin A5 Attenuates Pro-Inflammatory and Pro-Adhesive Effects of Activated Platelets and EVs

There is extensive evidence suggesting annexin A5's ability to bind to phosphatidylserine and to inhibit inflammation (Kenis *et al.,* 2006; Thiagarajan *et al.,* 1991; Arnold *et al.,* 2014). Our goal was to study the effects of annexin A5 on endothelial inflammatory response induced by LPS-activated platelets.

To simulate the *in vivo* setting, cultured endothelial cells were incubated with LPS-activated platelets or EVs in the absence or presence of annexin A5 for 4 hours. Under this condition we found that annexin A5 (1 μg/ml) treatment led to a significantly lower expression of inflammatory cytokines (TNFα and IL-6) and adhesion molecules (ICAM-1 and E-Selectin) when compared to cells incubated with LPS-activated platelets or EVs alone. Just as importantly, we showed that treatment of annexin A5 alone had no effect on endothelial cytokine expression.

Activated platelets release growth factors, chemokines, and cytokines-like factors (Gawaz *et al.,* 2005), as well as TGF- β (Wassmer *et al.,* 2006). TGF- β has been shown to increase expression of TNFα (Wassmer *et al.,* 2006). TNFα secretion can activate NF-κB leading to further chemokine and cytokine production within the endothelial cells (Blaser *et al.,* 2016), as mentioned above. Activated platelets bind to endothelial cells using externalized phosphatidylserine (Tong *et al.,* 2018), with annexin A5 binding, this avenue of binding is inhibited, reducing the activated platelet presence around the endothelial cells, and decreasing endothelial cell and NF-κB activation.

EVs may be eliciting similar effects through internalization into endothelial cells via phosphatidylserine mediated endocytosis. In this case, once internalized the EVs can alter expression of various inflammatory cytokines and adhesion molecules, as shown through liposome

internalization (Cauvi *et al.*, 2019). Binding of annexin A5 to the externalized phosphatidylserine on the EVs may attenuate internalization into endothelial cells.

4.5 Annexin A5 Maintains Endothelial Monolayer Structural Integrity in Septic Conditions

As sepsis develops, the structural integrity of the endothelial monolayer is compromised, the endothelial cells lining the vasculature become leaky, causing fluid and blood cells to escape from the vasculature in a process called extravasation (Granja *et al.*, 2019). Extravasation can lead to ischemic and inflammatory organ damage (Granja *et al.*, 2019). This subsequent organ damage is what leads to death in sepsis patients (Vincent and Moreno, 2000). In this experiment, endothelial cells were challenged with three separate septic conditions; LPS-activated platelets, LPS-activated EVs, or LPS (4 µg/ml) directly onto the endothelial cells with or without annexin A5 (1 µg/ml) treatment.

One of the factors contributing to the increased extravasation during a septic response is shedding of the gel-like layer that lines the endothelial cells called the endothelial glycocalyx (Ince *et al.*, 2016). In our study design, we first wanted to verify that our septic conditions could contribute to reduced structural integrity of the endothelial monolayer. To quantify this, we measure the trans-endothelial electrical resistance of the endothelial monolayer using an EVOM 3 machine with STX2 chopstick electrodes (Florida, USA). In our measurements, the higher the resistance reading, the more intact the endothelial monolayer. We found that all three septic conditions (LPS, LPS-activated platelets, and LPS-activated EVs) all led to dramatic reduction in the endothelial monolayer integrity. We then compared the resistance in these conditions in the absence and presence of annexin A5 (1 µg/ml) for a period of 24 hours. We found that in all three

septic conditions, annexin A5 treatment significantly improved endothelial monolayer resistance at various timepoints throughout the experiment.

It is well-established that during a septic response the endothelial glycocalyx can shed from the endothelial cells, leading to compromised structural integrity and leakage out of the vasculature, ultimately leading to organ failure (Becker *et al.,* 2010). As discussed above (Section 1.6) heparan sulfate makes up the majority of the endothelial glycocalyx and annexin A5 binds to heparan sulfate in a calcium specific manner (Capila *et al.,* 2001). Heparanase is an enzyme that cleaves heparan sulfate in inflammatory conditions, leading to exposed surface selectins on endothelial cells as well as increased extravasation (Iba *et al.,* 2018). Previous research has shown heparanase levels are elevated in inflammatory conditions (Garsen *et al.,* 2016). Our data show that annexin A5 decreased heparanase expression in endothelial cells challenged with LPS-activated platelets (Figure 3.6). However, whether this leads to reduced heparan sulfate cleavage in septic conditions remains to be investigated.

