The role of tetraspanins in neutrophil survival and phagocytosis

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Abstract

Inflammation is a beneficial process in the body that involves immune cell migration in the tissue in response to injury or infection. Failure to resolve inflammation is seen in chronic inflammatory diseases, such as Chronic Obstructive Pulmonary Disease (COPD). Neutrophils are at the very centre of this process, where they persist in the tissue due to ongoing recruitment and delayed apoptosis. Furthermore, failure to phagocytose and kill important clinical pathogens such as the multi-antibiotic resistant *Staphylococcus aureus,* compounds the pathogenesis of inflammatory disease and patients succumb to life-threatening infections. The aim of this thesis was to determine whether tetraspanins, a family of transmembrane receptors that play roles in cell survival and immune functions, are involved in neutrophil lifespan and phagocytosis.

Primary human neutrophils were isolated from the blood of healthy subjects and COPD patients. Anti-CD63 antibodies and Fab fragments delayed constitutive neutrophil apoptosis of both healthy and COPD neutrophils as indicated by morphology and Annexin-V staining. Donors varied in their responsiveness to anti-CD63 antibodies. All subjects expressed CD63 and survival rates did not correlate with CD63 regulation.

Anti-CD151 antibodies significantly reduced neutrophil phagocytosis of heat killed and live *S. aureus* but not *S. pneumoniae* or *H. influenzae*. Gentamicin protection assays confirmed that anti-CD151 antibodies reduced the number of intracellular viable *S. aureus*. Fluorescent microscopy showed that anti-CD151 antibodies reduced the numbers of pHrodo stained *S. aureus* present in the acidified phagosome.

The data presents novel and potential roles for tetraspanins in pathways underpinning neutrophil survival and interaction with *S. aureus*. Our findings suggest that CD63 may play a role in neutrophil survival while CD151 may play a key role in neutrophil interaction with *S. aureus*. The study provides two potential molecular targets for modulating neutrophil lifespan and *S. aureus* neutrophil interactions.

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Abbreviation list

+ve- positive

ADAM10- metalloproteinase domain-containing protein 10

AMP- antimicrobial peptides

AP- adaptor protein

Apaf-1- apoptotic-protease-activating factor-1

APC- allophycocyanin

APS- ammonium persulfate

ARDS- acute respiratory distress syndrome

ARF1/6- ADP-ribosylation factor 1/6

Bad- Bcl-2 associated death promoter

Bak- Bcl-2 associated antagonist/killer

Bax- Bcl-2 associated x protein

Bcl-2- B cell lymphoma 2

BH 1-4- BH domains 1-4

BHI- brain heart infusion

Bid- BH3 interacting-domain death agonist

Bik- Bcl-2 interacting killer

Bim- Bcl-2 like 11

BPI- bactericidal permeability increasing protein

BSA- bovine serum albumin

CCG- Cys-Cys-Gly

CCL2/12/5/1- C-C motif chemokine ligand 2

CD- cluster of differentiation

CFU- colony forming unit

CGD- chronic granulomatosis disease

CHIPS- chemotaxis inhibitory protein of *Staphylococcus*

Chr-chromosomal

COPD- chronic obstructive pulmonary disease

CR3- complement receptor 3

CXCL12/1/2- chemokine ligand 12/1/2/5

CXCR4/2- chemokine receptor 4/2

DAG- diacylgycerol

DAPI- 4, 6-diamidino-2-phenyl-indolehydrochloride

DAMP- damage associated molecular patters

DED- FADD N-terminal the death effector domain

DISC- death-inducing signalling complex

DMEM- Dulbecco's modified eagle medium

DPX- distrene-80 plasticiser xylene

DTT- dithiothreitol

EAP- extracellular adherence protein

EC1/2- extracellular domain 1/2

E. coli- Escherichia coli

Em- emission wavelength

ESAM- endothelial cell-selective adhesion molecule

ESL-1- E-selectin ligand-1

ER- endoplasmic reticulum

Ex- excitation wavelength

Fab- fragment antigen binding

FACS- fluorescence-activated cell sorting

FADD- Fas-associated death-domain containing protein

FasL- Fas ligand

FBS- fetal bovine serum

FcR- Fc receptor

FITC- fluorescein 5-isothiocynate

FLIPr/FLIPr-like- FPR-1 like inhibitory proteins

fMLP- N-formyl-methionyl-leucyl-phenalanine

fnbA & B- fibronectin binding protein A & B

FPR- formyl peptide receptor

FSC- forward scatter

GAB2- GRB2-associated-binding protein 2

GAP- GTPase-activating protein

G-CSF- granulocyte colony stimulating growth factor

GEF- guanine nucleotide exchange factor

GFP- green fluorescent protein

GM-CSF- granulocyte monocyte colony stimulating factor

GPCR- G-protein coupled receptor

GRB2- growth factor receptor-bound protein 2

GST- glutathione S-transferase

h- hour

H. influenzae- Haemophilus influenzae

HBSS- Hanks Buffered Saline solution

HC- healthy control

HCl- hydrochloric acid

H_2O_2- hydrogen peroxide

HK- heat killed

HPV16-human papilloma virus type 16

Hsp70- heat shock protein 70

ICAM-1/2- Intracellular adhesion molecule-1/2

IgG- immunoglobulin G;

