Role of platelets during innate immunity to *Streptococcus pneumoniae* pneumonia

Kristoffer Dreimanis

Supervisor: Stina Ekman, professor, DVM, Dipl. ECVP
Department of biomedicine & Veterinary public health
division of pathology, pharmacology & toxicology
Assistant supervisor: James D. Moffatt
Lecturer in pharmacology Veterinary Basic Sciences
The Royal Veterinary College

Sveriges lantbruksuniversitet
Fakulteten för veterinärmedicin och husdjursvetenskap
Veterinärprogrammet

Examensarbete 2008:23
ISSN 1652-8697
Uppsala 2008

Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Anmial Sciences
Veterinary Medicine Programme

Degree project 2008:23
ISSN 1652-8697
Uppsala 2008

“I declare that this work has been composed entirely by myself based on my own data. Where I have mentioned materials or results generated by fellow researchers, I have acknowledged them appropriately”

ACKNOWLEDGMENTS

I wish to thank:

My supervisors: Dr Jeremy Brown and Dr James Moffatt for making it all possible and critically reviewing my manuscript. I especially wish to thank them both for taking my ideas seriously!

Dr José Yuste for technical support with all animal work!

Staff at The Centre for Respiratory Research!

Dr Rebecca Lever and Dr Simon Pitchfold for supplying blood and antibodies!

Dr Dirk Werling course director and Lisa Harber for always being there!

Professor Stina Ekman, Mr Ronny Fransson-Steen and especially AstraZeneca Sweden for giving Swedish students this opportunity and together with Wellcome Trust funding this year!

Royal Veterinary College

AstraZeneca, Sweden

Centre for Respiratory Research

Wellcome Trust

Swedish University of Agricultural Sciences, SLU

TABLE OF CONTENTS

SAMMANFATTNING

Trombocytopeni är en vanlig företeelse i samband med svår infektion och graden av trombocytopeni hos akut sjuka patienter har visats vara korrelerad till klinisk prognos. Detta till trots är patofysiologin till detta fenomen ännu inte helt kartlagd. Det senaste årtiondets forskning har visat att trombocyter utöver sina uppgifter i koagulationen även äger flera antimikrobiska egenskaper vilket väckt frågan om trombocyter också spelar en ännu okänd roll i det cellmedierade immunförsvaret. Det har även visats att trombocyter som utsätts för stimuli som lipopolysaccharider (LPS) och septikemi sequestrerar ut i lungvävnad *in vivo*. *Streptococcus pneumoniae* är en vanlig etiologisk orsak till förvärvad pneumoni och septikemi. I detta arbete visar jag att trombocyter *in vitro* blir aktiverade av *S. pneumoniae* och frigör antimikrobiska substanser mot *S. pneumoniae* efter trombin stimulering, detta utan interaktioner från andra celler. *In vivo* visar jag att trombocytopeni under infektion leder till en snabbare bakteriell tillväxt i lungorna på möss i en modell av *S. pneumoniae* infektion. Histopatologisk undersökning av dessa lungor indikerar att trombocyter samlokaliserar med bakterier –och därmed eventuellt fångar bakterier i lungvävnad innan kolonisation av vävnad hinner ske. Slutsatsen är att trombocyter medverkar i det cellmedierade immunförsvaret mot *S. pneumoniae* pneumoni vilket potentiellt kan utgöra ett terapeutiskt mål för patienter med svår infektion.

ABSTRACT

Thrombocytopenia is a common feature in severe infection and the extent of the thrombocytopenia has been correlated to the clinical prognosis. However the pathophysiology behind this phenomenon is not completely understood. Research in the last decade has shown that thrombocytes possess several antimicrobial properties, suggesting that they might play a still unknown role in innate immunity. Moreover platelets are known to sequestrate out into lung tissue in response to stimuli like lipopolysaccharide (LPS) and septicaemia. *Streptococcus pneumoniae* is a common cause of severe community aquired pneumonia and septicaemia. In this project I show that platelets *in vitro* are activated by *S. pneumoniae* and can release antimicrobial peptides in response to thrombin. This occurs independent of interactions with other cells. *In vivo*, I show that platelet deficiency results in increased bacterial numbers within the lungs in a mouse model of *S. pneumoniae* pneumonia. Histopathological examination of the lungs indicates that platelets co-localise with the bacteria –and may "trap" them within lung tissue. I conclude that platelets contribute to innate immunity to *S. pneumoniae* pneumonia identifying a potential therapeutic target for patients with severe infection.

ABBREVIATIONS

APC	activated protein C
AT	antithrombin
ADP	adenosine diphosphate
BAL	broncoalveolar lavage
BALF	broncoalveolar lavage fluid
cfu	colony forming unit
dH_2O	distilled water
g	gram
GP	glycoprotein
IL	interleukin
i.p.	intraperitoneally
μ	micro- (suffix)
m	milli- (suffix)
M	molar
n	nano- (suffix)
μl	microlitre
pH	-log[H+]
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate-buffered saline
PPP	platelet poor plasma
PRP	platelet rich plasma
TAT	thrombin-antithrombin complex
TBS	Tris-buffered saline
TFPI	tissue factor pathway inhibitor
Tf	tissue factor
TF	TFLLR-NH_3 a PAR_1 agonist
TLR	toll like receptor
TM	thrombomodulin
tPA	tissue plasminogen activator
TXA_2	thromboxane A_2
vWF	von Willebrand factor

1. INTRODUCTION

1.1 Platelets

The first known description of platelets[1] is from 1865 by Max Johann Sigismund Schultze (Sigismund, 1865) and the clotting properties of platelets were discovered in the late 19th century (Bizzozero, 1882). Platelets are the smallest (2-5 μm in diameter) of the blood cells. In a normal person they range in number between 1.5–4 x 10^8/ml blood compared to the normal range of leukocytes of 4-11 x 10^6/ml blood. The average lifespan of platelets differ between mammals but normally range between 5-7 days in humans (McMichael, 2005). Platelets are produced in the bone marrow from megakaryocytes in response to thrombopoetin, produced in the liver and kidneys, and also in response to some interleukins (IL-3, IL-6, IL-11) (B. F. Feldman *et al.,* 2000 pp 446; Nemi, 1993 pp 115-116).
Thrombopoietin stimulates platelet progenitor cells (megakaryocytes) mainly by increasing the viability, the rate of differentiation and by stimulating endomitosis, resulting in increased ploidy and cell volume (Kaushansky, 1995; Nemi, 1993 pp 116). Thrombopoietin also stimulates megakaryocytes to release proplatelets that fragment into platelets. Free thrombopoietin levels in serum are determined by platelet mass (Li *et al.*, 1999). Thrombopoietin is bound by high affinity receptors (c-mpl) and cleared by platelets. In normal conditions the serum level of thrombopoetin is low. However when platelet count goes down the amount of c-mpl receptors decreases and free thrombopoetin levels in plasma rise acting as a stimuli to megakaryocytes and platelet production (B. F. Feldman *et al.,* 2000 pp 72; Kaushansky, 1995).
There are thought to be two rapidly mobilizable pools of platelets in humans and animals. One is in the spleen and the other still remains unknown. The dual pool thesis is based on: i) an increase in platelet numbers after epinephrine injection in normal individuals but not after splenectomy ii) after mild exercise a spleen independent increase of platelet-count is seen. From where this latter increase is mobilized is not known but the lung, liver, heart and bone marrow are proposed sources (Nemi, 1993 pp 116). The spleen pool of platelets might explain thrombocytosis post splenectomy as their loss leads to reduced binding of thrombopoetin. This results in elevated levels of thrombopoeitin that promotes megakaryocyte and platelet production. A decrease in an unknown megakaryocyte inhibitor produced by the spleen is another possible cause (Nemi, 1993 pp 116). Senescent or damage platelets are phagocytosed by the mononuclear phagocyte system mainly in the spleen and liver but also in the bone marrow (Nemi, 1993 pp 116).

1.2 Platelet structure

Platelets are anuclear and when inactive are discoid shaped cells. They contain RNA, mitocondria, α-granula, dense bodies, endoplasmic reticulum, golgi apparatus, actin and myosin molecules and an open canicular system (consisting of membrane invaginations). The larger α-granula are exocytosed from activated

[1] The terms “platelet” and “thrombocyte” are, in this project, used interchangeably although originally thrombocyte was introduced to describe the nucleated cell of lower vertebrates, the counterpart of the non-nucleated mammalian platelet (Meseguer *et al.*, 2002).

platelets and contain: coagulation factors (albumin, fibrinogen, factor V, von Willebrand factor [vWF]), platelet-specific proteins (β-thromboglobulin and platelet factor 4) growth factors (platlet-derived growth factor [PDGF], transforming growth factor β [TGF-β], epidermal growth factor [EGF] and endothelial cell growth factor [ECGF]), thrombospondin and fibronectin. The smaller dense bodies are also exocytosed in response to activation and contain ADP, ATP, serotonin, magnesium and calcium (B. F. Feldman *et al.*, 2000 pp 451; Thrall *et al*., 2004 pp 183). On the surface platelets express integrins made up of subunits α_2, α_5, α_6, β_1 and β_3. Glycoprotein IIb/IIIa **(**GPIIb/IIIa consisting of integrin $\alpha_{IIb}\beta_3$**)** is a megakaryocyte cell line specific receptor for fibrinogen, which is important for the aggregation of platelets and the formation of platelet plugs. Platelets also express a leucine-rich glycoprotein family, which mainly consist of the GP Ib-IX-V complex and is important during early hemostasis and platelet adhesion to damaged endothelia by binding to vWF. P-selectin is an α-granula membrane protein that is only expressed on the platelet surface after α-granula secretion. Hence P-selectin can be used as a marker of platelet activation (B. F. Feldman *et al.*, 2000 pp448-451). The platelet cytoskeleton consists of actin filaments that, in association with, myosin regulates platelet shape, extension of pseudopodia, platelet aggregation and secretion of granula (Guyton & Hall, 2006 pp 457-458).

1.3 Platelet function

1.3.1 Normal condition

Inactivated platelets are prevented from adhering to normal endothelium through four processes: i) electronegative charges on both the endothelium and platelet membranes repel one another ii) constant production of nitric oxide (NO) from endothelia down regulates free cytoplasmic Ca^{2+} in platelets and make them less prone to adhere iii) release of endothelial prostaglandin (PGI_2) in response to platelet agonists, which reduces expression of GPIIb/IIIa, decreases intracellular Ca^{2+} concentration and acts as a vasodilator (B. F. Feldman *et al.*, 2000 pp 457; McMichael, 2005)and iv) endothelial cell expression of substances like ADPases, thrombomodulin, heparan sulfate that inhibit platelet agonist functions (McMichael, 2005; Ruggeri, 2003).

1.3.2 Hemostasis

Hemostasis according to Saunders Comprehensive Veterinary dictionary 3rd edition (D.C. Blood, V.P. Studdert, C.C. Gay) is defined as the "arrest of the escape of blood by either natural (clot formation or vessel spasm) or artificial (compression or ligation) means…". Natural hemostasis involves several steps: 1) vascular constriction; 2) primary hemostasis (platelet clot formation); 3) secondary hemostasis (reinforcement of platelet clot by crosslinked fibrin); 4) endothelial regrowth or fibrous sealing of damage vessels (Guyton & Hall, 2006 pp.457). Vessel contraction immediately after trauma results from local myogenic spasm, nervous reflexes and contractile factors such as thromboxane A_2 (TXA_2) released from damaged endothelia and activated platelets. This contraction can last for minutes to hours during which primary and secondary hemostasis can take place (Guyton & Hall, 2006 pp 457). Platelets are the first cell to respond to

endothelial damage. In the normal endothelial turnover, platelets play a role in damage repair on a daily basis. Small holes are filled by platelets fusing with endothelial membrane. In patients with platelet deficiencies erythrocytes can be forced out through these holes and petechiae becomes a clinical sign (Guyton & Hall, 2006 pp 458). There are different mechanisms for adhesion of platelets to damage endothelia in low shear and high shear conditions (McMichael, 2005; Savage *et al.*, 1996). In high shear conditions (small and medium sized arteries) the initiation of platelet adhesion mainly occurs through von Willebrand factor (vWF) bound to collagen. vWF binds to exposed subendothelial collagen that makes the vWF molecule stabile and less prone to uncoil. This complex then works as an anchor point for the GP Ibα receptor on the platelets (McMichael, 2005). The binding of platelets to vWF is not irreversible, and has a fast dissociation rate and a short halflife. Together with P-selectin from the endothelia the platelet binding to vWF slows down the platelets under high shear conditions (Ruggeri *et al.*, 1999). The first interaction between GP Ibα and vWF might activate platelets to a certain extent but collagen plays a more important activating role. Activated platelets go through a conformational change and the expression and activity of integrin $\alpha_{IIb}\beta_3$ increases on the cell surface (B. F. Feldman *et al.*, 2000pp 459; McMichael, 2005). The slower moving platelets give the activated integrin $\alpha_{IIb}\beta_3$ time to bind to a second domain on vWF and/or substances in matrix like fibrinogen, fibronectin and vibronectin. This stabilises the platelet adhesion to the damaged endothelium (Ruggeri *et al*., 1999). Activated platelets also release α-granula and dense bodies that increase the activation of surrounding platelets (mainly ADP in high shear condition) (McMichael, 2005). Adhered platelets bind circulating vWF in the plasma via both GP Ib and integrin $\alpha_{IIb}\beta_3$. Together with fibrinogen this makes up a surface to which more circulating platelets can adhere and lead to the formation of a platelet aggregate (McMichael, 2005; Ruggeri, 2003).

In low shear circulation (capillaries and veins) platelets adhere to subendothelium via collagen, fibronectin and laminin. Adhesion occurs mainly through integrin $\alpha_{IIb}\beta_3$. vWF seems to play a less important role under these circumstances both for the initial adhesion and for the platelet aggregation (McMichael, 2005). vWF has other additional procoagulatory properties. In plasma it binds to and protects the important coagulation factor VIII and when vWF binds to collagen fVIII is released and help in maintaining the coagulation cascade (Ruggeri, 2003). Unlike in high shear condition it is TXA_2 that activates surrounding platelets under low shear conditions. Thrombin is a potent activator during both high and low shear conditions (McMichael, 2005).

1.3.3 Coagulation cascade –the forming of fibrin

Tissue factor (Tf) is expressed on the surface of cells delinating organ boundaries like epithelial cells, outer prekeratinised layers of the epidermis, squamous cell epithelia and myoepithelia encapsualating many organs in healthy tissue. One exception is vessel endothelium, that doesn't express Tf, but the tunica adventitia within the vessel does. Tf expression is a defence mechanism making sure that coagulation factor VII gets in contact with Tf in response to damage to the inner and outer boundaries of the body (McVey, 1999).
Tf exposure to blood leads to the contact with and binding of factor VIIa. The Tf-fVIIa complex catalyzes the conversion of factor X to Xa (Levi & van der Poll, 2005; McVey, 1999). Factor Xa converts prothrombin to thrombin. Without activated cofactor (factor Va) factor Xa only stimulates the formation of barely detectable levels of thrombin. However this low level of thrombin is enough for the activation of circulating or platelet derived fV to fVa. FVa and Xa together form the prothrombinase complex that has a much more potent prothrombin converting activity (Price *et al.*, 2004).
Tf-fVIIa also activates fIX to its active form fIXa. FVIII released from vWF becomes activated by thrombin and binds to the formed fIXa. These two factors form the tenase complex (fVIIIa-fIXa) and work together with prothrombinase complex in the activation of fX. Through both pathways enough fXa is formed to induce a thrombin burst at the site of injury (McVey, 1999; Price *et al*., 2004). To underline the importance of these complexes, deficiency of fVIII or fIX both result in severe bleeding disorder, haemophilia A for the former and haemophilia B for the latter. Thrombin activates platelets and converts soluble fibrinogen to insoluble fibrin polymers. Local secretion of fibrinogen from platelet a-granula can contribute to even more local formation of fibrin. The fibrin formed act as a support for the primary clot (B. F. Feldman *et al.*, 2000 pp462).
To work properly both prothrombinase and tenase complexes need to be associated to a surface of negatively charged phospholipids (Monroe & Hoffman, 2006). Very few normal cell membranes express these negatively charge phospholipids on their outer leaflets. However platelets upon activation go through conformational changes and the negatively charged molecules get exported from the inner to the outer leaflet of the membrane (Price *et al*., 2004). Transportation through the membrane requires a strong activator (thrombin or collagen) while a weaker activator (ADP, TXA_2) will not give this kind of conformational shift (Esmon, 2004). Thrombin activated platelets not only support the catalytic surface for the coagulation cascade, they also express plasminogen activator inhibitor (PAI-1). PAI-1 inhibits tissue plasminogen activator (tPA) and thereby inhibits fibrinolysis and gives the fibrin meshwork time to form (Podor *et al.*, 2002).