As neutrophils adhere to activated endothelial cells they release superoxide anions, hydrogen peroxide and, and granule enzymes (Varani and Ward, 1994). As these oxidants enter the endothelial cells, they force the cell to contain iron in its reduced form which will combine with neutrophil derived hydrogen peroxide to form a highly reactive and toxic hydroxyl radical. This hydroxyl radical is likely to be a direct mediator of endothelial injury (Varani and Ward, 1994). Zhu *et al.* (2019) has also shown that reducing TNFα and IL-6 expression may serve to protect endothelial cells against apoptosis, and oxidative stress. Thus, the anti-inflammatory properties of annexin A5 shown in Figures 3.3 and 3.4 provide evidence that annexin A5 can serve a protective role for endothelial cells.

4.6 Annexin A5 Reduced Monocyte Adhesion in Septic Conditions

In previous experiments, we have shown that mRNA expression of adhesion molecules is increased in endotoxic conditions and that these increases can be attenuated with annexin A5 supplementation. However, changes in mRNA expression levels do not always equate to changes in protein expression or to functional changes physiologically. In order to test if the changes observed in adhesion molecule expression led to any functional changes in adhesion to the endothelial monolayer, we ran adhesion assays to quantify monocyte and platelet adhesion in various conditions.

During the septic response, the endothelial glycocalyx sheds due to inflammation, leading to exposure of adhesion molecules on endothelial cells allowing leukocytes to bind (Ince *et al.,* 2016). Increased inflammatory cytokines secretion, as seen in sepsis, can lead to increased ICAM-1 exposure on endothelial cells (Scheller *et al.,* 2011). Exposed adhesion molecules on the endothelium initiate leukocyte adhesion which can ultimately lead to leukocyte infiltration into the tissue. Once white blood cells transmigrate into the local tissue, they release inflammatory cytokines and reactive molecules that aid in killing the pathogen, while at the same time raise the risk for tissue death (Ince *et al.,* 2016). The anti-inflammatory effects of annexin A5 may contribute to reduced ICAM-1 expression on the membrane of endothelial cells. Physiologically, reduced monocyte adhesion to the endothelium can prevent tissue inflammation and sepsis-related organ damage.

4.7 Annexin A5 Reduced Platelet Adhesion in Septic Conditions

Platelet adhesion to the endothelium during a septic response contributes to "immune thrombocytopenia" (platelet count <150x10^9/ dL blood). (Vardon-Bounces *et al.*, 2019). Immune thrombocytopenia is when platelets are destructed and the production of new platelets is inhibited leading to high risk of bleeding (Cuker and Cines, 2013). Severe drops in platelet counts can partly be attributed to increased adhesion of activated platelets to endothelial cells leading to hemophagocytosis (phagocytosis of platelet progenitor cells by mononuclear cells) (Levi & van der Poll, 2017). As platelet count decreases in a septic patient the severity of prognosis increases (Levi & van der Poll, 2017), this highlights the importance of controlling coagulation and platelet adhesion to the endothelium during sepsis.

Annexin A5's ability to bind to the externalized phosphatidylserine on platelets may be partly responsible for the decreased adhesion seen in our experiment shown above. Also, similar to the results described in our monocyte adhesion assay, platelet adhesion in the LPS challenged cells was significantly higher than adhesion in all other conditions. Once again, cells treated with annexin A5 showed no difference in adhesion compared to the control groups, but significantly attenuated platelet adhesion induced by LPS. In tandem, these adhesion experiments add validity to our early experiments in which we showed annexin A5's ability to reduce adhesion molecule expression in endotoxic conditions.

4.8 Annexin A5 as a Potential Therapeutic in COVID-19

The COVID-19 pandemic has become a worldwide issue, with more than 267 million cases and around 5.3 million deaths related to the virus across 224 countries, as of December 8th, 2021 (COVID Live Update, 2021). Although viral and bacterial sepsis can employ different

mechanisms, there are many parallels in inflammation and coagulation leading to multi-organ failure (DeMerle *et al.,* 2021). Elevated IL-6 levels have been discovered to be a strong predictor of mortality and lung damage in COVID-19 patients (Levi *et al.,* 2020). Annexin A5 has been shown to reduce IL-6 expression in hyperinflammatory conditions as well as reduction of IL-1β expression in macrophages (Sanches *et al.,* 2020) potentially leading to protection against pyroptosis (Figure 4.8).