IL-18/1β/8/6/10- Interleukin-18/1β/8/6/10

Ins(1,4,5)P_3- inositol-1,4,5-trisphosphate

IsdA- iron-regulated surface determinant protein A precursor

ITAM- immunoreceptor tyrosine-based motif

JAM-A/B/C- junctional adhesion molecule-A/B/C

LAMP-1- lysososmal-associated membrane protein 1

LEAF- Low Endotoxin, Azide-Free

LFA-1- lymphocyte function-associated antigen 1

LPS- lipopolysaccharide

LPS-RS- lipopolysaccharide from *Rhodobacter sphaeroides*

Mac-1- macrophage-1 antigen

Max- maxima

Mcl-1- myeloid leukemia cell differentiation protein

MDM- monocyte derived machrophages

MFI- mean fluorescence intensity

MHC- major histocompatibility complex

Mins- minutes

MMP- matrix metalloproteinase

MOI- multiplicity of infection

MRSA- methicillin resistant *S. aureus*

NaCl- sodium chloride

NAD- Nicotinamide adenine dinucleotide

NADPH- nicotinamide adenine dinucleotide phosphate

NaOH-sodium hydroxide;

NET- neutrophil extracellular traps

NLR- Nucleotide-binding oligomerization domain protein-like receptor

NMR- nuclear magnetic resonance

NOD- nucleotide-binding oligomerization domain protein

NOS- nitric oxide synthase

NTHi- *Nontypeable Haemophilus Influenzae*

O_2^--singlet oxygen

PAMP- pathogen-associated molecular pattern

PBMCs- peripheral blood mononuclear cells

PBS- phosphate buffer saline

PE-phycoerythrin

PECAM-1- platelet endothelial cell adhesion molecule 1

Pen/Strep- penicillin-streptomycin

PFA- paraformaldehyde

PI- phagocytic index

PI3/4-K- phospahatidylinositol 3/4-kinase

PKC- protein kinase C

PLCγ- phospholipase Cγ

PPP- platelet poor plasma

PRP- platelet rich plasma

PRR- pattern recognition receptors

PS- phospatidylserine

PSGL-1- P-selectin ligand-1

PtdIns(4,5)P_2- phosphatidylinositol -4,5 bisphosphate

PtdIns(3,4,5)P_3- phosphatidylinositol -3,4,5 trisphosphate

PVDF- Polyvinylidene difluoride

PVL- panton valentine leukocidin

Pyo- pyocyanin

Rab-5/7- Ras-related protein 5/7

RBC- red blood cell

RDS- retinal degeneration slow

RPMI- Roswell park Memorial Institute

ROS- reactive oxygen species

RT- room temperature

SAK- staphylokinase

S. aureus- Staphylococcus aureus

Sbi- staphylococcal IgG-binding molecule

SCIN- staphylococcal complement inhibitor

SD- standard deviation

SFK- Src-family kinases

SDS- sodium dodecyl sulfate

SDS-PAGE- sodium dodecyl sulfate- polyacrylamide gel electrophoresis

siRNA- small interfering RNA

SpA- staphylococcal protein A

*S. pneumoniae- Streptococcus pneumonia*e

SOK- surface factor promoting resistance to oxidative killing

SSC- side scatter

SSL5/3/7- staphylococcal superantigen-like 5/3/7

TB- trypan blue

TBS- Tris-buffered saline

TEM- tetraspanin enriched microdomains

TEMED- N,N,N',N'-Tetramethylethylenediamine'

TGF-β- transforming growth factor-β

TIMP-1- tissue inhibitor metalloproteinase-1

TLR- Toll-like receptors

TNFα- Tumor necrosis factor α

TNF-R1- TNF-receptor 1

TRAIL-R1/2- TNF-related apoptosis-inducing ligand receptor 1/2

TSPAN- tetraspanin

VEGF- vascular endothelial growth factor

WASP- Wiskott–Aldrich Syndrome protein

WHO- world health organization

WT- wild type

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Chapter 1-Introduction

1.1. Mammalian immunity

The immune system has a key role in maintaining homeostasis and protecting the organism against invading pathogens. The mammalian immune system can be further divided into innate and adaptive immunity. The innate immune system is mediated primarily by professional phagocytes such as neutrophils and macrophages and represents the first cellular line of defense against intruding pathogens (Silva, 2010). An important feature of the innate immune system is that it uses highly conserved receptors amongst the animal and plant kingdom to recognise specific pathogen structures. Adaptive immunity is considered to be more complex and is mediated largely by B and T lymphocytes (Iwasaki and Medzhitov, 2010, 2015). Lymphocytes express highly specific target receptors and utilise clonal selection and expansion to protect against later stages of infection, as well as maintaining an 'immunological memory' in case the threat is reencountered (Zinkernagel et al., 1996).