1.3.4 Regulation of hemostasis

The blood contains enough coagulation factors to convert the fibrinogen to fibrin 100 times over (Esmon, 2004) therefore close regulation of the pro- and anticoagulative forces is vital. The regulatory system is complex and in this chapter there is only room to discuss some of the important regulators associated with platelets (for summary see picture 1.3.1).
vWF is a glycoprotein built up of identical subunits forming large multimeres. Larger multimeres have higher affinity for platelets (Chauhan *et al.*, 2007).

Damaged endothelia release large vWF multimeres from preformed Weibel-Palade bodies (Chauhan *et al.*, 2007; McMichael, 2005). These large multimeres are broken down to smaller multimeres by plasma proteases. Platelet derived thrombospondin-1 is one of these proteases regulating the size of vWF multimeres and thereby the strength of vWF binding to GP Ib and the size of the formed platelet aggregate (Ruggeri *et al*., 1999).

Thrombin has multiple procoagulation functions. It activates coagulation factor V (fVa), factor VIII (fVIIIa) and platelets. Thrombin also converts fibrinogen to fibrin and activates factor XIII (fXIIIa) that crosslinks fibrin. During active coagulation, thrombin levels are elevated and in response to the high thrombin levels endothelial cells upregulate their expression of thrombomodulin (TM). The active site of thrombin binds to TM and thereby loses its procoagulatory properties. This highly reactive thrombin-TM complex then converts plasma protein C to activated protein C (APC). APC cleaves and inactivates fVIIIa and fVa, so providing negative feedback for thrombin formation and activation of the coagulation cascade (Dahlbäck & Villoutreix, 2005). The thrombin-TM complex also forms thrombin activatable fibrinolysis inhibitor (TAFI), which inhibits fibrinolysis and protects the formed fibrin clot (McVey, 1999; Monroe & Hoffman, 2006).

Tissue factor pathway inhibitor (TFPI) is a molecule associated with vascular endothelia, circulating in plasma or released by platelets after stimulation with thrombin. TFPI binds to and inactivates fXa (especially fXa bound to prothrombinase complex). This complex inhibits the formed TF-FVIIa complex and shifts the formation of Xa from the extrinsic to the lower part of the classical intrinsic pathway. This involves activation of fX from the fVIIIa-fIXa complex (Lwaleed & Bass, 2006). *In vivo* TFPI is also bound to heparan sulfate on the endothelial surface and is released in response to heparin injection (McVey, 1999) and might be responsible for some of the anticoagulative effect of heparin (Lwaleed & Bass, 2006). TFPI is a potent inhibitor of TF-fVIIa and thereby inhibits activation of factor X, thrombin formation and the coagulation cascade (Esmon, 2001; McVey, 1999).

Anti thrombin (AT) is formed in the liver and in endothelial cells and binds to and inhibits thrombin. AT and thrombin form Thrombin-AT (TAT) complexes which are cleared by the mononuclear phagocytic system. The formation of TAT complexes is catalyzed by heparin or heparansulfate (HS), which is normally bound to the surface of endothelia cells but is released in areas of injury to form high local levels. AT also has the ability to bind to and inactivate fXa and fIXa in a similar way (B. F. Feldman *et al.,* 2000 pp 538-539).

Central to the fibrinolytic system is the conversion of plasminogen to the active serine protease plasmin, catalyzed by tissue plasminogen activator (tPA). tPA is released from damaged endothelia, and binding to fibrin enhances its activity. Plasmin degrades fibrin and removes formed clots (B. F. Feldman *et al.*, 2000 pp 544-546).

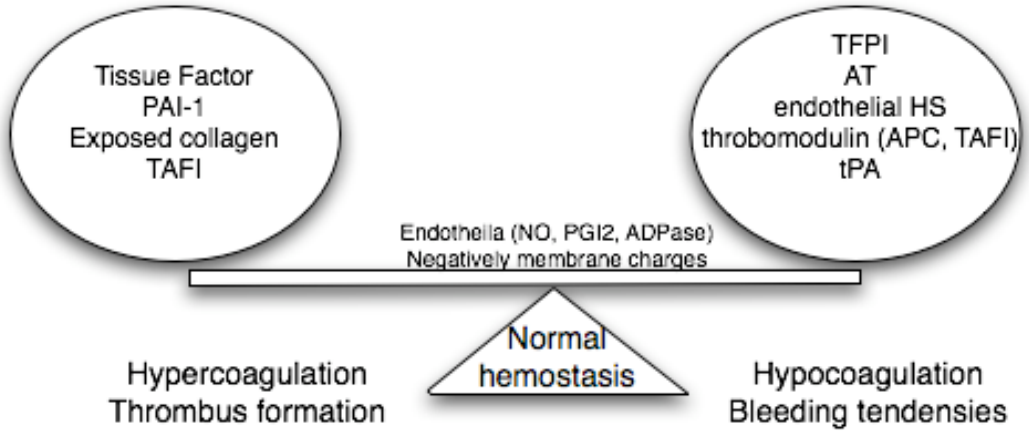


Figure 1.3.1. *Simplified view of some of the mentioned important factors in the balance of hemostasis during normal conditions. Different inhibiting pathways strictly control the fibrin formation.*

1.3.5 Tissue repair

Platelets contain different growth factors that are released upon activation, including PDGF, EGF and TGF-β. At sites of aggregation the levels of these factors can become thousand times greater than the circulating plasma concentration. PDGF and EGF stimulate mitogenesis of smooth muscle cells and fibroblasts (B. F. Feldman *et al.*, 2000 pp 465), whereas TGF-β inhibits proliferation of many cell types including endothelial cells (Giannouli & Kletsas, 2006). TGF-β is therefore thought to play more of a remodelling role of the extracellular matrix and thereby promoting wound healing (B. F. Feldman *et al*., 2000 pp 465).

1.4 Inflammation and coagulation are connected

1.4.1 Platelets activated through inflammation promotes coagulation

Increased proinflammatory cytokines during infections induces the mononuclear cell expression of Tf. IL-6 seems to be the most important of these cytokines as specific inhibition of IL-6 inhibits thrombin formation through this pathway (van der Poll *et al.*, 1994). fVIIa from blood and Tf form tenase and prothrombinase complexes on the surface of activated platelets, which mediate the formation of fibrin (see hemostasis). Platelets become activated by proinflammotory stimuli like PAF and thereby increase the inflammatory stimulation of coagulation. Activated platelets might also increase the expression of Tf on mononuclear cells by adhering through P-selectin onto these cells. This binding can cause Nuclear Factor κB activation and the expression of Tf on the cell surface (Levi & van der Poll, 2005). Endothelial cells also increase the expression of Tf on cell surfaces in response to certain stimuli such as platelet-derived serotonin. Activated platelets releases serotonin, which leads to an increased expression of Tf, which then activates the coagulation cascade and thereby creates thrombin, which in turn activates platelets in a positive feedback mechanism (Kawano *et al.*, 2001). Regulation of this pathway is vital also showed by Tf antibodies inhibiting lethality in a baboon sepsis model *in vivo* (Taylor *et al.*, 1991).

1.4.2 Inflammation interfere with anticoagulant pathways

Inflammation not only enhances coagulation it also affects the anticoagulant pathways. Endothelial function is impaired in inflammation. Thrombomodulin is down regulated from cell surfaces in response to the increased levels of TNF-α and IL-1b (Nawroth & Stern, 1986). A low level of thrombomodulin decreases the levels of activated protein C (APC), which is also confirmed in infectious models *in vivo* (Faust *et al.*, 2001). APC inhibits coagulation through inactivation of fVIIIa and fVa. Depleting animals of APC makes endotoxin models more severe with higher mortality while treatment with APC in baboons challenged with endotoxin protects the animals with a lower mortality and less severe organ dysfunction (Taylor *et al.*, 1987). APC has also gone through medical trials (PROWESS) and is now used in the clinic in treatment of sepsis.

Serum antithrombin levels also decrease in severely infected patients due to negative acute phase response (less production), degradation of elastase from activated neutrophils and, quantitatively most importantly, consumption in the clearing of formed thrombin (Levi *et al.*, 2004). Proinflammatory cytokines can also downregulate synthesis of glycosaminoglycans (GAGs) on endothelial surfaces. Normally these GAGs act, like heparin, as cofactors to antithrombin catalyzing the formation of TAT complexes and thereby facilitating the clearing of thrombin. This function is impaired in systemic inflammation with more active thrombin as a consequence (Kobayashi *et al.*, 1990).

Fibrinolytic proteins like tPA and uPA are released in response to TNF-α and IL-1b from their storage sites in vascular endothelia. The forming of plasmin is increased but overall the fibrinolytic effect stays unaltered due to a delayed but sustained increase in plasminogen activator inhibitor type 1 PAI-1. Fibrinolysis is inhibited in needed areas and a clearance of fibrin is not succeeded giving microvasculature thrombosis (Levi *et al*., 2004).

Inflammation affects the Tf and protein C pathways, thrombin and fibrinolysis leading to a local increase in fibrin formation (Schultz *et al.*, 2006). The effect of inflammation on fibrin formation might cause physiological effect of containing the inflammation/infection locally by trapping pathogens in fibrin. Supporting this many bacteria express or bind host plasmin. Y. pestis, Borrelia, and group A streptococci have shown increased virulence when expressing these fibrinolytic proteins (Lähteenmäki *et al.*, 2001).

1.4.3 Coagulation regulates inflammation

It is well known that inflammatory processes activate coagulation. In systemic inflammation this can be very severe with depletion of coagulation factors and DIC as a consequence. A lesser-known fact is that coagulation also activates inflammation. Coagulation of whole blood results in upregulation of IL-1 mRNA in blood cells (Mileno *et al.*, 1995) and thrombin, factor Xa and fibrin directly stimulate the endothelial and mononuclear cell leading to production of IL-6 and IL-8 (van der Poll *et al.*, 2001). This crosstalk between the two systems seems to be important in many pathological conditions, and especially in systemic inflammatory responses with multi systemic organ failure (Levi & van der Poll, 2005). APC has also been shown to have a binding site on mononuclear cells. Binding of APC to mononuclear cells can inhibit lipopolysaccharide induced NFκB translocation into the nucleus and thereby preventing the production of proinflammatory cytokines and expression of adhesive molecules by mononuclear

cells (White *et al.*, 2000). This could account for some of APCs anti-inflammatory properties.
Thrombin increases the endotoxin induced IL-1 activity by cultured Macrophages. Thrombin, together with fXa and fibrin, directly stimulates mononuclear and endothelial cell to produce IL-6 and IL-8 (van der Poll *et al*., 2001). High thrombin levels also increase the mRNA levels of E-selectin in cultured endothelial cells and promote TNF-α induced E-selectin expression. This gives a surface to which neutrophils can adhere (Levi & van der Poll, 2005). Thrombomudulin-thrombin complex also induces formation of thrombin activatable fibronolytic inhibitor (TAFI) (Bajzar *et al.*, 1996). TAFI has recently been implicated as the main inhibitor of complement c5b and can thereby protect the microvasculature from complement-induced damage (Levi & van der Poll, 2005). Thrombomodulin also inhibits neutrophil adhesion to endothelial cells and impair the migration of neutrophils out from the vessels into the tissue (Van de Wouwer *et al.*, 2004).
TFPI is the main inhibitor of TF-fVIIa complex. Its role and antiinflammation properties have not been elucidated. It has been shown though that administration decreases mortality in a baboon sepsis models with decreased systemical levels of IL-6 and thrombin-antithrombin complexes (Carr *et al.*, 1994). Whether this is a direct effect from the molecules or a result from the decreased activity of Tf remains to be answered. IL-6 increases the platelet count production and the newly formed platelets are more active (Burstein, 1994). Platelet activation indirectly increases inflammation through being a catalytic surface for the formation of coagulation proteases and thrombin. Platelets also release proinflammatory mediators, like CD40 ligands, which induce tissue factor syntheses and increase cytokines like IL-6 (Henn *et al.*, 1998). Thus, inflammatory mediators lead to increased platelet numbers and activation that through multiple positive feedback pathways, form platelet-formed-coagulation-proteases thus increasing the inflammation.

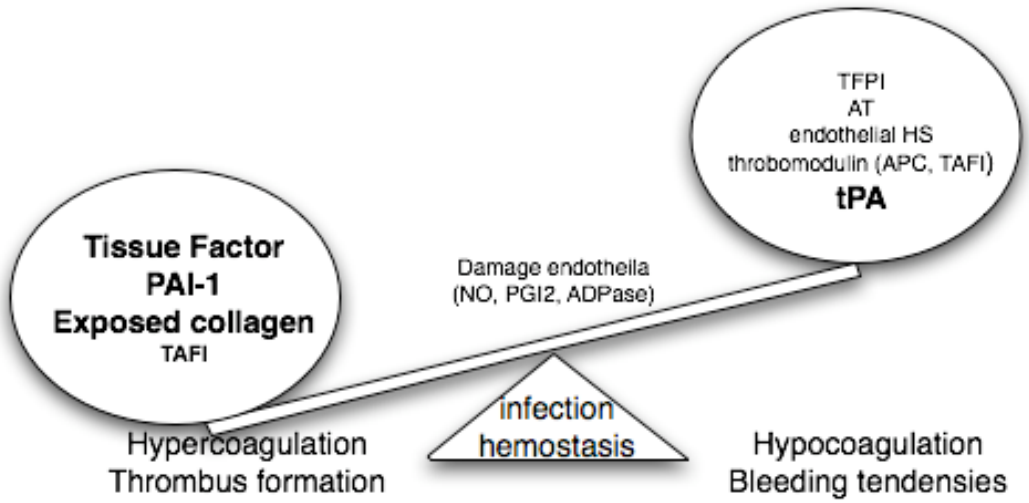


Figure 1.4. *In inflammation the balance of the hemostasis is disturbed ending in a hypercoagulative state.*

1.5 Project background

Thrombocytopenia, a reduction in circulating platelets, is a common feature in sepsis and severe infection. The extent of the thrombocytopenia has been shown to be correlated to severity of infection and is used in clinical scoring systems for sepsis (Knaus *et al.*, 1985; Ratanarat *et al.*, 2005). A decrease in platelet count is one of the earliest signs of disseminated intravascular coagulation (DIC)(Saba & Morelli, 2006; Ten Cate, 2003), and patients with thrombocytopenia have a higher mortality in intensive care units than patients with normal platelet counts (C. Feldman *et al.*, 1989). Even though thrombocytopenia is of prognostic importance the pathophysiology behind the phenomenon is not completely understood. Previously studies have proposed that thrombocytopenia is due to an activation of the Tissue factor pathway in inflammation (Ten Cate, 2003). Thrombin formed in this pathway is a potent platelet activator and results in the aggregation and adherence of activated thrombocytes to each other and endothelia respectively in the hypercoagulative state of DIC (Saba & Morelli, 2006; Ten Cate, 2003; Ware *et al.*, 2006). However, it has now been established that platelets express Toll like receptors and these receptors have been shown to play an important role in the development of LPS induced thrombocytopenia (Andonegui *et al.*, 2005). Platelets become activated through TLRs and as a response sequestrate out into the lung microvasculature. This sequestration occurs within minutes after LPS challenge and platelets demonstrate a neutrophil-like rolling behaviour along capillary endothelium in lung tissue (Kiefmann *et al.*, 2006; Yu *et al.*, 2005). *In vitro*, thrombin stimulated platelets release a cationic protein (platelet microbicidal protein [PMP]), which is antimicrobial to some strains of *Staphylococcus aureus* and *Candida albicans* (Yeaman *et al.*, 1997). Data has shown that resistance of C. albicans to PMP increases the severity of experimental *C. albicans* endocarditis (Yeaman *et al.*, 1996). Inflammation increases the expression of tissue factor and thereby increases the local production of thrombin stimulating platelet activation (Ten Cate, 2003) suggesting that PMP might be released at all sites of infection *in vivo*. Moreover activated platelets facilitate formation of fibrin, which might retain bacteria locally. Bacteria that express fibrinolytic peptides on their surface have been shown to increased virulence compared to their counterparts that lack the fibrinolytic properties (Lähteenmäki *et al*., 2001). Neutrophils, in contact with bacteria, release a sticky "net" of DNA and histones, which traps and kills pathogens even after the neutrophils lifespan (Fuchs *et al.*, 2007; Urban *et al.*, 2006). Platelets have recently been shown to aid this process. In one model neutrophil extracellular trapping (NET) formation is platelet TLR-4 dependent and only occurs under extreme conditions like severe sepsis (Clark *et al.*, 2007). Clark *et al* (2007) also propose that platelets act as a threshold mechanism for neutrophil activation and NET formation, but that NET formation on endothelia leads to tissue damage. These facts make platelets able to react to microbes, to sequestrate into sites of infection and both directly and indirectly help in the killing of microbes. Thus platelets have been proposed to play a role in host defence against microbes (Yeaman & Bayer, 1999). However some of the platelets indirect antimicrobial properties, like promoting the formation of NETs, have been shown to occur with side effects like tissue damage for the host (Clark *et al*., 2007).