Also contributing to poorer prognosis in COVID-19 is venous thrombosis, elevated coagulation and disseminated intravascular coagulopathy (DIC) (Levi *et al.,* 2020). The anti-coagulant properties of heparin have been shown to provide survival benefit (Magro, 2020), we expect the anti-inflammatory and anti-coagulant activity of annexin A5 will also be able to provide benefit.

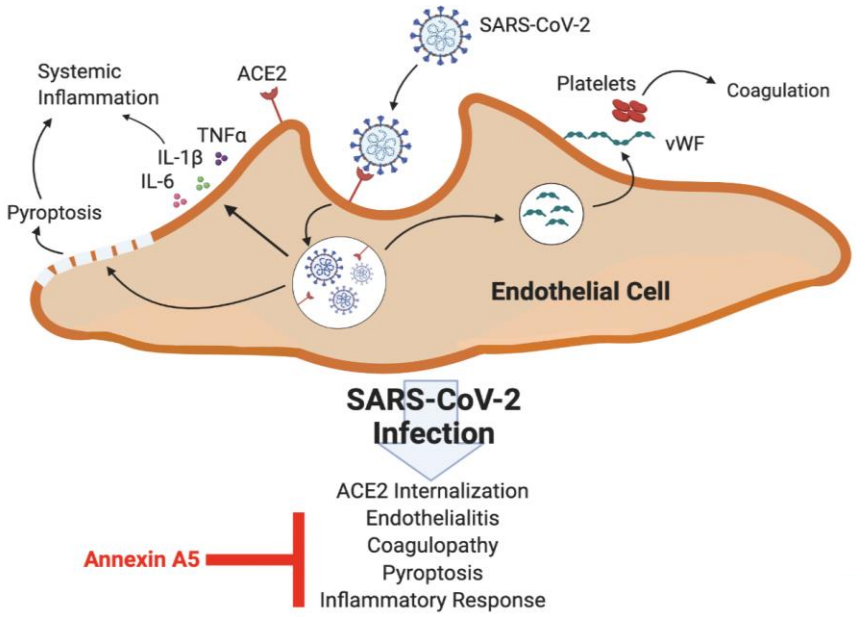


Figure 4.8. SARS CoV-2 Infection Pathways in endothelial cells. SARS-CoV-2 infection induces endothelialitis, coagulopathy, pyropotosis and inflammation, which may be inhibited by annexin A5 treatment. Modified from Mui, et al. (2021). *Frontiers in Pharmacology, 12,* 2377. Created in BioRender.com.

A randomized, doubled-blinded and placebo-controlled phase 2 clinical trial is currently underway to evaluate the safety, efficacy, and pharmacokinetics of recombinant human annexin A5 in critically ill COVID-19 patients with sepsis in London Health Sciences Centre (NCT04748757).

4.9 Future Directions

4.9.1 Investigate annexin A5's role in endothelial glycocalyx preservation in septic conditions.

We would like to provide some explanation for our results shown above. We have shown that in septic conditions, endothelial monolayers treated with annexin A5 show significantly higher electrical resistance (maintained structural integrity) when compared to groups without annexin A5 (figure 3.5.1-3.5.3). We have also shown that annexin A5 treated endothelial monolayers have significantly less adhered monocytes in septic conditions when compared to endothelial monolayers that were not treated with annexin A5. The endothelial glycocalyx, especially heparan sulfate, may be playing a significant role in the vascular pathogenesis of sepsis. In pro-inflammatory environments the endothelial glycocalyx can shed, contributing to increased vascular permeability and increased exposure of endothelial adhesion molecules (selectins) (Ince *et al.,* 2016). Heparan sulfate shedding during inflammatory diseases such as sepsis, leads to increased vascular permeability and neutrophil adhesion to the endothelium and protecting against heparan sulfate shedding has been shown to reverse these adverse effects (Garsen *et al.,* 2015; Kiyan *et al.,* 2019).

As mentioned above, heparan sulfate can be selectively bound by annexin A5 (Iba & Levy, 2019). Thus, we want to investigate if annexin A5 can attenuate heparan sulfate shedding.