1.1.1. Inflammation

The concept of inflammation and its clinical symptoms has been known for a long time (Rather, 1971). The first four 'cardinal' symptoms were first described by Roman Cornelius Celsus in the first century as: redness, swelling, heat and pain. However, only in the second century the fifth 'cardinal' sign of impaired function was added to the list of symptoms. The two great microbiologists Louis Pasteur and Robert Koch went on to hypothesise that microorganisms can cause inflammation. We now know that invading pathogens can cause inflammation as a result of the host trying to contain and clear the infection. This is a highly regulated process, at the base of which neutrophil infiltration has a primary role.

1.1.2. Neutrophils: role in immunity and inflammation

Neutrophils are terminally differentiated, polymorphonucleated leukocytes, first discovered by Elie Metchnikoff in starfish larvae as cells responding to inserted rose thorns (Cavaillon, 2011; Korchak et al., 1983). In humans, neutrophils are the largest group of cells

in the circulation, comprising up to 70% of the leukocyte pool (Smith, 1994). It takes 14 days for the myeloid stem cell progenitor to produce functionally differentiated neutrophils at a rate of 10^8 cells/min (Bainton et al., 1971; Haslett, 1999; Haylock et al., 1992). At any time, humans have up to 10^{10} neutrophils in the circulation but this number can increase 10-fold during stress and infection (Cannistra and Griffin, 1988; Summers et al., 2010). Neutrophils contain a number of granules with highly toxic contents, packed during neutrophil development, with which they can protect against pathogen threats (Borregaard and Cowland, 1997a).

1.1.2.1. Granulocytopoiesis

The production of mature neutrophils and other granule containing cells from the common myeloid progenitor stem cells in the bone marrow is called granulocytopoiesis. The differentiation process as well as release into the circulation is tightly regulated. It is mainly dictated by the rate of neutrophil apoptosis and it requires a number of transcription factors, with PU.1 having a key role in committing the lineage to neutrophil production, and a tight regulation of bone marrow stromal cell-myeloid progenitor cytokine production (Bjerregaard et al., 2003; Borregaard, 2010; Lapidot and Kollet, 2002).

Differentiation of stem cells to neutrophils takes several stages to ensure that the cells are fully functional. The process starts at the myeloblast stage, progressing through promyelocyte, myelocyte, metamyelocyte, band cells, segmented cells and finally to fully functional neutrophils (Glasser and Fiederlein, 1987). During these stages, the neutrophil will acquire granules and granule proteins, they will stop proliferating and finally they will form the segmented nucleus (Borregaard and Cowland, 1997b; Glasser and Fiederlein, 1987). At the promyelocyte stage the azurophilic/primary granules are formed, containing myeloperoxidase, neutrophil elastase, defensins, serine proteases and antimicrobial peptides (Borregaard and Cowland, 1997b; Fouret et al., 1989). Secondary/specific granule containing lactoferrin, collagenase and haptoglobin as well as tertiary/gelatinase granules having gelatinase, ficolin-1 and arginase1 will form in myelocyte, metamyelocyte and band cells stages (Borregaard and Cowland, 1997b; Jacobsen et al., 2007; Rorvig et al., 2009; Theilgaard-Monch et al., 2006). During the segmented cell stage an additional set of

granules, called secretory vesicles are formed. These vesicles contain receptors involved in chemotaxis and recognition of conserved pathogen structures, such as complement receptor 3 (CR3), Fc receptors (FcR) and formyl peptide receptors (FPR) (Borregaard et al., 1992; Borregaard et al., 1987; Nordenfelt and Tapper, 2011).