Streptococcus pneumoniae is the most commonly isolated pathogen in community acquired pneumonia (Lauderdale *et al.*, 2005; López, 2006; Tajima *et al.*, 2006) and a common cause to severe sepsis in the developed world (Bernard *et al.*,

2001). During alveolar- and bronchopneumonia the local hemostatic balance is altered (Schütte *et al.*, 1996). Proinflammatory cytokines increase the endothelial and mononuclear cells expression of tissue factor, and inhibits fibrinolytic activity resulting in local fibrin deposition (Günther *et al.*, 2000). The role of sequestered platelets in fibrin formation is to date unclear. Nevertheless it has been proposed that platelet accumulation plays an important role in the development of endotoxin and sepsis induced acute lung injury, rationally as a consequence of platelet-endothelial interaction leading to an increased expression of P-selectin, which then aids neutrophil migration into interstitial tissue and therefore the development of lung injury (Kiefmann *et al.*, 2006). Cell wall components from *S. pneumoniae* are also known to work as a ligand activating TLRs (Yoshimura *et al.*, 1999). These ligands could also be responsible for the direct activation of platelets from some strains of *S. pneumoniae* seen *in vitro* (Zimmerman & Spiegelberg, 1975).
The primary aim of this study was to investigate the interactions between platelets and *S. pneumoniae* in a pneumonia and sepsis model for several reasons. Firstly, *S. pneumoniae* is known to possess surface endonucleases (EndA) that make it possible for the bacteria to degrade and escape the killing by NET (Beiter *et al.*, 2006). Secondly, platelet-activating factor released from platelets (PAF) plays an important role in the orchestration of different inflammatory reactions, including the release of cytokines, chemokines, and free radicals. The PAF signalling pathway is needed in some instances to direct neutrophils extravasation to the infectious foci (Moreno *et al.*, 2006). The receptor that induces that signalling, platelet activating factor receptor (PAFr), is also used by *S. pneumoniae* to mediate adhesion of the bacteria to endothelium and promote colonisation (van der Sluijs *et al.*, 2006). Finally, while platelet TLR-4 dependent NET formation has been shown to cause tissue damage *in vivo*, whether platelets limit infection locally and help in the killing of bacteria has not been investigated.

1.6 Hypothesis

Platelets aid neutrophils in the immune defence against pneumonia through mediating extravasation into lung tissue.

1.7 Aims

In vitro: determine whether bacteria activate platelets and if platelet derived products possess antimicrobial properties against *S. pneumoniae*.

In vivo: identify whether a depletion of circulating platelets affect *S. pneumoniae* replication and the neutrophil inflammatory response in pneumonia.

In vivo: investigate possible co-localisation of platelets and neutrophils in lung tissue during pneumonia.

2. MATERIALS AND METHODS

2.1 Bacterial strains

Different strains of *Streptococcus pneumoniae* were kindly provided by professors James Paton (University of Adelaide) and Brian Spratt (Imperial College) and stored at -80°C as single use aliquots in 10% glycerol until used.

2.2 Activation of platelets

2.2.1 Obtaining of platelet rich plasma

Platelet rich plasma (PRP) was prepared as previously described (Yeaman *et al.*, 1992). In short, 50 ml of blood was collected in 4 ml of acid citrate/dextrose (85 mM sodium citrate, 65 mM citric acid and 110 mM dextrose) from healthy volunteers that haven't used aspirin or other drugs for at least 14 days. The samples were centrifuged at 200 x g for 20 min and the top 2/3 of the platelet rich supernatant was obtained. The PRP were stored on ice until used typically not more than 2 hours later. 5µl of PRP was diluted in 95µl verbatim (100µl glacial acetic acid in 10ml saline) and after letting platelets setting for 10 minutes the platelet count manually obtained using haemocytometer**.** Platelet counts typically varied between 2-5 x 10^8 platelets/ml of PRP. Any PRP with signs of platelet aggregates was discarded.

2.2.2 Aggregation as a sign of activation

Aggregation was used as a measurement of activation as previously described (Bednar *et al.*, 1995). In short, 90 µl of PRP were mixed with 10µl of positive control (ADP 10mM or TFLLR-NH_3 [TF] 10mM, a PAR1 agonist), negative control (dH_2O) or different test substances in a 96 well microplate. The absorbance was measured at different time points (see results section) using Titertek Multiscan MCC/340 at an optical density of 620nm using the constant movement setting**.** Aggregation of platelets decreases the absorbance and the difference in absorbance from the start of the experiment compared to the end is a measurement of the degree of platelets activation. When Ca^{2+} was added to PRP this was before adding of test substances at a final concentration of 0.25 mM $CaCl_2$.

2.3 Antimicrobial proteins from platelet rich plasma (PRP)

2.3.1 Obtaining of antimicrobial proteins

PRP from the same sample was divided into two tubes. 10µl/ml PRP ADP 10mM, TF 10 mM or thrombin 0.2 mM was added in one of the tubes and 10µl dH_2O and prostaglandin E_1 40nM (Cayman chemical) was added into the other as a negative control. Samples were incubated for 20 minutes in 37°C. To confirm activation absorbance was measured using a Amersham Pharmacia biotech Utrospec 3000 at an optical density of 620 nm. Samples were centrifuged at 800 x g for 10 minutes to pellet platelets and the top 2/3 of the activated platelet supernatant (Aps) or control platelet supernatant (Cps) removed and stored in -80 °C until used.

2.3.2 Concentrated platelet supernatant

10 ml PRP with prostaglandin E_1 40nM (Cayman chemical) was centrifuged at 800 x g for 10 minutes to pellet platelets. 9 ml of platelet poor supernatant was removed after this centrifugation and used as the negative control. The pellet was then resuspended in the remaining plasma. This super platelet rich plasma (sPRP) was activated with 100 µl of ADP 10mM and incubated for 20 minutes at 37°C. The sPRP was centrifuged at 2000 x g for 10 minutes and the top 2/3 of the concentrated Aps was stored at -80 °C until used.

2.3.3 Antimicrobial proteins from platelet rich Tyrode solution (PRT)

PRP from the same sample was divided into two different tubes. Prostaglandin E_1 40nM (Cayman chemical) was added to the PRP in the control tube. The samples were then centrifuged at 800g x 10 min to pellet platelets. Platelet poor plasma (PPP) was removed. Pellet was resuspended in and washed with Tyrodes salt solution (0.08mM NaCl, 3.8 mM K_2HPO_4, 4 mM Na_2HPO_4, 2.8 mM glucose, 16.6 mM citric acid, 34 mM sodium citrate, pH adjusted to 6.8) two times. The pellet after the last wash was resuspended in Tyrodes solution containing 0.25mM $CaCl_2$. 10µl/ml PRT of control (dH_2O) or different agonists (ADP 10 mM, TF 10 mM or thrombin 0.2 mM) was added. Samples were incubated for 30 min in 37°C and then centrifuged at 2000 x g for 10 minutes. The top 2/3 of the supernatant were then removed and used like Aps or Cps.

2.3.4 Evaluation of antimicrobial properties

Either 200µl of activated platelet supernatant (Aps) or 200µl of Control platelet supernatant (Cps) were mixed with 200µl bacteria (2×10^8 cfu/ml) and incubated at 37°C for 90 minutes. Serial dilutions (in 10) of the reaction mix were then plated on Columbian agar with 5% defibrinated horseblood and after incubation in 37°C for 24 hours bacterial colony forming unit (cfu) counted to allow the calculation of bacterial cfu/ml of reaction mix.

2.4 Inhibition test

100µl *S. pneumoniae* (2×10^5 cfu/ml) was evenly distributed on a Columbia blood agar plate. Sterilised filter paper discs (0.3 cm diameter) that were pretreated either with concentrated Aps (ADP activated), Cps or PBS where placed on the blood agar plate. Plates were incubated in 37°C for 24 hours to look for inhibition zone around discs. If the Aps possessed bacteriostatic properties an inhibition zone around Aps discs were suspected.

2.5 Chemotaxic test

2.5.1 Obtaining of mouse PRP

Dr José Yuste obtained whole blood from female C57BL/6 wild type mice through cardiac puncture after euthanasia with CO_2 in accordance with the UK Home Office The Animals (Scientific Procedures) Act (1986)**.** Typically 800-1000µl blood was mixed with 100µl of acid citrate dextrose in tubes. The tubes

were stored on ice and within 1 hr centrifuged at 200g x 20 min. The top 2/3 of the PRP was obtained and stored on ice until usage (within 3 hrs).

2.5.2 Chemotaxic movement

Before performing tests $CaCl_2$ to a final concentration of 0.25 mM, was added to mouse PRP. 100µl mouse PRP was incubated with 100µl of *S. pneumoniae* (~2 x 10^7 cfu/ml) or 100 µl of TF 0.01mM in a 96 well plate**.** Time laps confocal microscopy, using a Zeiss LSM 510 Scanning laser confocal microscope objective Plan Neufloar 10x/0.3O, was then performed taking 2 pictures/min during 1 hr and 30 min. The pictures retained where then played in faster speed to see evidence of chemotaxic movement.

2.6 Pneumonia infection model

All experiments were conducted with full local Ethical approval and in accordance with the UK Home Office The Animals (Scientific Procedures) Act (1986). The mice were kept with ad lib water and food during the test period.
Dr José Yuste performed all live animal experiments. Female mice wild type C57BL/6 8-16 weeks were injected intra peritoneally (i.p.) with either 40µg/mouse platelet depletion antibody (purified rat monoclonal antibody against GP1ba/CD42b from Amfret Analytics, Germany) or 40 µg/mouse control antibody (mixture of non immune rat IgG with no cytotoxic effect on platelets from Amfret Analytics, Germany). After 1-2 hours the animals were anaesthetised with halothane and challenged with *S. pneumoniae* intranasally. In the low dose infection model 5 x 10^5 cfu/mouse was used and in the high dose 10^6 cfu/mouse was used. After 24 hours the mice were anaesthetised with injection i.p. of penthobarbital natrium and terminally bleed from the femoral artery. 5µl of blood was retained in 95µl of verbatim (100µl glacial acetic acid in 10ml saline) for platelet counts manually in haemocytometer and 30µl of blood was used for bacterial cfu. Spleens were taken and stored in filtered PBS on ice for calculation of bacterial cfu. Before bronco alveolar lavage (BAL) the diaphragm was perforated to release negative pressure. BAL was performed with 1 ml of PBS being repeatedly injected and aspirated three times through an endotracheal cannula, the final time typically getting back 0.7 ml for total cellcounts and bacterial cfu. The left lung lobe was taken for cfu while the right lung lobe was fixated in 3% paraformaldehyde for immunohistochemistry. 2 unchallenged mice went through the same processes as controls for immunohistochemistry.
Spleens and lungs were homogenized in PBS. Serial dilutions (in 10) of the homogenized mix, BALF and blood were then plated on Columbian agar with 5% defibrinated horseblood and after incubation in 37°C for 24 hours bacterial colony forming unit (cfu) counted to allow the calculation of bacterial cfu/ml of the target organs. 50µl of BALF was mixed in 50µl of Turk´s solution and total cellcount performed using haemocytometer. 100µl of BALF were cytospined at 1000 rpm for 5 minutes for quick differential staining and manual differential cell counts.

2.7 Immunohistochemistry

2.7.1 Tissue to sectioning

After 24 hours of fixation in paraformaldehyde, the right lung lobe was processed using a Leica TP 1050 vaccuinfiltration processer for histochemistry. The tissues were paraffin embedded and then cut in 3 μm thick sections in a Shandon AS325 rotary microtone.

2.7.2 Staining with antibody

The tissue was dewaxed using a Sakura Diversified stainer program 3. Antigen retrieval was performed through microwave treatment in citrate buffer (1.14g Sodium Citrate in 500 ml distilled water adjust pH to 6.0) for 2 x 5 minutes. After cooling for 5 minutes the slides were washed twice in TBS for 5 minutes. Blocking of non-specific secondary antibody binding to the tissue was performed by incubating the slides with 1:6 normal Rabbit serum in TBS containing 4 drops/ml avidin blocking solution for 30 minutes. Thereafter the slides were incubated with the primary antibody (Polyclonal goat IgG antiintegrin α_{IIb} from Santa Cruz Biotechnology) in TBS containing 1% bovine serum albumin (BSA), 1% rabbit serum and 4 drops/ml biotin blocking solution over night at 4°C. Slides were washed twice for 5 minutes in TBS and incubated with the secondary FITC-conjugated rabbit anti-goat antibodies (from DAKO cytomation) at room temperature for one hour. The secondary antibody was premixed with 1/10 normal mouse sera to avoid unspecific binding. The secondary antibody was washed off using TBS (x2 5 minute washes). DNA was stained by incubating with propidium iodide 0.05mg/ml for 5 minutes. After final wash in TBS for 5 minutes the slides were blotted and mounted with Gelvatol.

2.7.3 Control of antibody staining

The following controls were used: i) polyclonal goat IgG (Santa Cruz Biotechnology) instead of the primary antibody; ii) primary antibody preincubated with bacteria and then centrifuged (2000g x 10 min); iii) cytospins of platelet rich plasma (100μl PRP cytospined in 1000 rpm x 5 min) were stained using the above method to asses the specificity of primary antibody and propodium iodide.

2.8 Imaging

Pictures were taken using a Zeiss LSM 510 Scanning laser confocal microscope with the Plan Neufloar 10x/0.3O objective.

2.9 Statistical analyses

Statistical analyses of the aggregation and antimicrobial properties tests were performed using the Students t-test. Differences were assumed to be statistically significant at the 5% level.
Statistical analyses of bacterial cfu in target organs and cell counts were performed using Mann-Whitney t-test. Differences were assumed to be statistically significant at the 5% level.

3. RESULTS

3.1 Platelet activation

3.1.1 Activation in response to bacteria

For this thesis I investigated whether bacteria activate platelets *in vitro* using optical density of platelet rich plasma after incubation with different strains of *S. pneumoniae*. In addition I assessed, whether interaction with other cells was required. Decrease of absorbance is a sign of aggregation and therefore platelet activation (Bednar *et al*., 1995). The absorbance of the PRP decreased statistically compared to controls only when incubated with *S. pneumoniae* strain 14, although there was a statistically non-significant trend for activation with other strains as well (figure 3.1.1 A). The anticoagulant (acid citrate) diminishes Ca^{2+} stores in the plasma and when Ca^{2+} was added to the PRP the ST6B and D39 *S. pneumoniae* strains also activated platelets statistically (figure 3.1.1 B). In severe sepsis high levels of bacteria can be found in the circulation, and could lead to platelet activation when lower numbers of bacteria does not. I therefore investigated whether a high ratio of bacteria to platelets increased platelet activation. Platelet activation occurred with the higher ratio of bacteria to platelets to statistically significant levels in all strains investigated (D39, ST6B, 14, 18C and 19F) (figure 3.1.2). Time course assays showed the aggregation started after 7 minutes (data not shown) and was statistically significant by 15 minutes (figure 3.1.3). Heat deactivated *S. pneumoniae* also caused platelet aggregation but to a lesser extent than that of live bacteria (figure 3.1.4). I did not observe any chemotaxic movement of platelets towards *S. pneumoniae* (data not shown) still this technique needs refinement before any conclusions can be done.