In order to test annexin A5's ability to attenuate heparan sulfate shedding, we plan to challenge mice with LPS *in vivo* for four hours to induce endotoxemia with or without annexin A5 (10 μg/kg, i.v.) treatment prior to plasma isolation to measure heparan sulfate levels using ELISA.

4.9.2 Investigate Annexin A5's Effects on EV Clearance

Previous research has shown that annexin A5 treated EVs are taken up by THP1 immune cells at a higher rate than EVs not coated with annexin A5 (Tontanahal *et al.,* 2021). This increased uptake led to reduced circulating levels of EVs and ultimately a dose dependent delay in disease onset (Tontanahal *et al.,* 2021). Interestingly, Tontanahal, (2021), found that increased uptake was only observed if the EVs were treated with annexin A5 or if the EVs and THP1 cells were pretreated with annexin A5 but not if the THP1 cells alone were pretreated with annexin A5. They have hypothesized that this is due to annexin A5 binding to the externalized phosphatidylserine on the EVs leading to binding with phagocytic cells.

In our study we will treat endothelial cells with LPS for 3-hours (as in the adhesion experiment) followed by addition of tdTomato labelled EVs for 3-hours. In our EV treatment groups, one group will be supplemented with annexin A5 (1 μg/ml), whereas the other group will contain only EVs. We expect to see more uptake of EVs shown by greater tdTomato signal in the endothelial cells treated with EVs alone, partially explaining the decreased inflammatory cytokine expression in annexin A5 treated endothelial cells (Figure 3.4).

4.10 Study Limitations

In this study, RT-qPCR was used to characterize the endotoxic response to LPS stimulation and to measure the anti-inflammatory and anti-adhesion effects of annexin A5. With the knowledge that mRNA expression does not always translate to protein expression, activity, or physiological changes within an organism. In order to address this shortcoming, we followed up much of our mRNA expression data with functional assays, for example we followed up our adhesion molecule expression data (Figures 3.3 and 3.4) with functional measures of monocyte and platelet adhesion (Figures 3.7.2 and 3.8.2, respectively).

As discussed in section 1.1, sepsis can arise from many sources, including bacterial, fungal, or viral, with around one third of sepsis cases originating from gram-negative bacteria (van der Poll & Opal., 2008). In this study, LPS was used to induce endotoxemia. LPS was used in our study for several reasons, first off, the use of LPS allowed us to induce a very controlled and consistent endotoxic response as we have established standard doses throughout my experimentation. LPS also activates platelets, EVs and endothelial cells in an *in vitro* setting through addition of 1 μg/ml doses into cell culture media or PBS suspension. However, endotoxemia models of sepsis via LPS stimulation causes high, and rapid increases in cytokines which differs from a human sepsis response, thus, the endotoxemia response often lacks some of the complexities seen in human responses to septic conditions (Dejager *et al.*, 2011). For the purposes of focusing on the pro-inflammatory and procoagulant nature of a septic response, we decided LPS stimulation was suitable to investigate annexin A5's effects in isolated pathways and responses. Opposed to cecal ligation and puncture (CLP) which provides a more accurate representation of a human sepsis response but can be much more inconsistent in severity depending

on the surgical procedure (Dejager *et al.,* 2011) and does not allow for *in vitro* activation assays as performed in this study.

4.11 Conclusion

Sepsis is the leading cause of mortality globally, accounting for 11 million deaths in 2017. To date, no therapeutics are available to treat the underlying septic response. We hypothesized that annexin A5 will block the pro-inflammatory response induced by the activated platelets and EVs in vascular endothelial cells under septic conditions. We showed that treatment with annexin A5 lowered expression of inflammatory cytokines and adhesion molecules induced by LPS-activated platelets or EVs in endothelial cells. Furthermore, annexin A5 treatment improved endothelial cell structural integrity and reduced monocyte and platelet adhesion to endothelial cells in septic conditions. We have now begun to characterize EV trafficking into endothelial cells to get a better idea of the role of annexin A5 in EV clearance during sepsis.

Our study shows that annexin A5 inhibits endothelial cell inflammation in septic conditions, suggesting its potential as a treatment for sepsis. Severe COVID-19 patients develop sepsis. A phase 2 clinical trial on the effects of annexin A5 in critically ill COVID-19 patients with sepsis is currently underway at London Health Sciences Centre. Results from this research, as well as this trial may help us better understand the role of annexin A5 in sepsis and COVID-19 as well as provide a better treatment option for the leading cause of death globally.