1.1.2.2. Neutrophil release in the circulation

Once they mature, neutrophils are released from the bone marrow into the circulation in response to cytokine cues. Chemokine receptor 4 (CXCR4), expressed by both mature neutrophils and progenitors, has a key role in retaining the cell in the bone marrow. CXCR4 binds to chemokine ligand 12 (CXCL12) on bone marrow stromal cells and is important in regulating neutrophil homeostasis (Lapidot and Kollet, 2002). Deletion of CXCR4 in mice resulted in failure of neutrophil recruitment in response to stimuli while genetic mutations in the CXCR4 chemokine receptor gene have been positively correlated with neutropenia and WHIM immunodeficiency syndrome (Eash et al., 2009; Hernandez et al., 2003). CXCR2 expression on myeloid cells also has a key role in homing the cells to the bone marrow, as shown by knockout studies where a constitutive mobilisation of neutrophils in circulation was noticed (Eash et al., 2010). Release of mature neutrophils from the bone marrow is pivotally orchestrated by granulocyte colony stimulating growth factor (G-CSF) levels (Cannistra and Griffin, 1988). G-CSF production is downregulated in response to neutrophil apoptosis as a result of a feedback mechanism. Neutrophil clearance results in interleukin-23 (IL-23) decreased levels that promote IL-17A downregulation which ultimately induces G-CSF decreased production (Cannistra and Griffin, 1988; Schwarzenberger et al., 2000). The cytokine has two opposing roles: to disrupt the neutrophil bone marrow retention mechanism, by downregulating CXCR4 and its CXCL12 ligand, and to promote neutrophil migration by upregulating the production of CXCR2 ligands, CXCL1 and CXCL2, on bone marrow endothelium (Eash et al., 2010; Kim et al., 2006; Semerad et al., 2005; Wengner et al., 2008).

1.1.3. Neutrophil recruitment to the site of inflammation and bacterial clearance

Under normal circumstances, neutrophils are released from the bone marrow in an inactivated state which is maintained until the cells are primed by a stimulus, making them ready to elicit an antimicrobial and inflammatory function (Southgate et al., 2008). Neutrophil priming often results in a mixed population containing both inactive cells as well as partially or fully activated neutrophils, as shown by both *in vitro* and *in vivo* studies (Gallin, 1984). Neutrophil priming is an essential step in neutrophil recruitment and migration to the site of infection/inflammation and is often done by inflammatory cytokines released from tissue such as granulocyte macrophage- colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) and IL-1α (Ekpenyong et al., 2015). This is, however, not enough to elicit full neutrophil killing potential and a second activating agent or agonist is required, usually a bacterial product such as lipopolysaccharide (LPS) (Ekpenyong et al., 2015).

1.1.3.1. Movement of neutrophils to the site of inflammation

Neutrophil priming is enough to start neutrophil adhesion to the blood vessel endothelium and subsequent leukocyte extravasation, a process often referred to as the neutrophil adhesion cascade (Ley et al., 2007). The first step that allows initiation of neutrophil adhesion cascade is the activation of the endothelium. This is driven by either cytokines (such as IL-1β, TNF-α, IL-8) released from tissue inflammatory cells, regarded as a slow activation, or by inflammatory stimuli ligation (such as histamines, thrombin and bradykinin) to the specific endothelial cell receptors, typically to G-protein coupled receptors (GPCRs), known as fast activation (Pober and Sessa, 2007). Activated endothelium expresses P- and E-selectin to the cell luminal surface, two molecules involved in neutrophil adhesion (Pober and Sessa, 2007). Neutrophils then undergo the initial steps of tethering and rolling on the endothelial membrane, which requires binding of P-selectin ligand-1 (PSGL-1), CD44 and E-selectin ligand-1 (ESL-1) expressed by neutrophils to P-selectin and E-selectin on endothelial cells (Buscher et al., 2010; Hidalgo et al., 2007). The subsequent neutrophil firm adhesion step is mediated by the lymphocyte function-associated antigen 1 (LFA-1) and macrophage-1 antigen (Mac-1)/ αMβ2 integrin binding on neutrophils to

Intracellular adhesion molecule-1 and 2 (ICAM-1 or 2) on endothelial cells (Woodfin et al., 2010; Zimmerman et al., 1992). Neutrophil L-selectin was shown to have role in this process, although this is controversial. *In vivo* study shows that inhibition of L-selectin shedding by neutrophils resulted in increased neutrophil firm adhesion and extravasation while *in vitro* study showed opposing result (Allport et al., 1997; Hafezi-Moghadam et al., 2001). Tetraspanins CD9 and CD151 can play a role in the endothelial cell membrane structural organisation to facilitate this process (Barreiro et al., 2008). This firm adhesion step coincides with activation of downstream signalling involved in neutrophil formation of the lamellipodium, which is required for the physical transmigration through the tissue, and also recruitment of receptors, needed for bacterial recognition and phagocytosis, to the surface of the cell (Borregaard, 2010; Van Keymeulen et al., 2006). The neutrophil transendothelial migration (i.e. movement through the endothelial layer) can be either paracellular, the most common form of extravasation involving movement between epithelial cells, or transcellular, which is more uncommon and involves penetration of the cell (Woodfin et al., 2010). Paracellular migration is mediated by platelet endothelial cell adhesion molecule 1 (PECAM-1), junctional adhesion molecule (JAM) –A,-B and –C; endothelial cell-selective adhesion molecule (ESAM), CD99, VE-cadherins (all of these have roles in stabilising the endothelial cell-cell junction), while Mac-1, LFA-1 interaction with ICAM-1, ICAM-2 are involved in the deeper migration of the neutrophils between the endothelium (Alcaide et al., 2009; Diamond et al., 1990; Muller et al., 1993; Woodfin et al., 2009). The last step is neutrophil passage through the perivascular basement membrane into the tissue is mediated by CD99-CD99L2 and CD177-PECAM-1 interactions with the help of neutrophil stored matrix metalloproteinases (MMPs), which cleave the basement membrane (Bayat et al., 2010; Bixel et al., 2010; Kang et al., 2001).