3.1.2 Activation in response to LPS

Platelets are known to express TLR4 but little is known whether binding of TLR4 ligands by platelets has functional consequences. Hence I investigated whether platelets could be activated through LPS stimulation. The results show that in this assay platelets do aggregate in response to LPS but only after exposure to high doses (20μg/ml) (figure 3.1.5).

3.1.3 Activation in response to neutrophils and neutrophil derived products

Recent work has described a neutrophil-platelet interaction and I therefore investigated whether neutrophils may enhance platelet activation by *S. pneumoniae.* Supernatant from activated neutrophils did not increase platelet aggregation (figure 3.1.6 A). Preliminary data also suggests that direct platelet-neutrophil interaction do not have any effect on platelets tendency to aggregate in response to bacteria (data not shown).

3.1.4 Platelets are important in the early fibrin formation with increased absorbance as a consequence

The absorbance assay is based on a relative change in absorbance and since activated platelets enhance the formation of fibrin I wanted to investigate how much this fibrin formation could interfere with the detection of a decrease in

absorbance due to platelet aggregation early in our assay. Fibrin formation causes an increase in absorbance within minutes after platelet stimulation with thrombin (figure 3.1.7).

3.2 Antimicrobial properties

Platelets are known to release different antimicrobial proteins in response to different stimuli (Yeaman & Bayer, 1999). To investigate if these antimicrobial proteins had any bactericidal or bacteriostatic properties against *S. pneumoniae* the supernatant from ADP- or thrombin-stimulated platelets was incubated with *S. pneumoniae* and the bacterial cfu calculated by plating serial dilutions. The supernatant from thrombin- but not ADP-stimulated platelets reduced *S. pneumoniae* cfu compared to un-stimulated supernatant (figure 3.2.1 and 3.2.2). Locally concentrations of antimicrobial proteins might be high *in vivo* but even concentrated ADP-stimulated supernatant failed to show antimicrobial properties (data not shown). Even more inhibition test with ADP activate supernatant did not show any bacteriostatic properties (data not shown). Thrombin stimulates platelets through PAR1 and PAR4 (Kahn *et al.*, 1998), but primary data suggests that supernatant from platelets stimulated with the PAR1 agonist (TFLLR-NH_3) do not possess antimicrobial properties (data not shown) suggesting PAR4 or other thrombin receptors are required for these effects.

3.3 *In vivo* pneumonia model in mice challenged with 5 x 10^5 *S. pneumoniae* strain D39 intra nasally

3.3.1 Platelet depletion

To assess whether platelets are important for the control of *S. pneumoniae* pneumonia, bacterial cfu in target organs was investigated in thrombocytopenic mice infected by intranasal inoculation with *S. pneumoniae* D39. The bacterial strain were chosen since it causes an infectious model which is well characterised. Thrombocytopenia was induced as previously described (Elzey *et al.*, 2003) by parenteral administration of a rat monoclonal antibody against GP1ba/CD42b 1 or 2 hours prior to inoculation with *S. pneumoniae*. Platelet counts at the point when the animals were culled 24 hours after inoculation showed >90 % of platelets were depleted in the treatment group (figure 3.3.1).

3.3.2 Blood contamination of bronchoalveolar lavage fluid (BALF)

Visually the BALF was contaminated by blood in the platelet-depleted group (picture 3.3). The quantity of blood in BALF was calculated through erythrocyte counts (table 3.3), and the extra degree of blood contamination determined by the equation (median BALF erthrocyte $count_{depleted}$ − median BALF erthrocyte $count_{control}$/ normal murine blood erythrocyte count [9 x 10^9 erythrocytes/ml] (B. F. Feldman *et al.*, 2000)). Using this formula results in that there was <0.5% blood contamination of the BALF from platelet-depleted animals.

3.3.2 Platelets effect on the neutrophil influx into BALF

Whether platelets are important for the neutrophil sequestration into BALF during *S. pneumoniae* pneumonia is unknown. During pneumonia the total leukocyte and neutrophil cell count in the BALF is elevated in platelet-depleted groups (figure 3.3.3 A, B). However the ratio of bacteria to neutrophil counts in the BALF was higher in platelet-depleted groups, suggesting that possibly there might be a relative impairment of neutrophil recruitment for the level of infection in these mice (figure 3.3.4).

3.3.3 Amount of bacteria in tissue

Platelet depleted mice had higher numbers of *S. pneumoniae* cfu in all tissues (BALF, lung, blood and spleen) 24 hours after inoculation compared to control group (figure 3.3.5). Statistical analysis (Mann Whitney U test) demonstrated that these differences were all significant (spleen P value 0.0327, blood P value 0.0079, lung P value 0.0111 and BALF P value 0.003). The ratio of bacterial cfu between the lung and the blood was similar between platelet-depleted and control mice (figure 3.4.6). Infection using a higher dose challenge (1×10^6 bacteria intranasally) at a shorter time after treatment with the anti-platelet antibody (1 hr rather than 2 hours) did not result in statistically significant differences in *S. pneumoniae* cfu recovered from target organs, although there was possibly a trend towards higher bacterial counts in depleted groups (figure 3.4.7).

3.4 Platelet form aggregate around bacteria *in vivo*

3.4.1 Staining controls

The interaction of platelets with *S. pneumoniae* during pneumonia infection was investigated using histology and specific staining of platelets and bacteria using polyclonal goat IgG anti integrin α_{IIb} to mark the platelets and propodium iodide to mark DNA. Difference in size makes bacterial DNA easily distinguishable from host cell DNA.
With the primary antibody replaced with a polyclonal goat IgG no green fluorescent can be seen (figure 3.4.1), indicating that the secondary antibody did not react with bacteria. As expected platelets were only stained with fluorescent green indicating that propodium iodide did not stain the platelets (figure 3.4.2). Antibody preincubated with *S. pneumoniae* did not lose its ability to stain platelet complexes (data not shown). Hence goat IgG anti integrin α_{IIb} stains platelets specifically.

3.4.2 Platelets from aggregate around bacteria in pneumonia

Platelet-bacteria interactions were readily observable in the air spaces of mice with pneumonia (figure 3.4.3). Several such instances of platelets aggregating around bacteria could be found in any given tissue section in control mice (figure 3.4.4). No neutrophils, which are easily identified in tissue sections based on their obvious polymorphonuclear appearance, could be found in close proximity to the platelet-bacteria interactions. Platelets in the platelet depleted mice lungs also seemed to form aggregates around bacteria although the platelets were very rare

and a large numbers of “untrapped” bacteria were seen (figure 3.4.5). Interestingly in unchallenged mice a few platelets were found in the alveolar spaces but no aggregates were seen (figure 3.4.6). In the high dose challenged mice platelets could also be seen forming aggregates around bacteria even in the platelet-depleted group (figure 3.4.7). These data suggest that during *S. pneumoniae* pneumonia platelets form neutrophil-independent aggregates, which are intimately related to the sites of bacterial infection.

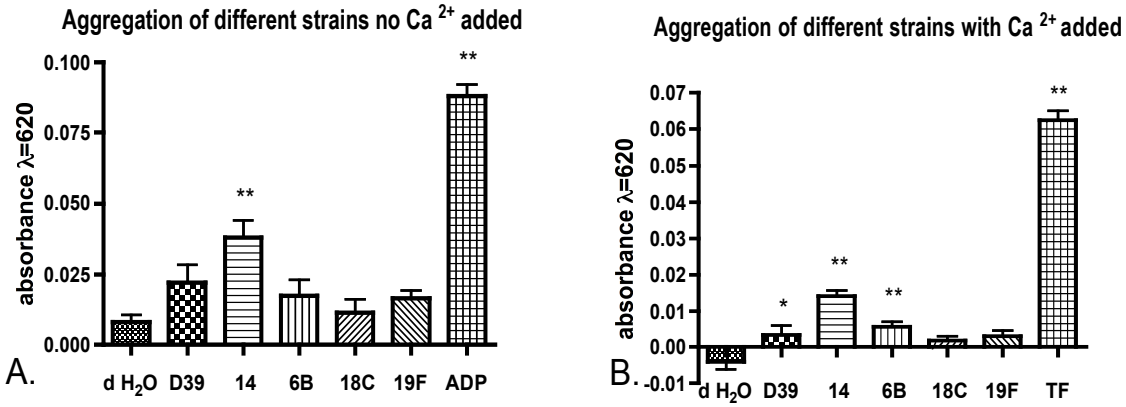


Figure 3.1.1 *Change in absorbance (start absorbance - absorbance after 90 minutes) of 90µl platelet rich plasma (PRP) in response to 10µl of different strains of* S. pneumoniae *(10^7 cfu/ml).* ***A.*** S. pneumoniae *strain 14 (final concentration 10^6 cfu/ml) aggregates platelets (n=5).* ***B.*** *Change in absorbance after 90 minutes with 2.5µl 0.1 M $CaCl_2$ added to the sample. Aggregation of platelets in response to bacteria (n=8). * Indicates significant difference compared to dH_2O (one-way ANOVA with Dunnett´s post-test; P<0.05). ** Indicates significant difference compared to dH_2O (one-way ANOVA with Dunnett´s post-test; P<0.01). Values are representative of 3 experiments.*

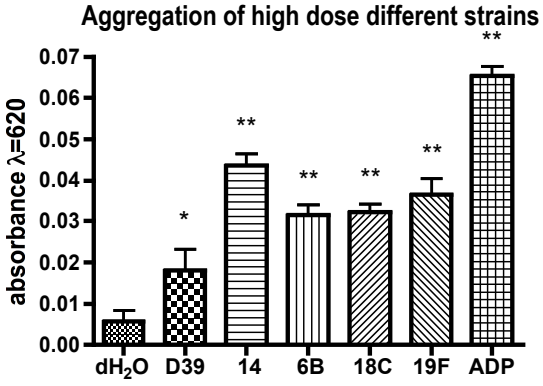


Figure 3.1.2 *Change in absorbance (start absorbance - absorbance after 90 minutes) of PRP incubated with different strains of* S. pneumoniae *(final concentration 5 x 10^6 cfu/ml) using dH_2O as negative control and ADP 1mM as positive control (n=6). * Indicates significant difference compared to dH_2O (one-way ANOVA with Dunnett´s post-test; P<0.05). ** Indicates significant difference compared to dH_2O (one-way ANOVA with Dunnett´s post-test; P<0.01).*

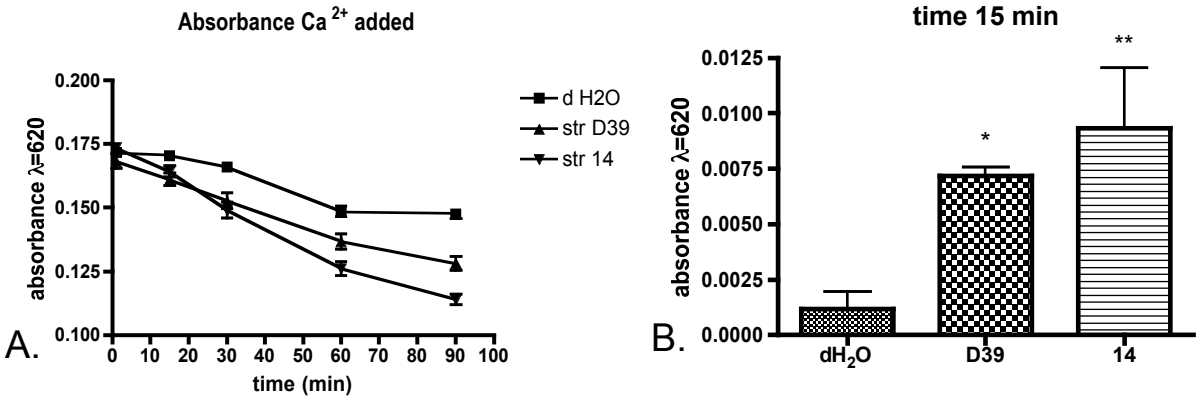


Figure 3.1.3 A. *Time-curve of absorbance (=SEM) of PRP under the influence of* S. pneumoniae *strain D39, strain 14 (final concentration 10^6 cfu/ml) or dH_2O all with 2.5µl 0.1 M $CaCl_2$ added (n=6). After 15 minutes there are statistical differences in the group that has been in contact with bacteria compared to the control groups.* ***B.*** *Change in absorbance (start absorbance - absorbance after 15 minutes) from A. * Indicates significant difference compared to dH_2O (one-way ANOVA with Dunnett´s post-test; $P<0.05$). ** Indicates significant difference compared to dH_2O (one-way ANOVA with Dunnett´s post-test; $P<0.01$). Values are representative of 2 experiments.*

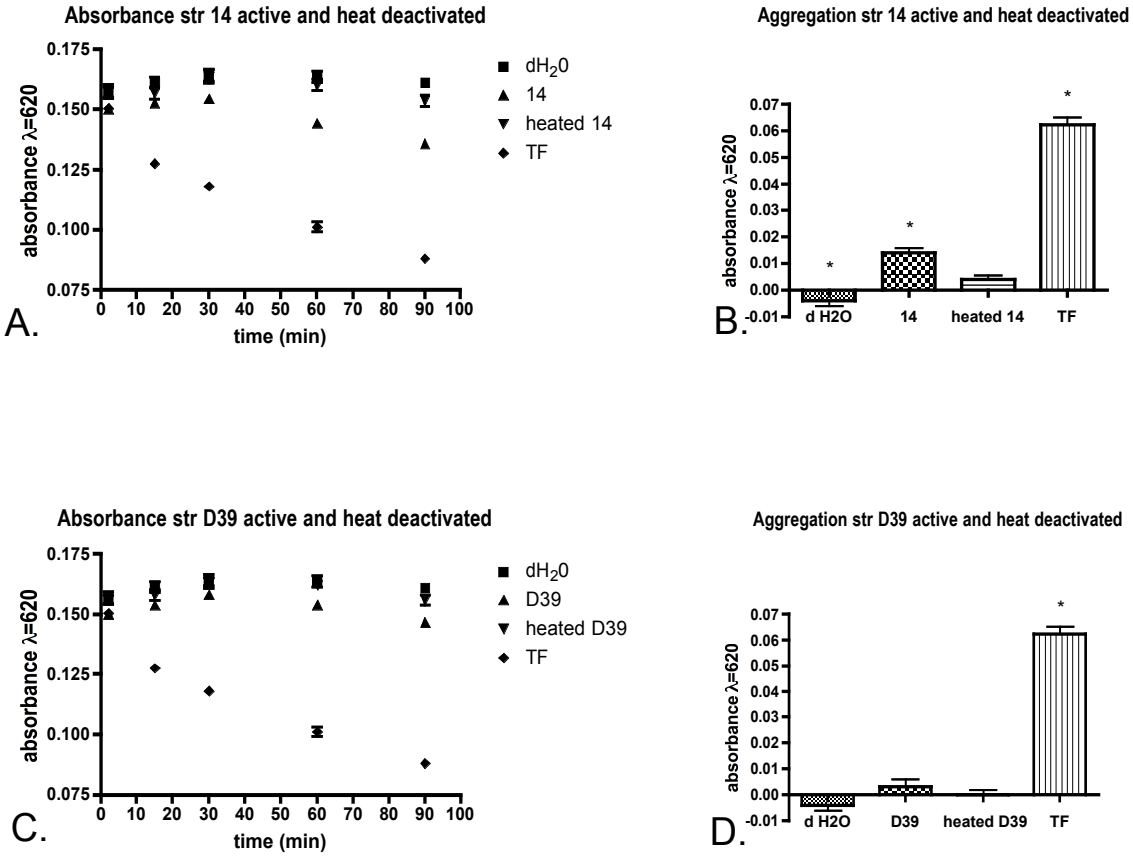


Figure 3.1.4 *Absorbance of PRP incubated with* S. pneumoniae *(final concentration 10^6 cfu/ml) heated to 100°C for 10 minutes, TF (1mM) as positive control and dH_2O as negative control.* ***A.*** *Time curve of PRP absorbance after exposure to heat-treated* S. pneumoniae *strain 14.* ***B.*** *Change in absorbance (start absorbance - absorbance after 90 minutes), from A.* ***C.*** *Time curve of absorbance after exposure to heat-treated* S. pneumoniae *strain D39 (final concetration 10^6 cfu/ml).* ***D.*** *Change in absorbance (start absorbance -end absorbance) from C. Same trend as in A and B but no statistical difference. * Indicates significant difference compared to deactivated bacteria (one-way ANOVA with Neuman-Keuls post-test; $P<0.05$, $n=8$ for each group).*