Chapter 5

5. Supplementary Data

5.1 Isolectin B4 Labelled Endothelial Cells

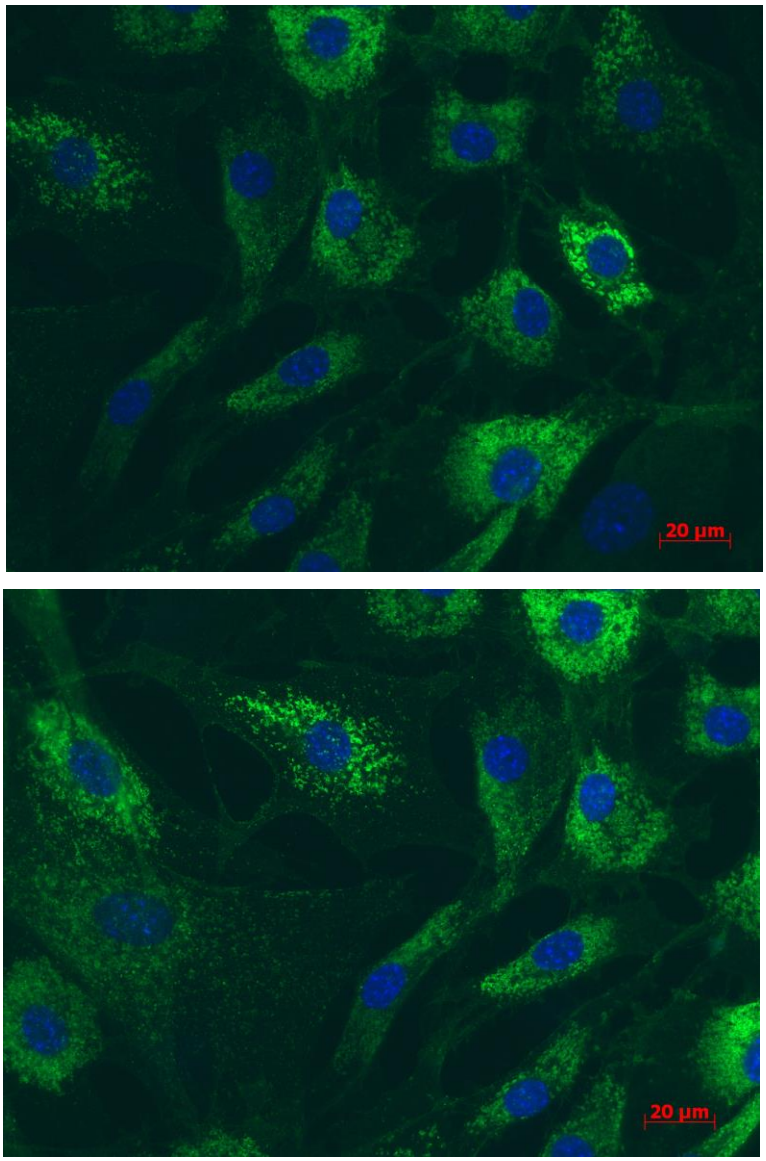


Figure 5.1. Fluorescein labelled isolectin B4. (Vector, Burlington, Ontario) used to label the glycocalyx of endothelial cells (green). Isolectin B4 was added to PBS suspended, PFA fixed cells overnight. Hoechst stain was used to label nuclei (blue). Hoechst stain was added to cells for 10 minutes.