1.1.3.2. Bacterial recognition by neutrophils

Once in the tissue, neutrophil migration to the site of infection is mediated by receptors that recognise both conserved bacterial structures and chemoattractants but also chemoattractants produced by the organism in response to the infection, such as the complement C5a (Foxman et al., 1999; Hopken et al., 1996). Chemoattractants can also fully

activate the neutrophil, that is why this process takes place proximal to the infection site to prevent unnecessary self-cell damage (Swain et al., 2002). These receptors are called 'pattern recognition receptors' (PRR) and neutrophils contain a wide variety of such receptors.

1.1.3.2a. Formyl peptide receptors

One type of PRR receptor that neutrophils express are FPRs, involved in the chemotaxis and amplification of the phagocytic process. The receptors recognise formylated bacterial peptides, such as N-formyl-methionyl-leucyl-phenalanine (fMLP), produced by bacteria in high amounts during exponential growth (Bardoel and van Strijp, 2011). Neutrophils also contain specific receptors that allow chemoattractant prioritisation and migration towards the highest concentration of bacteria chemoattractants (Heit et al., 2008). FMLP binding to the FPR, a GPCR, results in Rho/Rac/Cdc42 signalling pathway activation and ROS production (Bokoch, 2005). What is more, this interaction is the prerequisite for neutrophil migration towards the fMLP gradient because it activates p38 which redistributes the chemoattractant prioritisation receptor around the cell, thus eliminating cell polarity and allowing specific neutrophil migration (Billadeau, 2008).

1.1.3.2b. Toll-like and NOD-like receptors

Toll-like receptors (TLRs) are amongst the most studied PRRs and are involved in the recognition of conserved bacterial structures called pathogen-associated molecular patterns (PAMPs). Neutrophils express all known TLRs, except TLR3 (Gordon, 2002; Hayashi et al., 2003; Takeuchi and Akira, 2010). The most studied is TLR4 which mediates recognition of LPS, a Gram-negative outer membrane glycoprotein, with the help of CD14 LPS-binding protein and myeloid differentiation factor 2 (Hayashi et al., 2003; Lugtenberg and Vanalphen, 1983; Takeuchi and Akira, 2010). TLR4 knockout mouse models show failure of neutrophil recruitment in response to LPS (Zhou et al., 2009). TLR2/TLR1 heterodimers can recognise diacylated and triacylated lipopeptides, found on the membrane of Gram negative and positive bacteria, while TLR2/TLR6 heterodimerisation allows recognition only of tryacilated lipopeptides (Bardoel and van Strijp, 2011; Schenk et al., 2009). Downstream

signal of TLR4 and TLR2 heterodimers results in the activation of TNFα, IL-1β and IL-6, cytokines involved in priming and recruitment of innate immune cells at the site of inflammation (Hayashi et al., 2003). TLR5 recognises flagellin, a conserved flagella component (Andersen-Nissen et al., 2007). TLR5 knockout studies have shown important role of the receptor in neutrophil recruitment and pneumonia development in response to *Legionella pneumophila* (Hawn et al., 2007). TLR7, TLR8 and TLR9, unlike the receptors mentioned above, are expressed in the endoplasmic reticulum (ER) (Takeuchi and Akira, 2010). While TLR9 is involved in recognising unmethylated, CpG motif containing DNA and DNA sugar backbone 2' deoxyribose, TLR8 and TLR9 recognise single stranded RNA (Haas et al., 2008; Heil et al., 2004; Takeuchi and Akira, 2010).

Nucleotide-binding oligomerization domain protein (NOD)-like receptors (NLRs) are another type of receptor found in neutrophils, involved in the recognition of muramyl dipeptide peptidoglycan and g-D-glutamyl-mesodiaminopimelic bacterial structures (Berrington et al., 2010; Theivanthiran et al., 2012). Activation of NLRs will trigger increased phagocytosis, oxidative burst and cytokine production (Ekman and Cardell, 2010; Frutuoso et al., 2010).