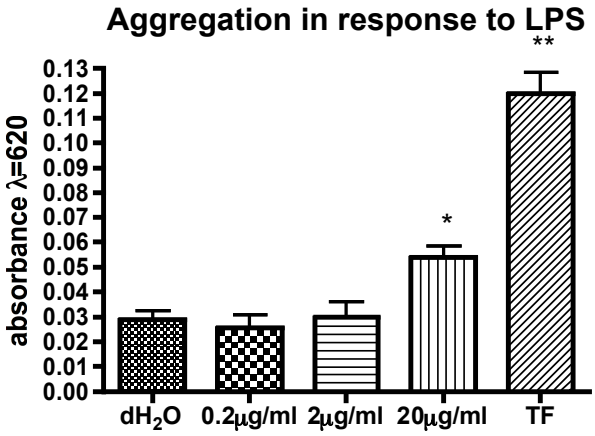


Figure 3.1.5 *Change in absorbance (start absorbance – absorbance after 90 minutes) of PRP after addition of different concentrations of LPS (n=4). * Indicates significant difference compared to dH_2O (one-way ANOVA with Dunnett´s post-test; P<0.05). ** Indicates significant difference compared to dH_2O (one-way ANOVA with Dunnett´s post-test; P<0.01). Values are representative of 2 experiments.*

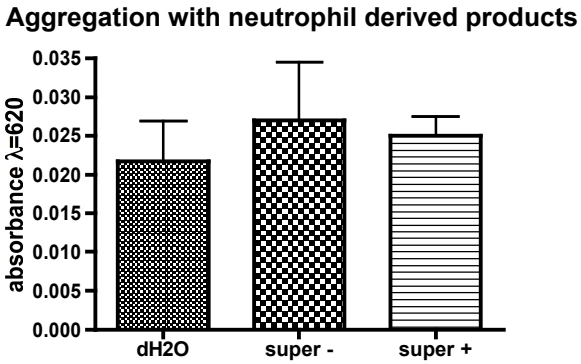


Figure 3.1.6 *Change in absorbance (start absorbance – absorbance after 45 minutes) of PRP after addition of supernatant from activated (super+) or unactivated neutrophils (super -) (n=3).*

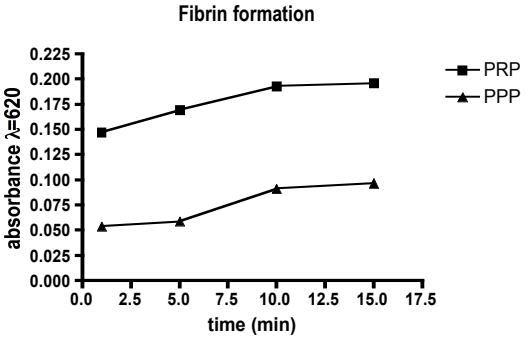


Figure 3.1.7 *Absorbance of platelet rich (PRP) or poor (PPP) plasma after addition of thrombin (final concentration 0.5μM) and $CaCl_2$ (final concentration 0.25mM).*

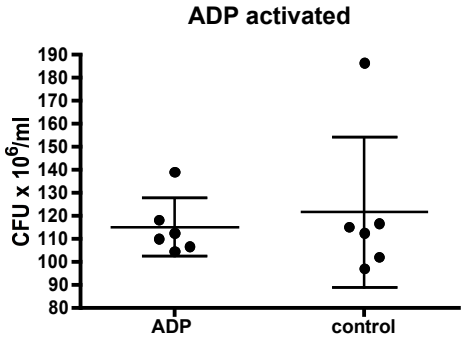


Figure 3.2.1 *Cfu of* S. pneumoniae *D39 after incubation with supernatant from ADP activated platelets in 37°C for 1.5 hr (n=6). Values are representative of 4 experiments.*

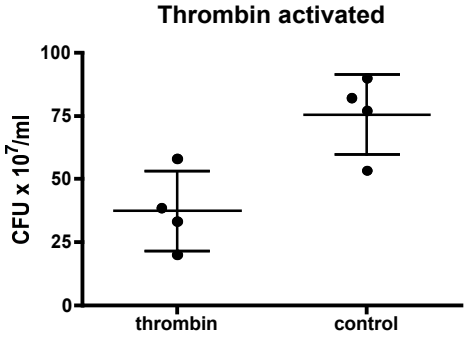


Figure 3.2.2 *Cfu of* S. pneumoniae *D39 after incubation with supernatant from thrombin activated platelets in 37°C for 1.5 hr (n=4) (ttest P value=0.0139). Values are representative of 2 experiments.*

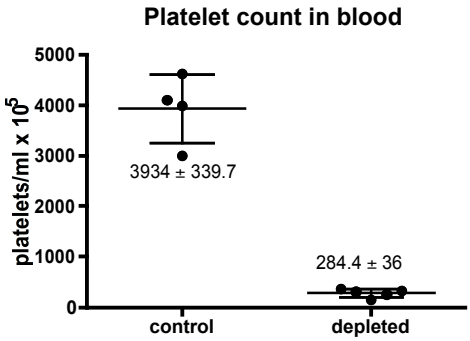
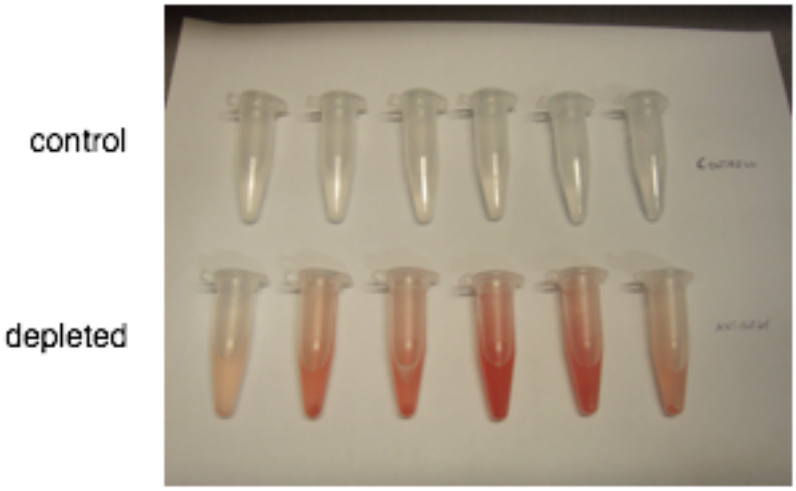


Figure 3.3.1 *Platelet count in the blood of mice treated with purified rat monoclonal antiplatelet antibody against GP1ba/CD42b and used for the infection experiments.*



Picture 3.3 *Haemorrhage in BALF in platelet depleted group after 24 hours.*

Table 3.3 *Erythrocyte counts in BALF counted using a haemocytometer*

Erythrocytes x 10^4 in BAL/ml		
Animal	control	platelet depleted
1	60	1970
2	70	5130
3	20	440
4	40	1110
5	20	4000
6	30	6000
Median	35	2985

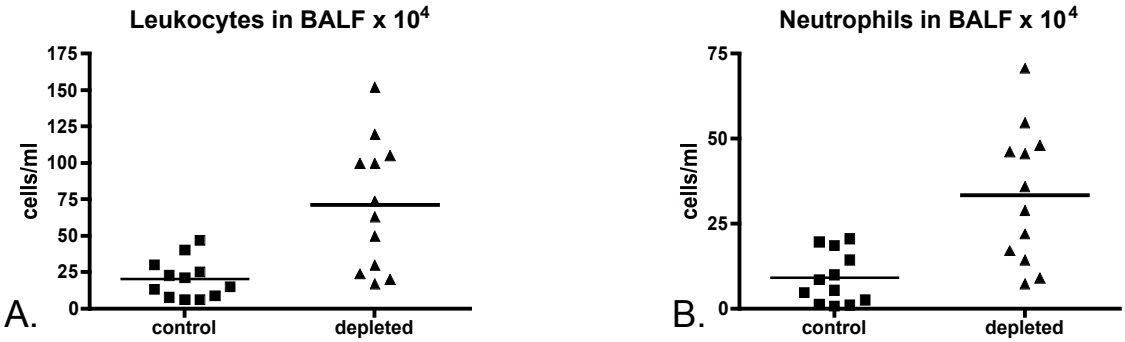


Figure 3.3.3 A. *Leukocyte cell count in BALF (n=12) (ttest P value=0.001).* ***B.*** *Neutrophil cell count in BALF performed in haemocytometer (n=12) (ttest P value=0.0007).*

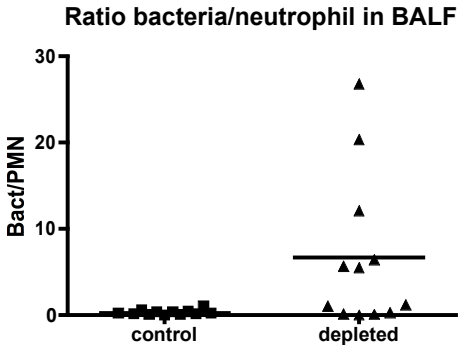


Figure 3.3.4 *Ratio of bacteria to neutrophil in BALF (n=12) (ttest P value=0.021).*

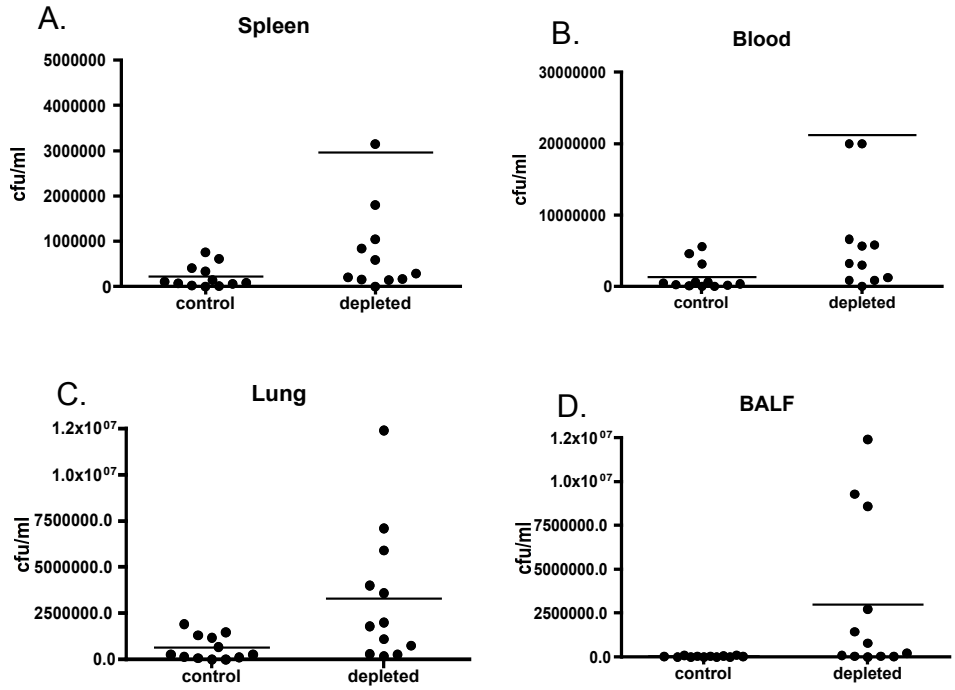


Figure 3.3.5 S. pneumoniae *cfu/ml of target organs 24 hours after challenge with 5 x 10^5 bacteria i.n. 2 hours after i.p. injection of antiplatelet or control antibody. Comparison between control and platelet depleted (depleted) groups (n=12). Statistical analysis performed with Mann Whitney test.* ***A.*** *Homogenised spleen (P value 0.0327).* ***B.*** *Blood (P value 0.0079).* ***C.*** *Lung (P value 0.0111).* ***D.*** *BALF (P value 0.003).*

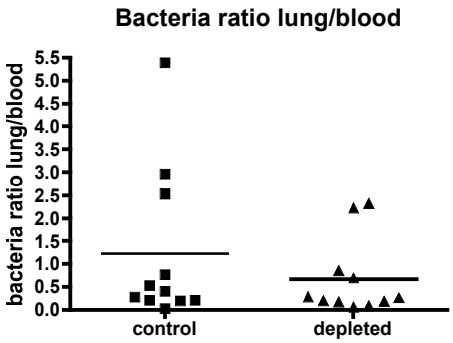


Figure 3.3.6 *A measurement of the spreading of bacteria from lung tissue to blood in the low dose infection (n=12). No statistical difference between the two groups.*

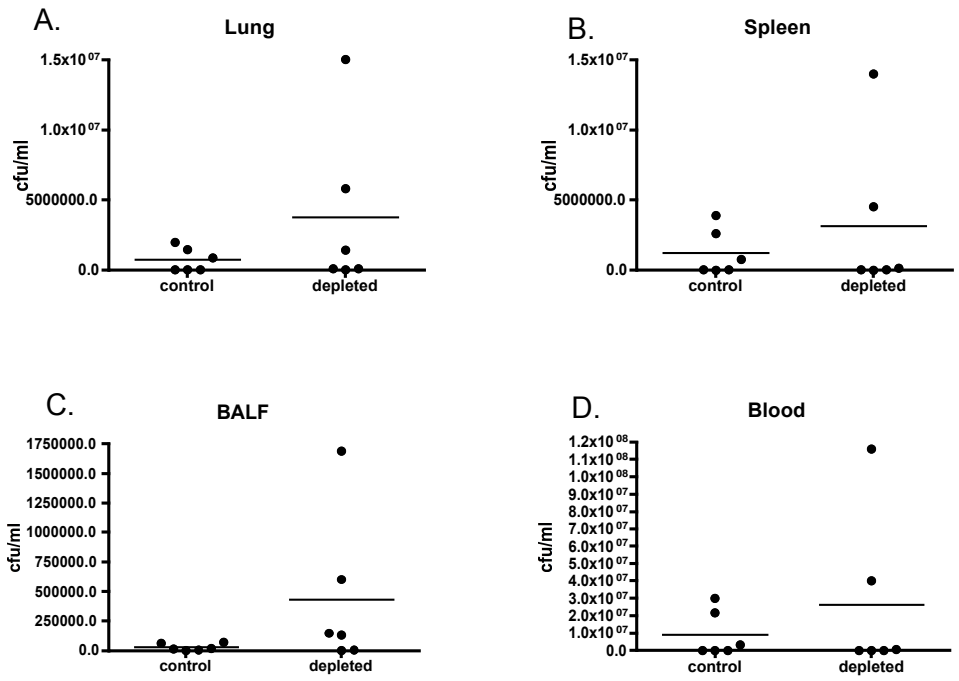


Figure 3.3.7 S. pneumoniae *cfu/ml of target organs 24 hours after challenge with 10^6 bacteria i.n. 1 hour after i.p. injection of antiplatelet or control antibody. Comparison between control and platelet depleted (depleted) groups (n=6). No statistically significant difference.*