5.2 Calcein AM-Labelled Platelets

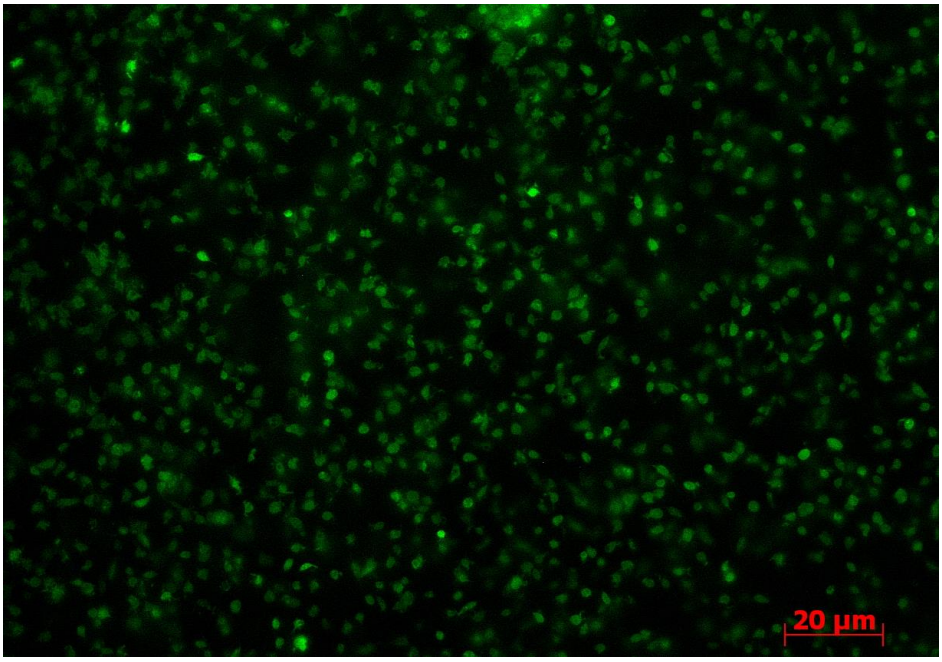


Figure 5.2. Platelets isolated from WT mice and labelled with calcein AM. (Thermo Scientific, Waltham, United States). Non-fluorescent calcein AM is converted into green florescence by live cell through acetoxymethyl ester hydrolysis by intracellular esterases.

5.3 EVs Isolated from tdTomato-Labelled mTmG Transgenic Mouse

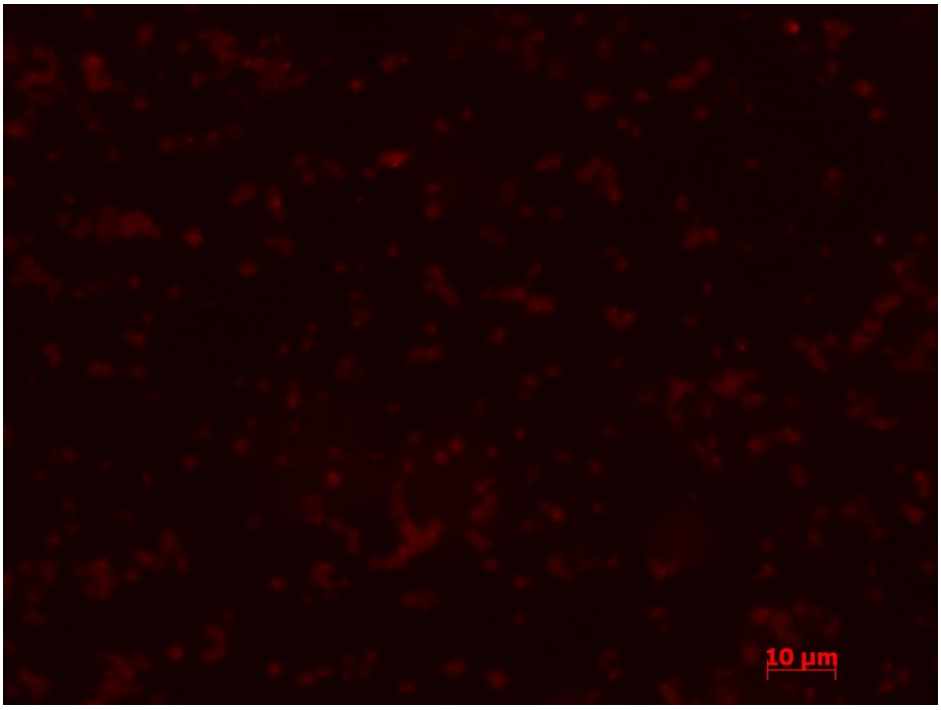


Figure 5.3 tdTomato-labelled EVs. EVs isolated from tdTomato-labelled mTmG transgenic mouse and viewed under fluorescent microscope to confirm positive red florescent signal before endothelial cell treatment.