1.1.3.2c. Phagocytic/Opsonin receptors

Neutrophils also express opsonin receptors: FcγRIIa, FcγRIIIb and CR3 (Fleit et al., 1982; Witko-Sarsat et al., 2000). While FcγRIIIb has a role in recruitment and clustering of FcγRIIa, both recognising IgG coated bacteria, the latter receptor has an important role in activating downstream signalling processes involved in bacteria engulfment and killing, being the most predominant phagocytic receptor in immune complexes (Chuang et al., 2000; Witko-Sarsat et al., 2000). CR3 recognises C3bi coated bacteria but does not directly trigger changes involved in bacterial killing and phagocytosis, however it promotes FcγRIIa cytoplasmic domain phosphorylation to bring about those functions (Schymeinsky et al., 2007; Todd, 1996). Neutrophils also contain CR1 receptor, recognising C3b, and CR4, recognising C3bi, with functions in amplify bacterial phagocytosis and in promoting killing through ROS production and enzyme degranulation (Ross et al., 1978). In addition, neutrophils contain C-type lectin receptors that bind to and recognise conserved pathogen carbohydrate

structures, such as the dectin-1 lectin receptor that recognises fungal β-glucans (Brown, 2006; McGreal et al., 2004).

1.1.3.3. Neutrophil phagosome formation

Neutrophils can kill bacteria through two main mechanisms: the first is phagocytosis which involves engulfment of bacteria and killing in a contained intracellular environment and the second is via neutrophil extracellular traps (NETs) whereby neutrophils trap and kill extracellular bacteria by exuding genetic contents in conjunction with cytosolic and antimicrobial peptides (AMPs) (Brinkmann et al., 2004).

Phagocytosis is the most common mechanism by which neutrophils kill bacteria. It starts via FcγR ligation to the Fc region of IgG coated bacteria, triggering downstream signalling through the phosphorylation and dimerisation of the cytoplasmic tails containing the immunoreceptor tyrosine-based motif (ITAM) (Figure 1.1) (Cox and Greenberg, 2001; Swanson, 2008). The process involves a zipper model where the neutrophil membrane takes the shape of the ingested target. Initial steps in the process involves recruitment of Src-family kinases (SFK) to ITAM, which forms the platform for the recruitment of more kinases that will facilitate phagosome formation such as Syk, phospholipase Cγ (PLCγ), growth factor receptor-bound protein 2 (GRB2), GRB2-associated-binding protein 2 (GAB2) and phosphatidylinositol-3-kinase (PI3-K)(Figure 1.1) (Swanson, 2008). The end result is the conversion of the membrane lipid phosphatidylinositol -4,5 bisphosphate (PtdIns(4,5)P_2) by PI3-K into phosphatidylinositol -3,4,5 trisphosphate (PtdIns(3,4,5)P_3) (Figure 1.1) (Cox and Greenberg, 2001). The lipid has key role in recruiting kinases involved in actin polymerisation and phagosome shaping, including ADP-ribosylation factor 6 (ARF6)-guanine nucleotide exchange factor (GEF), ARF6 GTPase-activating protein (GAP), Rac1 and Cdc42 and Wiskott–Aldrich Syndrome protein (WASP) family, but also in the recruitment of myosin X, an important component in phagosome sealing (Figure 1.1) (Cox et al., 2002; Cox and Greenberg, 2001; Miki and Takenawa, 2003). PLCγ can also promote the conversion of PtdIns(4,5)P_2 to inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylgycerol (DAG) (Figure 1.1) (Swanson, 2008). DAG is a conical shaped lipid, able to flip across the membrane to promote membrane curving and advancement around the bacteria (Carrasco and Merida,

2007). Ins(1,4,5)P_3 allows release of calcium from the ER, a step necessary in phagosome maturation by being part of the actin dissolving process, once the phagosome is closed, to allow granule fusion (Nunes and Demaurex, 2010). If the target is too big neutrophils can bring membranes from other components such as specific and primary granules, late endosome and the ER to aid in the process (Swanson, 2008).

1.1.3.4. Neutrophil antimicrobial mechanism

To give the phagosome antimicrobial activity, the phagosome must undergo a drastic, progressive conversion, known as phagosome maturation. Early phagosome formation starts even before its sealing by fusing with early endosomes, a prerequisite in phagosome sealing (Bajno et al., 2000; Roberts et al., 2000). This then transitions through the late phagosome, characterised by late endosome fusion, to the fully mature phagolysosome where it attains catabolic function through its fusion with neutrophil granules (Borregaard and Cowland, 1997b; Harrison et al., 2003). Granule fusion is a calcium concentration dependent process, taking place in a pre-set order with secretory vesicles being the first ones to fuse with it, followed by tertiary granules, specific and primary granules (Figure 1.1) (Borregaard and Cowland, 1997b; Nunes and Demaurex, 2010; Sengelov et al., 1993). Granule fusion facilitates the assembly of the nicotinamide adenine dinucleotide phosphate (NADPH) complex which pumps protons out of the phagolysososme to promote an acidic pH environment and ROS generation (Kobayashi et al., 2005; Segal, 2005). It also allows protease activation, such as gelatinase and collagenase cleavage by elastase (Delclaux et al., 1996; Kobayashi et al., 2005; Segal, 2005).