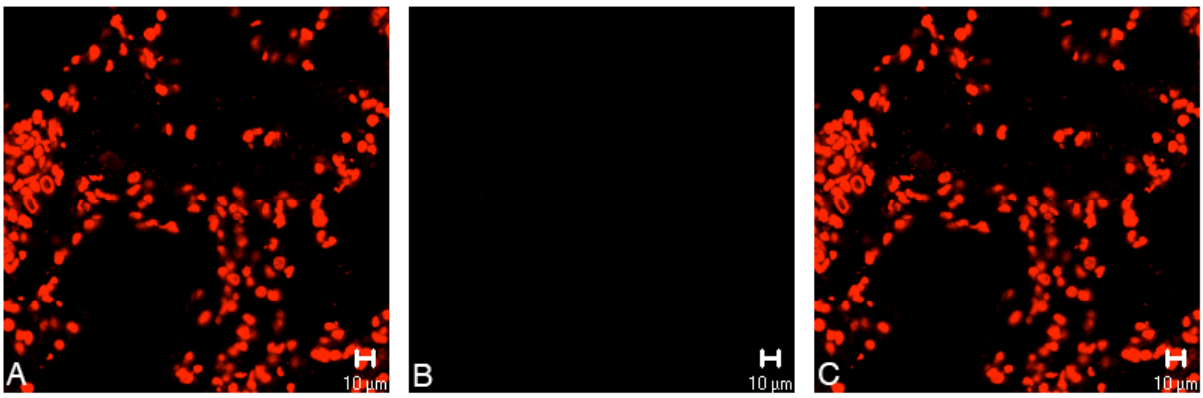


Figure 3.4.1 *Control of secondary antibody specificy.* ***A.*** *Red filter, nuclei and bacteria appear red* ***B.*** *Green filter, no unspecific green fluorescent is seen.* ***C.*** *A on B.*

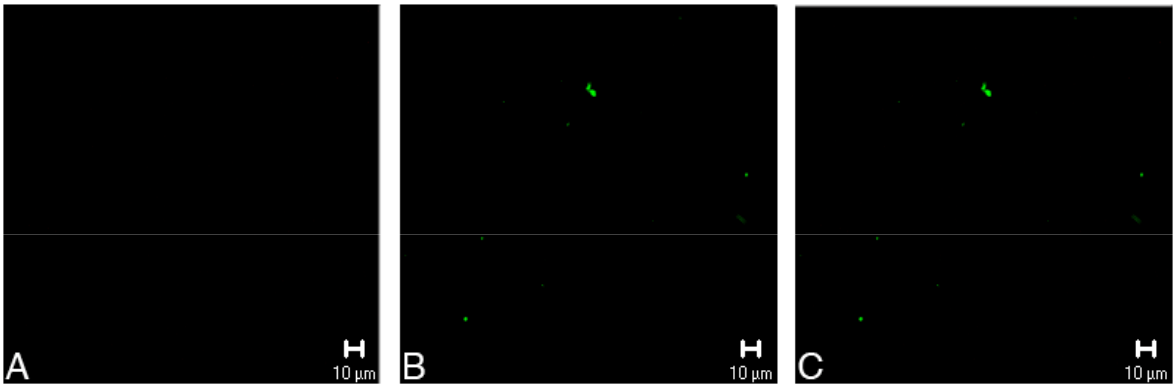


Figure 3.4.2 *Control of primary antibody and DNA staining.* ***A.*** *Red filter, platelets not stained by DNA staining.* ***B.*** *Green filter, platelets appear green.* ***C.*** *A on B.*

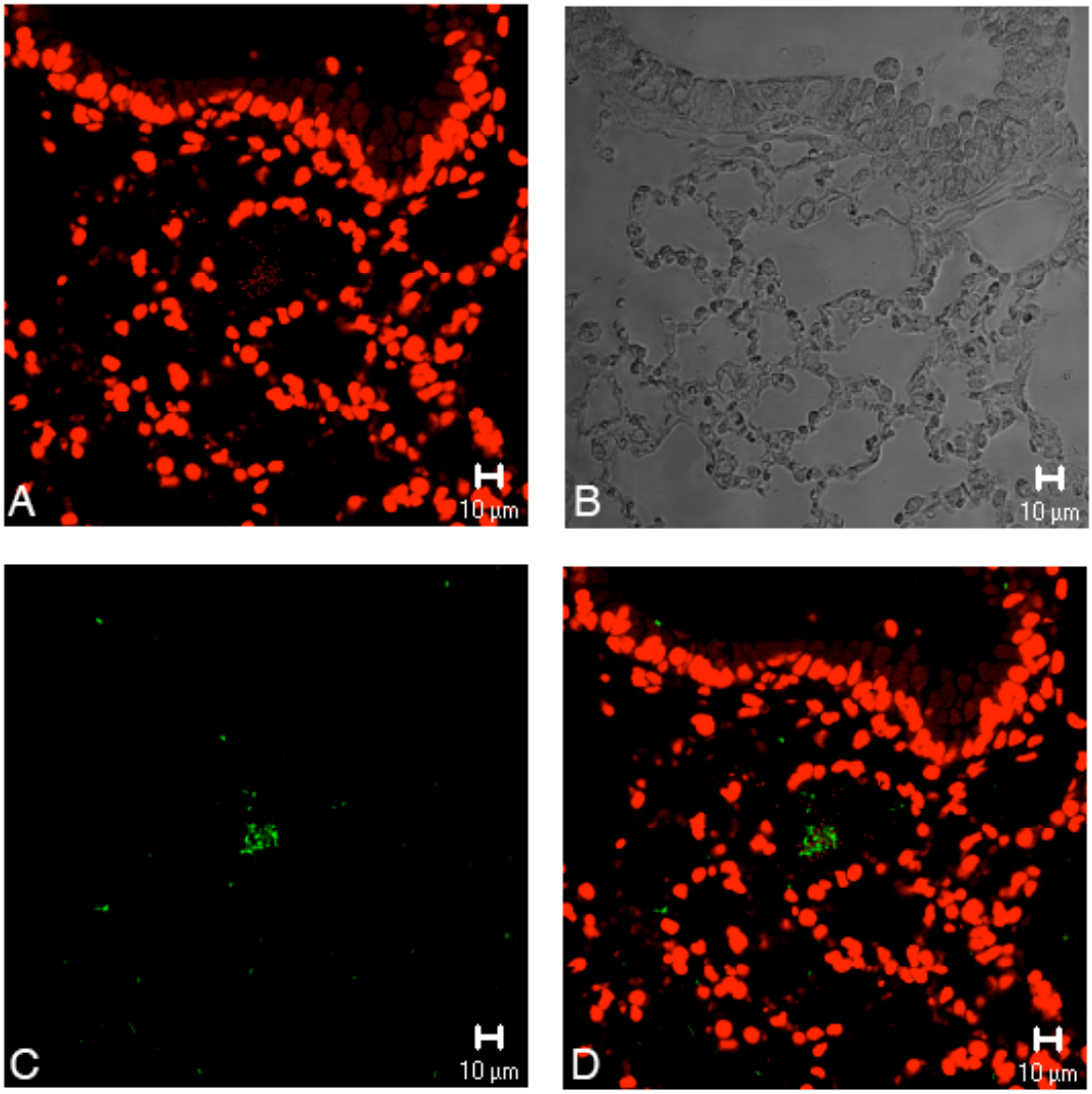


Figure 3.4.3 *Fluorescent microscopy showing relationship between platelets and* S. pneumoniae *during low dose lung infection (mouse 1).* ***A.*** *Red filter, bacterial aggregate centrally in the alveolar space.* ***B.*** *Light microscopy.* ***C.*** *Green filter, platelets aggregate centrally.* ***D.*** *A on C. Platelets are closely associated with bacteria in the alveolar space.*

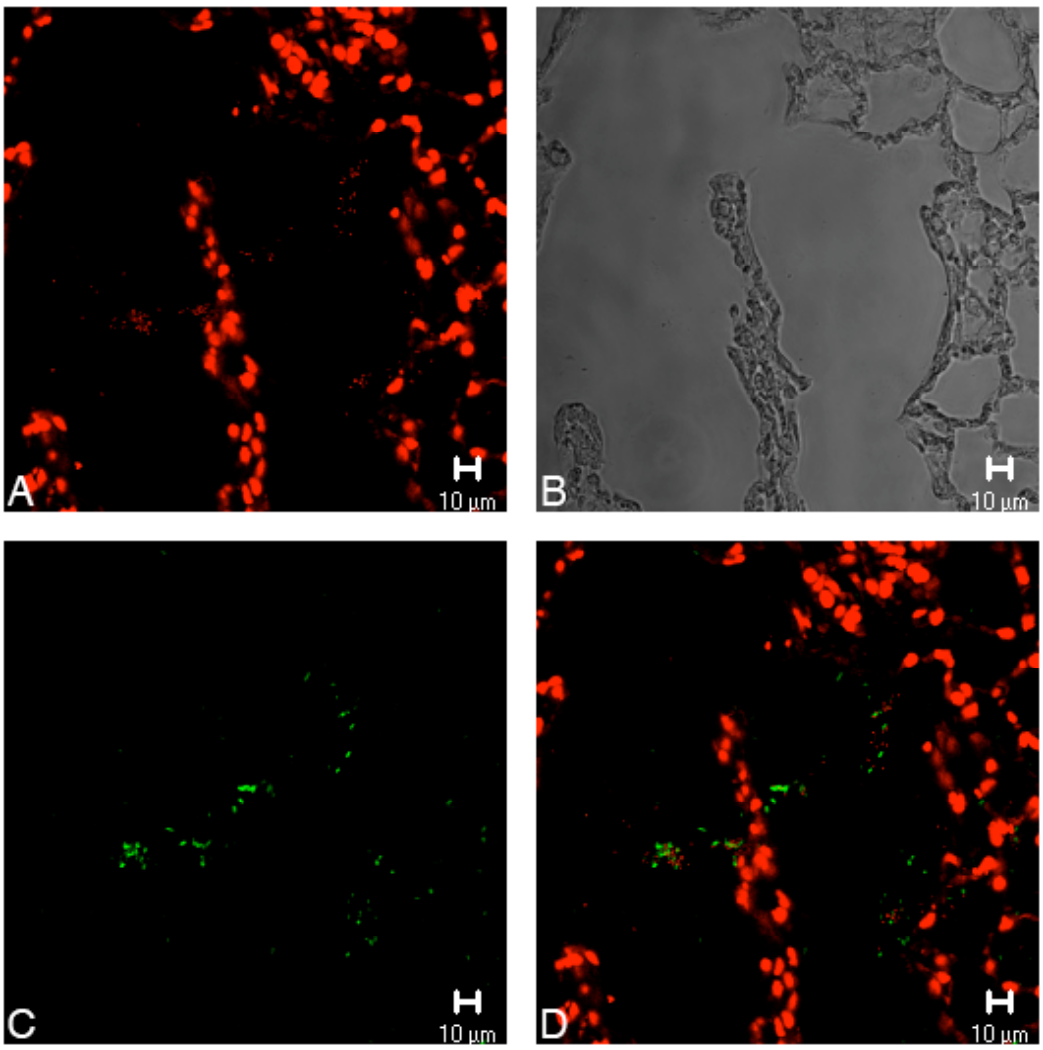


Figure 3.4.4 *Fluorescent microscopy showing relationship between platelets and* S. pneumoniae *during low dose lung infection (mouse 2).* ***A.*** *Red filter, multiple bacterial aggregates centrally in the alveolar space.* ***B.*** *Light microscopy.* ***C.*** *Green filter, multiple platelet aggregates centrally.* ***D.*** *A on C. Platelets are closely associated with bacteria in the alveolar spaces.*

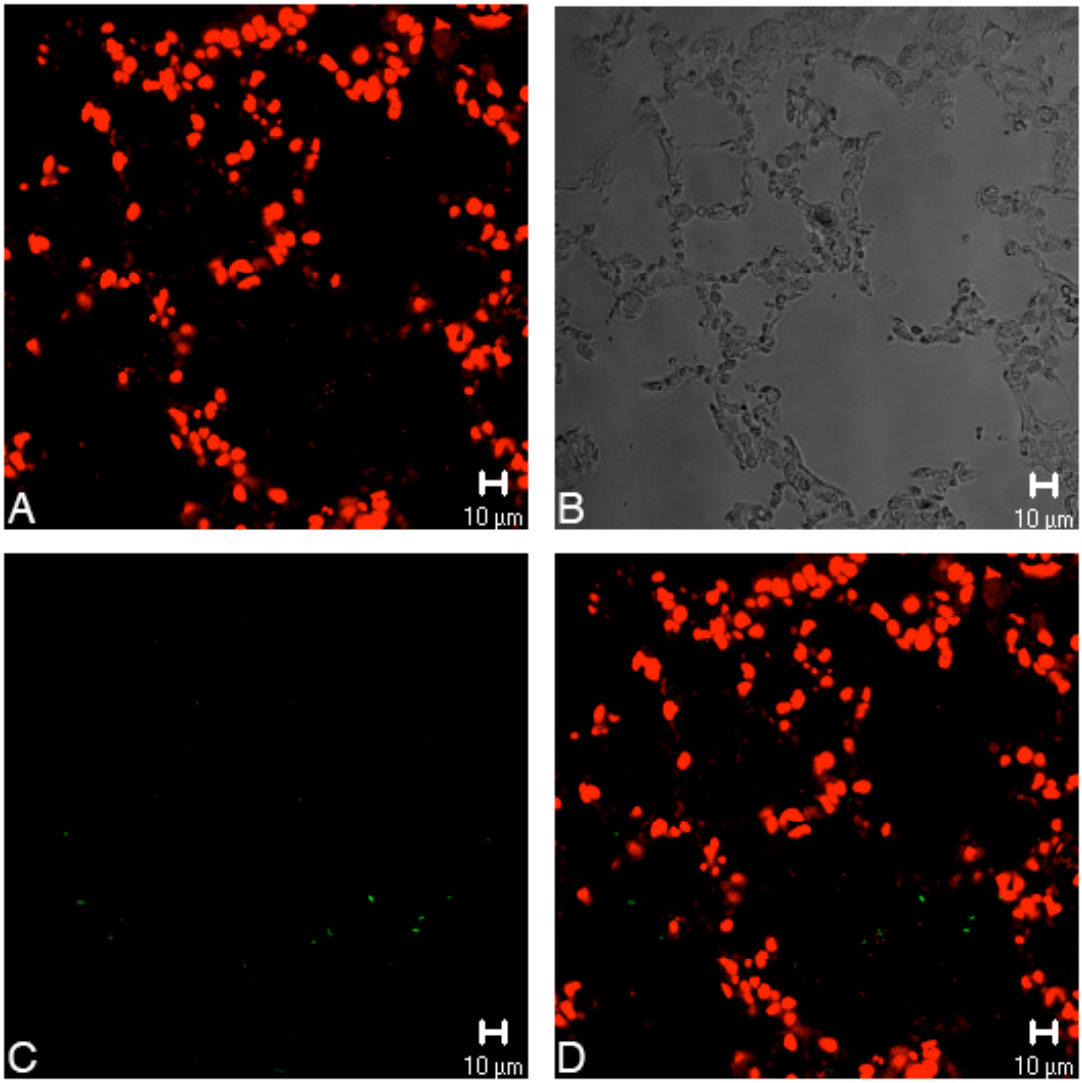


Figure 3.4.5 *Fluorescent microscopy showing relationship between platelets and* S. pneumoniae *during low dose lung infection (platelet depleted mouse).* ***A.*** *Red filter, bacterial aggregate in the alveolar space.* ***B.*** *Light microscopy.* ***C.*** *Green filter, platelets in alveolar spaces.* ***D.*** *A on C. Platelets are closely associated with bacteria in the alveolar space.*