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Appendix

Animal Use Protocol

Qingping Feng

From: eSirius3GWebServer <esirius3g@uwo.ca>
Sent: March 17, 2021 1:43 PM
To: Qingping Feng; eSirius 3G Administration
Cc: ACC Office
Subject: eSirius3G Notification -- 2020-128 New Protocol Approved

Western

AUP Number: 2020-128
PI Name: Feng, Qingping
AUP Title: Modulation of Myocardial Function in Myocardial Infarction, Sepsis and Diabetes
Approval Date: 03/01/2021

Official Notice of Animal Care Committee (ACC) Approval:

Your new Animal Use Protocol (AUP) 2020-128:1: entitled " Modulation of Myocardial Function in Myocardial Infarction, Sepsis and Diabetes" has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1. This Animal Use Protocol is in compliance with:
    - Western's Senate MAPP 7.12 [PDF]; and
    - Applicable Animal Care Committee policies and procedures.
2. Prior to initiating any study-related activities—as per institutional OH&S policies—all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have:
    - Completed the appropriate institutional OH&S training;
    - Completed the appropriate facility-level training; and
    - Reviewed related (M)SDS Sheets.

Submitted by: Copeman, Laura on behalf of the Animal Care Committee

Curriculum Vitae

Name:	Brent Jeffrey Tschirhart
Post-Secondary Education and Degrees:	University of Western Ontario London Ontario, Canada 2015-2019, BHSc.
Honours and Awards:	Western University Entrance Scholarship 2015
	Deans Honour List 2015-2019
	University of Western Ontario, BHSc Graduate with Distinction 2019
	Western Graduate Research Scholarship 2019-2021
	CSPT- Canadian Journal of Physiology and Pharmacology, Ken Piafsky Award 2021
	CSPT-ASPET Conference Travel Award 2021
	Malcolm Arnold Presentation Award, Department of Physiology and Pharmacology Research Day 2021
Related Work Experience	Research Volunteer 2018-2019
	Research Assistant University of Western Ontario 2019-2021
	Teaching Assistant- PHYS 2130 University of Western Ontario 2019-2021

Academic Courses/ Teaching Experiences

Molecular Techniques (9550)
2019

Communications and Critical Thinking (9551)
2020

Basic Knowledge in Physiology and Pharmacology (Online Modules)
2020

Animal Handling and Care (Online and in-person)
2020

IP/SQ Injection Training (Online and in-person)
2020

Department of Physiology and Pharmacology Tutor
2019-2021

Physiology 4980 Thesis Student Supervisor
2020-2021/2021-2022

Phys/Pharm Department Student Mentor
2020-2021/2021-2022

Publications

Tschirhart, B. J., & Ma, X. T. (2020). A closer look at physiological indicators of cardiovascular function post-transplantation. *The Journal of Physiology, 599*(4), 1031–1032. https://doi.org/10.1113/JP280523

Mui, L., Martin, C. M., Tschirhart, B. J., & Feng, Q. (2021). Therapeutic Potential of Annexins in Sepsis and COVID-19. *Frontiers in Pharmacology, 12*, 2377. https://doi.org/10.3389/fphar.2021.735472

Abstract 14208: Annexin A5 Inhibits Endothelial Inflammation Induced by Lipopolysaccharide-Activated Platelets and Extracellular Vesicles | *Circulation*. (2021). https://www.ahajournals.org/doi/abs/10.1161/circ.144.suppl_1.14208

Presentations:

The Department of Physiology and Pharmacology Research Day, London, ON, 2019.
Effects of Annexin A5 on Endothelial Inflammation Induced by Lipopolysaccharide-Activated Platelets and Microparticles in Sepsis.

Canadian Society of Pharmacology and Therapeutics Annual Conference, Poster. 2020.
Effects of Annexin A5 on Endothelial Inflammation Induced by Lipopolysaccharide-Activated Platelets and Microparticles in Sepsis.

The Department of Physiology and Pharmacology Research Day, London, ON, 2020.
Effects of Annexin A5 on Endothelial Inflammation Induced by Lipopolysaccharide-Activated Platelets and Microparticles in Sepsis.

London Health Research Day, London, ON, 2021.
Effects of Annexin A5 on Endothelial Inflammation Induced by Lipopolysaccharide-Activated Platelets and Microparticles in Sepsis.

Canadian Society of Pharmacology and Therapeutics Annual Conference, Oral. 2021.
Effects of Annexin A5 on Endothelial Inflammation Induced by Lipopolysaccharide-Activated Platelets and Microparticles.

The Department of Physiology and Pharmacology Research Day, London, ON, 2021.
Effects of Annexin A5 on Endothelial Inflammation Induced by Lipopolysaccharide-Activated Platelets and Extracellular Vesicles.

American Heart Association Annual Scientific Session, Boston, MA, 2021 → Online.
Effects of Annexin A5 on Endothelial Inflammation Induced by Lipopolysaccharide-Activated Platelets and Extracellular Vesicles.