As a result, granule fusion exposes the organism to a vast array of AMPs and ROS. AMPs catabolise bacteria by targeting key bacterial survival components or deactivating highly pathogenic bacterial toxins. For example, lysozyme cleaves the bacterial cell wall between N-acetylglucosamine and N-acetyl muramic acid while lactoferrin has the double role of iron chelation, an important nutrient for bacterial survival, and membrane permeabilisation by promoting LPS release (Jenssen and Hancock, 2009; Wecke et al., 1982). Bactericidal permeability increasing protein (BPI) and cationic defensins form pores in the bacterial membrane to allow cell content leakage (Ooi et al., 1987; White et al., 1995). Other proteins

such as elastase are able to degrade membrane protein A, an important protein involved in the pathogenesis of *Staphylococcus aureus* (Belaaouaj, 2002).

Neutrophils ROS vast array can induce severe microbial damage due to their ability to target key bacterial processes required for their survival (Hampton et al., 1998). The process of ROS production is driven by the NADPH complex, which upon assembly in the phagosome and plasma membrane, is able to transfer electrons to oxidise oxygen (Kobayashi et al., 2005). Superoxide can synergistically interact with hydrogen peroxide to induce iron release from the bacterial iron-sulfur clusters, while the iron can be oxidised further to produce additional DNA-damaging ROS products (Jang and Imlay, 2007; Keyer and Imlay, 1996). Myeloperoxidase catalyses the interaction between hydrogen peroxide and different ions to form highly potent acids such as hyperchloric acid (Aiken et al., 2012; Nauseef, 2007). The acid, as well as its precursors, can interact with other proteins to oxidise them or to abrogate bacterial energy transduction and protein biosynthesis at plasma membrane (Hurst, 2012; Witko-Sarsat et al., 2000). Furthermore, superoxide can interact with nitric oxide, produced by either nitric oxide synthase (NOS), or inducible NOS to form peroxynitrite, a key bactericidal molecule with multiple roles including: DNA damage, ion channel inactivation and disruption of signal transduction (Forstermann et al., 1991; Marletta, 1993; Ye et al., 1996).

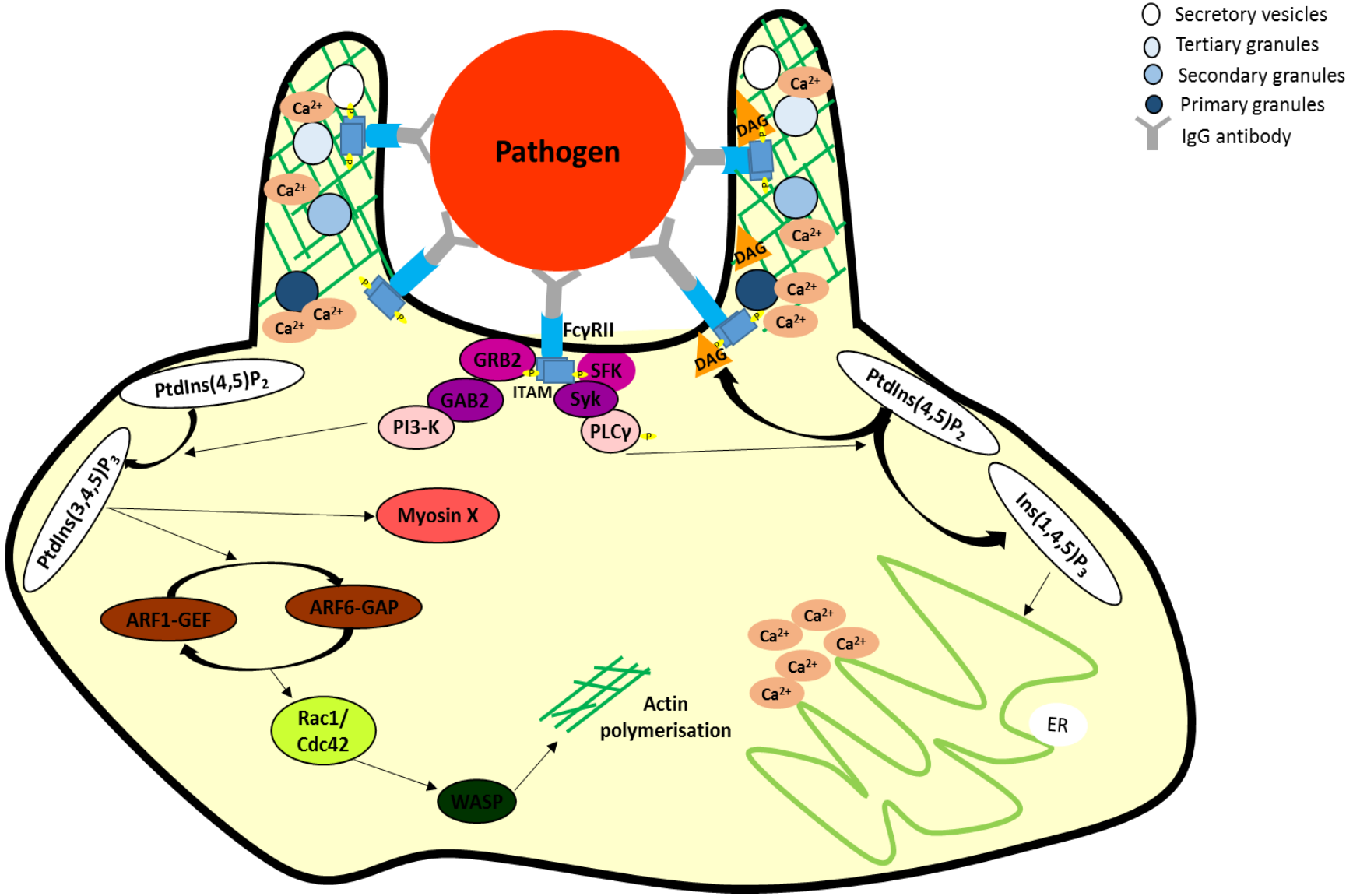
Secretory vesicles
Tertiary granules
Secondary granules
Primary granules
IgG antibody
Pathogen
FcγRII
ITAM
GRB2
SFK
GAB2
Syk
PI3-K
PLCγ
PtdIns(4,5)P2
PtdIns(3,4,5)P3
Ins(1,4,5)P3
DAG
Ca2+
Myosin X
ARF1-GEF
ARF6-GAP
Rac1/
Cdc42
WASP
Actin
polymerisation
ER