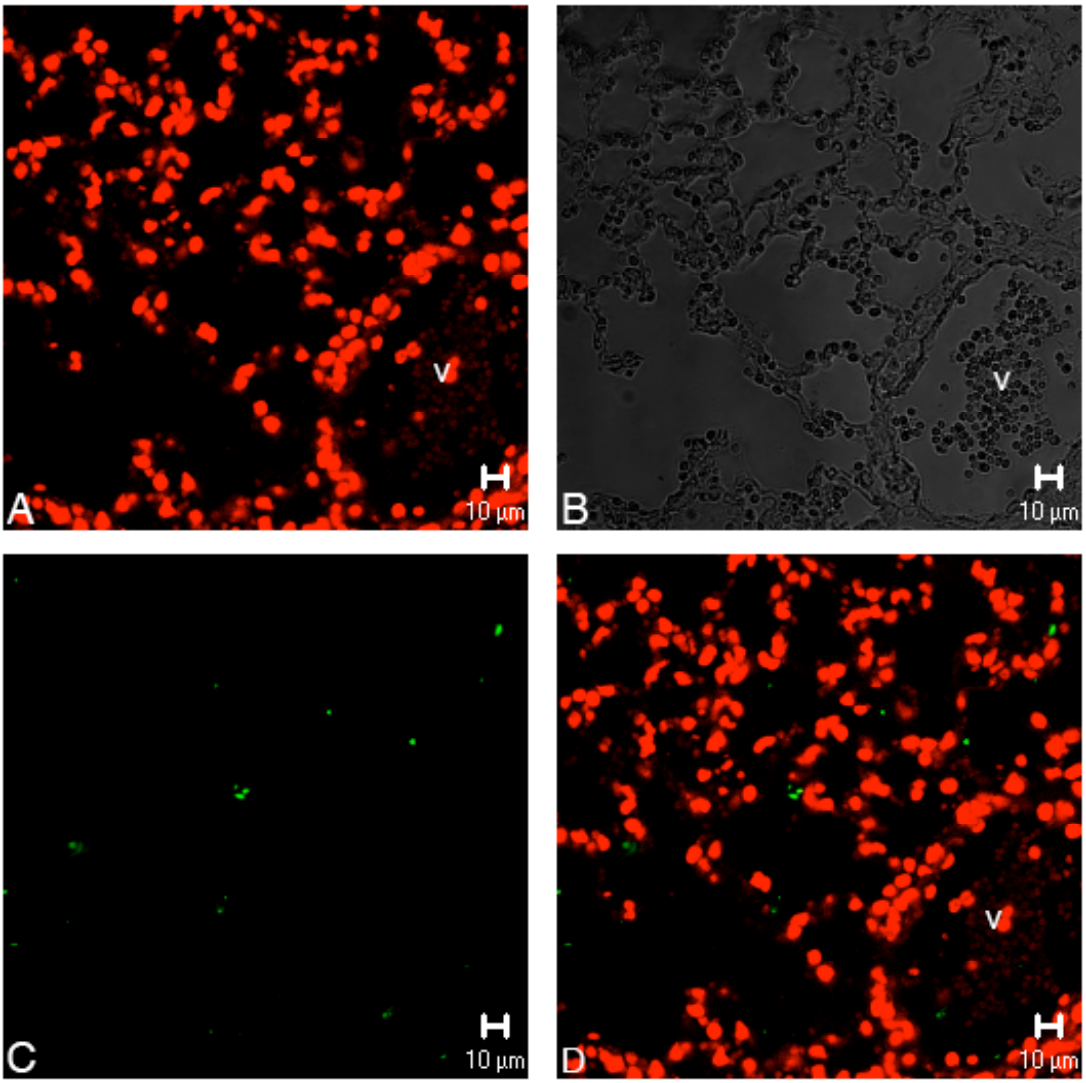


Figure 3.4.6 *Fluorescent microscopy of unchallenged control mouse* ***A.*** *Red filter, no bacterial found* ***B.*** *Light microscopy, no sign of erythrocytes outside blood vessels (v).* ***C.*** *Green filter, platelets outside blood vessels.* ***D.*** *A on C.*

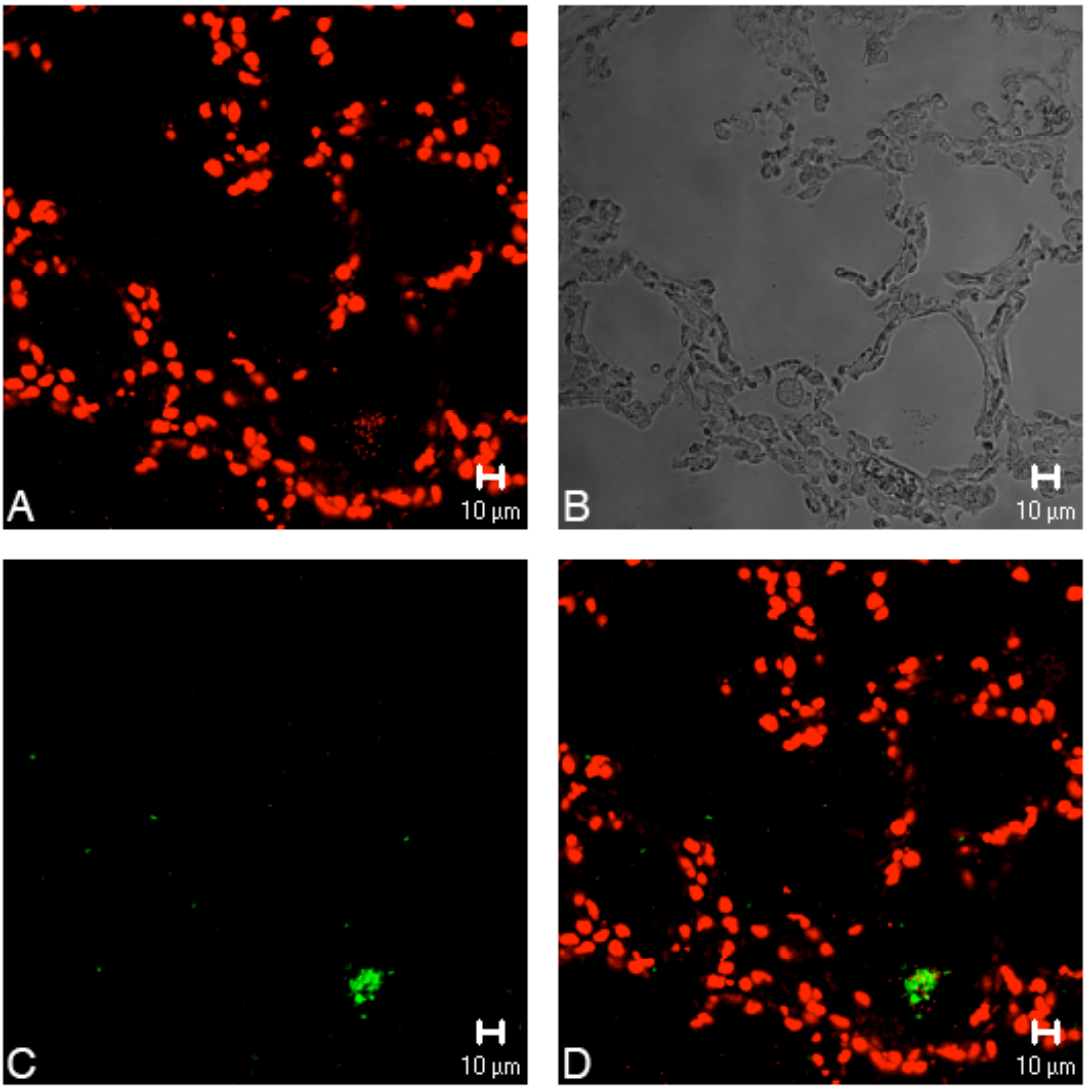


Figure 3.4.7 *Fluorescent microscopy showing relationship between platelets and* S. pneumoniae *during high dose lung infection (platelet depleted mouse).* ***A.*** *Red filter, bacterial aggregate in the alveolar space.* ***B.*** *Light microscopy.* ***C.*** *Green filter, platelets in alveolar spaces.* ***D.*** *A on C. Platelets are closely associated with bacteria in the alveolar space.*

4. DISCUSSION

Platelets have recently been shown to contribute to tissue damage in severe sepsis (Clark et al., 2007) and recent studies have shown that thrombocytes increase the severity of ALI (Kuebler, 2006). In the present study we show that platelets also play a beneficial role in the defence against *Streptococcus pneumoniae* infections. This is an important observation to consider when new treatment strategies affecting platelets are considered for patients with severe sepsis.

4.1 Platelets are activated when in contact with bacteria through a as yet unknown pathway

This project has shown that platelets are activated by *S. pneumoniae* bacteria without interactions with other cells, especially neutrophils or endothelial cells, which agrees with previous studies (Zimmerman & Spiegelberg, 1975). The extent of platelet activation is dependent on the bacteria/platelet ratio, with a higher ratio leading to stronger activation, suggesting that platelet activation may especially occur during severe septicaemia, which was confirmed in previously work (Clark et al., 2007). However the activation might also occur in local infectious foci with high bacterial counts. Platelet aggregation is to a certain extent Ca^{2+} dependent something that should be considered when evaluating anticoagulant (acid citrate, EDTA) treated platelets function *in vitro*. Even further, *in vitro*, the activation of platelets by bacteria in absorbance assays is neither as strong nor as fast as activation by agonists (ADP or TF). However under *in vivo* conditions the effects of bacteria on platelet activation will also be influenced by the presence of other platelet activating factors such as thrombin, levels of which are locally elevated during infection (Günther et al., 2000; Schultz et al., 2006). Activation of platelets *in vivo* has also been described to occur within minutes after LPS challenge (Kiefmann et al., 2006; Yu et al., 2005). This suggests that platelets become activated rapidly after bacterial stimuli under altered conditions and quickly can respond to infection. Platelets do aggregate in response to LPS but only in extreme doses in this assay, contradicting previous reports (Clark et al., 2007), which may not have used such a high dose of LPS. The activation by LPS supports the fact that platelets express functional TLR4 as previously described (Andonegui et al., 2005; Aslam *et al.*, 2006). *S. pneumoniae* express TLR2-ligands, (cell wall components) as well as pneumolysin a TLR4 agonist (Yoshimura et al., 1999). Murine and human platelets also express TLR2 (Aslam et al., 2006) suggesting a possible way of platelet activation by bacteria. However we have shown that heat deactivated *S. pneumoniae* didn't activate platelets to the same degree as live bacteria. The reason to this phenomena remains to be answered but some thesis are i) platelets might become activated through another process than that of TLR2 ii) live *S. pneumoniae* release staphylococcus like antiplatelet activator proteins (Sheu *et al.*, 2000) iii) bacteria binds platelets through platelet activating factor receptor (PAFR) promoting their aggregation iv) TLR2 ligands denature during the heat process, althoughTLR2 ligands have been shown to stimulate TLR2 signalling even after going through heat-treatment (Yoshimura et al., 1999).

4.2 Platelets release antimicrobial mediators that are bactericidal to *S. pneumoniae*

Platelets release antimicrobial proteins in response to different stimuli (Yeaman et al., 1997). We have shown that mediators released from ADP stimulated platelets did not possess any bactericidal or bacteriostatic properties to *S. pneumoniae*, while platelets activated by thrombin released antimicrobial proteins that are bactericidal to *S. pneumoniae.* Previous studies have shown that thrombin activates human platelets through PAR1 and PAR4 (Kahn et al., 1998) suggesting that the release of antimicrobial mediators is dependent on signalling through one of these receptors. However platelets stimulated with a strict PAR1 agonist did not in preliminary experiments release any antimicrobial mediators suggesting that the release of antimicrobial mediators is PAR1 independent. This implies that platelets require stimulation through PAR4 or other unidentified receptors to release antimicrobial proteins. However these experiments have only been performed once and therefore further investigation is needed. In addition even though thrombin itself did not possess any antimicrobial properties we can not exclude the possibility that thrombin, in association with platelets, may still promote platelet antimicrobial properties through other pathways.

4.3 Platelets have a protective role in *S. pneumoniae* infection in a mouse pneumonia model

We have shown that mice depleted of platelets have higher bacterial count in all targeted tissue samples 24 hours post challenge. However, bleeding in thrombocytopenic mice caused contamination of the BALF with blood. Since the bacterial cfu are higher in blood than BALF a quantification was performed to ensure that the differences in cfu in the BALF between control and thrombocytopenic mice were not due to this blood contamination. This showed that less than 0.5% of the volume of the BALF was due to blood contamination. In my experiment the mean cfu/ml blood in platelet-depleted mice were 2.118×10^7 resulting in $<1.1 \times 10^5$ cfu/ml BALF might be due to blood contamination. The mean cfu/ml BALF in depleted group were 2.966×10^6 cfu/ml and for the control group 2.544×10^4 cfu/ml thus the cfu differences in BALF between the groups was only in a minor extent due to the blood contamination. It has been suggested that neutrophil sequestration into the lung in acute lung injury is platelet dependent (Zarbock *et al.*, 2006), but this study has shown that the total amount of neutrophils is higher in BALF in the platelet-depleted group (Andonegui et al., 2005). We also show that even when possible contamination of the BALF with neutrophils from blood is considered the difference is still statistically significant. However the ratio of bacteria to neutrophil is higher in platelet-depleted mice suggesting that even though the total amount of neutrophils is higher in platelet-depleted groups there maybe some relative impairment of neutrophil recruitment in thrombocytopenic mice. Whether this is due to the depletion of platelets or the nonlinear relationship between bacterial amount and the neutrophil sequestration out into lung still needs to be answered. Surprisingly there was no differences in the spread of bacteria from lung tissue to the blood stream between platelet-depleted and control groups, suggesting that the platelets protective role in infection isn't due to a tissue-blood barrier function of the platelets.

4.4 Platelets trap bacteria in alveolar spaces without interaction with neutrophils

Formation of fibrin and local activation of the coagulation cascade is a common feature in acute lung injury (Schultz et al., 2006). It is also known that platelets are important in the early formation of fibrin. Furthermore platelets are known to sequestrate out into lung tissue in response to severe sepsis or endotoxinemia (Kiefmann et al., 2006; Yu et al., 2005) positioning them at the scene of fibrin formation. This project found that the amount of platelets associated with alveolar tissue in challenged controls where much higher than that of unchallenged controls indicating a gathering of platelets in response to *S. pneumoniae* pneumonia. A gathering of platelets in lung microvasculature was also seen within minutes after LPS challenge supporting that platelets quickly accumulate in lung in response to pathogens (Kiefmann et al., 2006; Yu et al., 2005). Fibrin formation might be a physiological attempt to keep infection local and we have, for the first time to our knowledge, shown that platelets form aggregates around *S. pneumoniae* in the alveolar spaces. This appearance suggests that platelets may trap the bacteria and thereby assist host defence against bacterial colonisation of the lung. We also found these aggregates in platelet-depleted groups but they were much smaller and less frequent than in the control groups. However we didn't observe this bacterial trapping *in vitro* suggesting that other cell interaction is needed to form these aggregates *in vivo*. Further more we failed to show that platelets move towards bacteria *in vitro* implying that signalling from other cell types, or directly from infectious foci, is needed for platelet aggregates to form. Interestingly the aggregates formed were, in contrast to the LPS model (Clark et al., 2007), not associated with neutrophils, suggesting that this trapping mechanism is different from that seen with NETs and gram negative bacteria. To support this we have shown that neutrophil derived products do not promote activation of platelets *in vitro*.

4.6 Platelets in high dose pneumonia model

No differences in cfu in high dose *S. pneumoniae* challenges were detected. Two experimental reasons may have caused this result. Firstly, higher cfu challenges allow the infection to progress more rapidly and will obscure differences between mouse groups. Secondly, during this experiment the bacterial challenge was performed 1 hour after injection of antiplatelet antibody thus the platelet pool within the lung tissue might not have been completely depleted during the early phase of infection.

4.7 Future directions

Future work could include more activation parameters in the assessment of platelets activation in response to bacteria. This would be to clarify platelet activation at early time points in infection that might be missed due to fibrin formation in the absorbance test. A less crude assay of activation is needed, possible alternatives could be: flow cytometry or the release of platelet factor 4 (PF-4). Cytokine assessments of the BALF and serum samples could be performed to assess possible modulation of immunity by platelets. To investigate if platelets are found in the normal lung tissue as a helper in the immune defence, immunohistochemistry of normal lung tissue, which has not been subjected to BAL, must be performed. If a platelet pool can be detected a high dose challenge might be reconsidered thus giving the antiplatelet antibody more time. This could be important as 1 hr from injection of antibody to challenge might not have been enough to deplete the platelet pool in the lung. An absorbance test of platelets under the influence of live bacteria suspended in heat deactivated bacteria supernatant would also be of value to rule out the possibility that *S. pneumoniae* release antiplatelet activator peptides.

4.8 Summary and conclusions

Overall the results in this thesis indicate that platelets play an important role in the immune defence against *S. pneumoniae*. This project has shown that platelets form aggregates around bacteria *in vivo,* seemingly without the influence of neutrophils. *In vitro* we have shown that platelets are activated by bacteria without other cell interactions. A higher ratio of bacteria to platelets leads to a stronger activation. We have also shown that platelets release antimicrobial proteins in response to thrombin, which suggests that in inflammatory foci, which will contain thrombin, the platelet aggregates may possess direct antimicrobial properties. My data provides more support that thrombocytes have a positive role in host immunity and the effects on these of potential anti platelet treatment strategies for sepsis need to be considered.

REFERENCES

Andonegui, G., Kerfoot, S., McNagny, K., Ebbert, K., Patel, K., & Kubes, P. (2005). Platelets express functional toll-like receptor-4. *Blood, 106*(7), 2417-2423.

Aslam, R., Speck, E., Kim, M., Crow, A., Bang, K., Nestel, F., et al. (2006). Platelet toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor-alpha production *in vivo*. *Blood, 107*(2), 637-641.

Bajzar, L., Morser, J., & Nesheim, M. (1996). Tafi, or plasma procarboxypeptidase b, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *J Biol Chem, 271*(28), 16603-16608.

Bednar, B., Condra, C., Gould, R., & Connolly, T. (1995). Platelet aggregation monitored in a 96 well microplate reader is useful for evaluation of platelet agonists and antagonists. *Thromb Res, 77*(5), 453-463.

Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A., & Henriques-Normark, B. (2006). An endonuclease allows streptococcus pneumoniae to escape from neutrophil extracellular traps. *Curr Biol, 16*(4), 401-407.

Bernard, G., Vincent, J., Laterre, P., LaRosa, S., Dhainaut, J., Lopez-Rodriguez, A., et al. (2001). Efficacy and safety of recombinant human activated protein c for severe sepsis. *N Engl J Med, 344*(10), 699-709.

Bizzozero, J. (1882). Über einen neuen forrnbestandteil des blutes und dessen rolle bei der thrombose und blutgerinnung. *Arch Pathol Anat Phys Klin Med*.

Burstein, S. (1994). Effects of interleukin 6 on megakaryocytes and on canine platelet function. *Stem Cells, 12*(4), 386-393.

Carr, C., Bild, G., Chang, A., Peer, G., Palmier, M., Frazier, R., et al. (1994). Recombinant e. Coli-derived tissue factor pathway inhibitor reduces coagulopathic and lethal effects in the baboon gram-negative model of septic shock. *Circ Shock, 44*(3), 126-137.

Chauhan, A., Goerge, T., Schneider, S., & Wagner, D. (2007). Formation of platelet strings and microthrombi in the presence of adamts-13 inhibitor does not require p-selectin or beta3 integrin. *J Thromb Haemost, 5*(3), 583-589.

Clark, S., Ma, A., Tavener, S., McDonald, B., Goodarzi, Z., Kelly, M., et al. (2007). Platelet tlr4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med, 13*(4), 463-469.

Dahlbäck, B., & Villoutreix, B. (2005). The anticoagulant protein c pathway. *FEBS Lett, 579*(15), 3310-3316.

Doerschuk, C., Downey, G., Doherty, D., English, D., Gie, R., Ohgami, M., et al. (1990). Leukocyte and platelet margination within microvasculature of rabbit lungs. *J Appl Physiol, 68*(5), 1956-1961.

Elzey, B., Tian, J., Jensen, R., Swanson, A., Lees, J., Lentz, S., et al. (2003). Platelet-mediated modulation of adaptive immunity. A communication link between innate and adaptive immune compartments. *Immunity, 19*(1), 9-19.

Esmon, C. (2001). Role of coagulation inhibitors in inflammation. *Thromb Haemost, 86*(1), 51-56.

Esmon, C. (2004). The impact of the inflammatory response on coagulation. *Thromb Res, 114*(5-6), 321-327.

Faust, S., Levin, M., Harrison, O., Goldin, R., Lockhart, M., Kondaveeti, S., et al. (2001). Dysfunction of endothelial protein c activation in severe meningococcal sepsis. *N Engl J Med, 345*(6), 408-416.

Feldman, B. F., Zinkl, J. G., & Jain, N. C. (2000). Schalm´s veterinary hematology 5th ed.

Feldman, C., Kallenbach, J., Levy, H., Reinach, S., Hurwitz, M., Thorburn, J., et al. (1989). Community-acquired pneumonia of diverse aetiology: Prognostic features in patients admitted to an intensive care unit and a "Severity of illness" Core. *Intensive care medicine, 15*(5), 302-307.

Fuchs, T., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., et al. (2007). Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol, 176*(2), 231-241.

Giannouli, C., & Kletsas, D. (2006). Tgf-beta regulates differentially the proliferation of fetal and adult human skin fibroblasts via the activation of pka and the autocrine action of fgf-2. *Cell Signal, 18*(9), 1417-1429.

Günther, A., Mosavi, P., Heinemann, S., Ruppert, C., Muth, H., Markart, P., et al. (2000). Alveolar fibrin formation caused by enhanced procoagulant and depressed fibrinolytic capacities in severe pneumonia. Comparison with the acute respiratory distress syndrome. *Am J Respir Crit Care Med, 161*(2 Pt 1), 454-462.

Guyton, A. C., & Hall, J. E. (2006). Textbook of medical physiology. (Eleventh edition).

Henn, V., Slupsky, J., Gräfe, M., Anagnostopoulos, I., Förster, R., Müller-Berghaus, G., et al. (1998). Cd40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature, 391*(6667), 591-594.

Kahn, M., Zheng, Y., Huang, W., Bigornia, V., Zeng, D., Moff, S., et al. (1998). A dual thrombin receptor system for platelet activation. *Nature, 394*(6694), 690-694.

Kaushansky, K. (1995). Thrombopoietin: The primary regulator of platelet production. *Blood, 86*(2), 419-431.

Kawano, H., Tsuji, H., Nishimura, H., Kimura, S., Yano, S., Ukimura, N., et al. (2001). Serotonin induces the expression of tissue factor and plasminogen activator inhibitor-1 in cultured rat aortic endothelial cells. *Blood, 97*(6), 1697-1702.

Kiefmann, R., Heckel, K., Schenkat, S., Dörger, M., & Goetz, A. (2006). Role of p-selectin in platelet sequestration in pulmonary capillaries during endotoxemia. *J Vasc Res, 43*(5), 473-481.

Knaus, W., Draper, E., Wagner, D., & Zimmerman, J. (1985). Apache ii: A severity of disease classification system. *Crit Care Med, 13*(10), 818-829.

Kobayashi, M., Shimada, K., & Ozawa, T. (1990). Human recombinant interleukin-1 beta- and tumor necrosis factor alpha-mediated suppression of heparin-like compounds on cultured porcine aortic endothelial cells. *J Cell Physiol, 144*(3), 383-390.

Kuebler, W. (2006). Selectins revisited: The emerging role of platelets in inflammatory lung disease. *J Clin Invest, 116*(12), 3106-3108.

Lähteenmäki, K., Kuusela, P., & Korhonen, T. (2001). Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev, 25*(5), 531-552.

Lauderdale, T., Chang, F., Ben, R., Yin, H., Ni, Y., Tsai, J., et al. (2005). Etiology of community acquired pneumonia among adult patients requiring hospitalization in taiwan. *Respiratory medicine, 99*(9), 1079-1086.

Levi, M., & van der Poll, T. (2005). Two-way interactions between inflammation and coagulation. *Trends Cardiovasc Med, 15*(7), 254-259.

Levi, M., van der Poll, T., & Büller, H. (2004). Bidirectional relation between inflammation and coagulation. *Circulation, 109*(22), 2698-2704.

Li, J., Xia, Y., & Kuter, D. (1999). Interaction of thrombopoietin with the platelet c-mpl receptor in plasma: Binding, internalization, stability and pharmacokinetics. *Br J Haematol, 106*(2), 345-356.

López, R. (2006). Pneumococcus: The sugar-coated bacteria. *Int Microbiol, 9*(3), 179-190.

Lwaleed, B., & Bass, P. (2006). Tissue factor pathway inhibitor: Structure, biology and involvement in disease. *J Pathol, 208*(3), 327-339.

McMichael, M. (2005). Primary hemostasis. Journal of veterinary emergency and critical care, 15(1), 1-8.

McVey, J. (1999). Tissue factor pathway. *Baillieres Best Pract Res Clin Haematol, 12*(3), 361-372.

Meseguer, J., Esteban, M., & Rodríguez, A. (2002). Are thrombocytes and platelets true phagocytes? *Microsc Res Tech, 57*(6), 491-497.

Mileno, M., Margolis, N., Clark, B., Dinarello, C., Burke, J., & Gelfand, J. (1995). Coagulation of whole blood stimulates interleukin-1 beta gene expression. *J Infect Dis, 172*(1), 308-311.

Monroe, D., & Hoffman, M. (2006). What does it take to make the perfect clot? *Arterioscler Thromb Vasc Biol, 26*(1), 41-48.

Moreno, S., Alves-Filho, J., Rios-Santos, F., Silva, J., Ferreira, S., Cunha, F., et al. (2006). Signaling via platelet-activating factor receptors accounts for the impairment of neutrophil migration in polymicrobial sepsis. *J Immunol, 177*(2), 1264-1271.

Nawroth, P., & Stern, D. (1986). Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J Exp Med, 163*(3), 740-745.

Nemi, C. J. (1993). Essentials of veterinary hematology.

Podor, T., Singh, D., Chindemi, P., Foulon, D., McKelvie, R., Weitz, J., et al. (2002). Vimentin exposed on activated platelets and platelet microparticles localizes vitronectin and plasminogen activator inhibitor complexes on their surface. *J Biol Chem, 277*(9), 7529-7539.

Price, G., Thompson, S., & Kam, P. (2004). Tissue factor and tissue factor pathway inhibitor. *Anaesthesia, 59*(5), 483-492.

Ratanarat, R., Thanakittiwirun, M., Vilaichone, W., Thongyoo, S., & Permpikul, C. (2005). Prediction of mortality by using the standard scoring systems in a medical intensive care unit in thailand. *Journal of the Medical Association of Thailand = Chotmaihet thangphaet, 88*(7), 949-955.

Ruggeri, Z. (2003). Von willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost, 1*(7), 1335-1342.

Ruggeri, Z., Dent, J., & Saldívar, E. (1999). Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood, 94*(1), 172-178.

Saba, H., & Morelli, G. (2006). The pathogenesis and management of disseminated intravascular coagulation. *Clinical advances in hematology & oncology : H&O, 4*(12), 919-926.

Savage, B., Saldívar, E., & Ruggeri, Z. (1996). Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von willebrand factor. *Cell, 84*(2), 289-297.

Schultz, M., Haitsma, J., Zhang, H., & Slutsky, A. (2006). Pulmonary coagulopathy as a new target in therapeutic studies of acute lung injury or pneumonia--a review. *Crit Care Med, 34*(3), 871-877.

Schütte, H., Lohmeyer, J., Rosseau, S., Ziegler, S., Siebert, C., Kielisch, H., et al. (1996). Bronchoalveolar and systemic cytokine profiles in patients with ards, severe pneumonia and cardiogenic pulmonary oedema. *Eur Respir J, 9*(9), 1858-1867.

Sheu, J., Hsiao, G., Lee, C., Chang, W., Lee, L., Su, C., et al. (2000). Antiplatelet activity of staphylococcus aureus lipoteichoic acid is mediated through a cyclic amp pathway. *Thromb Res, 99*(3), 249-258.

Sigismund, M. J. (1865). Ein heizbarer objecttisch und seine vermendung bei untersuchungen des blutes.

Tajima, T., Nakayama, E., Kondo, Y., Hirai, F., Ito, H., Iitsuka, T., et al. (2006). Etiology and clinical study of community-acquired pneumonia in 157 hospitalized children. *J Infect Chemother, 12*(6), 372-379.

Taylor, F., Chang, A., Esmon, C., D'Angelo, A., Vigano-D'Angelo, S., & Blick, K. (1987). Protein c prevents the coagulopathic and lethal effects of escherichia coli infusion in the baboon. *J Clin Invest, 79*(3), 918-925.

Taylor, F., Chang, A., Ruf, W., Morrissey, J., Hinshaw, L., Catlett, R., et al. (1991). Lethal e. Coli septic shock is prevented by blocking tissue factor with monoclonal antibody. *Circ Shock, 33*(3), 127-134.

Ten Cate, H. (2003). Trombocytopenia: One of the markers of disseminated intravascular coagulation. *Pathophysiol Haemost Thromb, 33*(5-6), 413-416.

Thrall, M. A., Baker, D. C., Campbell, T. W., DeNicola, D., Fettman, M. J., Lassen, E. D., et al. (2004). Veterinary hematology and clinical chemistry.

Urban, C., Lourido, S., & Zychlinsky, A. (2006). How do microbes evade neutrophil killing? *Cell Microbiol, 8*(11), 1687-1696.

Van de Wouwer, M., Collen, D., & Conway, E. (2004). Thrombomodulin-protein c-epcr system: Integrated to regulate coagulation and inflammation. *Arterioscler Thromb Vasc Biol, 24*(8), 1374-1383.

van der Poll, T., de Jonge, E., & Levi, M. (2001). Regulatory role of cytokines in disseminated intravascular coagulation. *Semin Thromb Hemost, 27*(6), 639-651.

van der Poll, T., Levi, M., Hack, C., ten Cate, H., van Deventer, S., Eerenberg, A., et al. (1994). Elimination of interleukin 6 attenuates coagulation activation in experimental endotoxemia in chimpanzees. *J Exp Med, 179*(4), 1253-1259.

van der Sluijs, K., van Elden, L., Nijhuis, M., Schuurman, R., Florquin, S., Shimizu, T., et al. (2006). Involvement of the platelet-activating factor receptor in host defense against streptococcus pneumoniae during postinfluenza pneumonia. *Am J Physiol Lung Cell Mol Physiol, 290*(1), L194-199.

Ware, L., Camerer, E., Welty-Wolf, K., Schultz, M., & Matthay, M. (2006). Bench to bedside: Targeting coagulation and fibrinolysis in acute lung injury. *Am J Physiol Lung Cell Mol Physiol, 291*(3), L307-311.

White, B., Schmidt, M., Murphy, C., Livingstone, W., O'Toole, D., Lawler, M., et al. (2000). Activated protein c inhibits lipopolysaccharide-induced nuclear translocation of nuclear factor kappab (nf-kappab) and tumour necrosis factor alpha (tnf-alpha) production in the thp-1 monocytic cell line. *Br J Haematol, 110*(1), 130-134.

Yeaman, M., & Bayer, A. (1999). Antimicrobial peptides from platelets. *Drug Resist Updat, 2*(2), 116-126.

Yeaman, M., Puentes, S., Norman, D., & Bayer, A. (1992). Partial characterization and staphylocidal activity of thrombin-induced platelet microbicidal protein. *Infect Immun, 60*(3), 1202-1209.

Yeaman, M., Soldan, S., Ghannoum, M., Edwards, J., Filler, S., & Bayer, A. (1996). Resistance to platelet microbicidal protein results in increased severity of experimental candida albicans endocarditis. *Infect Immun, 64*(4), 1379-1384.

Yeaman, M., Tang, Y., Shen, A., Bayer, A., & Selsted, M. (1997). Purification and *in vitro* activities of rabbit platelet microbicidal proteins. *Infect Immun, 65*(3), 1023-1031.

Yoshimura, A., Lien, E., Ingalls, R., Tuomanen, E., Dziarski, R., & Golenbock, D. (1999). Cutting edge: Recognition of gram-positive bacterial cell wall components by the innate immune system occurs via toll-like receptor 2. *J Immunol, 163*(1), 1-5.

Yu, Z., Ohtaki, Y., Kai, K., Sasano, T., Shimauchi, H., Yokochi, T., et al. (2005). Critical roles of platelets in lipopolysaccharide-induced lethality: Effects of glycyrrhizin and possible strategy for acute respiratory distress syndrome. *Int Immunopharmacol, 5*(3), 571-580.

Zarbock, A., Singbartl, K., & Ley, K. (2006). Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. *J Clin Invest, 116*(12), 3211-3219.

Zimmerman, T., & Spiegelberg, H. (1975). Pneumococcus-induced serotonin release from human platelets. Identification of the participating plasma/serum factor as immunoglobulin. *J Clin Invest, 56*(4), 828-834.