Figure 1.1: Bacterial recognition and phagocytosis by neutrophils.

The IgG opsonised pathogen is recognised by FcγRII dimers which recruit SFK via ITAM motifs. This then forms a platform for the recruitment of the Syk kinase which phosphorylates (yellow dot) and activates the ITAM. Recruitment of the GRB2 and GAB2 then takes place to the ITAM region. These kinases activate PI3-K which catalyses the conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. The lipid will recruit myosin X as well as ARF1-GEF and ARF6-GAP. These regulatory proteins in turn promote the activation/ deactivation of Rac1/Cdc42 to recruit WASP family kinases. This will have the end result of actin polymerisation and membrane advancement around the target. Syk also phosphorylates and recruit PLCγ which promotes the conversion of PtdIns(4,5)P_2 into Ins(1,4,5)P_3 and DAG. The latter lipid inserts into the neutrophil membrane and promotes its curving round the target while the latter promotes Ca^{2+} release from the ER. The Ca^{2+} will then promote granule fusion with the phagosome to form the phagolysosome. This will take place in a Ca^{2+} concentration dependent manner, with secretory vesicles being the first to fuse, requiring the least amount of Ca^{2+}, followed by tertiary, secondary and primary granules.

1.1.4. The molecular mechanism of neutrophil apoptosis

Kerr et al., 1972 described for the first time the process of apoptosis as a series of events comprising of chromatin and cytoplasmic condensation, DNA fragmentation and membrane blebbing, allowing subsequent clearance of the cells by macrophages (Bratton and Henson, 2011). Neutrophils are short-lived cells with a half-life in the circulation of 8 hours or, if recruited to the tissue, lifespan can be increased up to 2 days (Bainton et al., 1971; Cronkite and Fliedner, 1964; Elbim and Estaquier, 2010; Kim et al., 2011). However, an alternative study demonstrated that neutrophil lifespan in circulation is 5.4 days, although this discovery is controversial (Pillay et al., 2010; Tak et al., 2013). The main purpose of neutrophil apoptosis is to maintain homeostasis and to allow a quick resolution of inflammation, therefore it happens even in the absence of extracellular stimuli (Filep and El Kebir, 2009). Failure to do so can result in the development of inflammatory diseases which will be described later in this chapter.

The key features that can be observed in apoptotic neutrophils are that they are unresponsive to external stimuli, they are unable to phagocytose and that they express 'find me' and 'eat me' signals to allow macrophage recognition and clearance (Bratton and Henson, 2011; Haslett et al., 1994; Savill and Haslett, 1995). Diseases such as Kostmann syndrome and chronic congenital neutropenia, whereby neutrophil number is dramatically reduced, have shown that an imbalance in neutrophil number by accelerated apoptosis results in failure of the organism to mount an appropriate immune response against invading pathogens (Carlsson et al., 2004; Klein et al., 2007; Rosenberg et al., 2006). This suggests that for a system to function adequately and maintain homeostasis, neutrophil clearance and turnover must be in perfect equilibrium.

Neutrophil apoptosis can be induced via an extrinsic pathway, involving extracellular signals and death receptors activation, or via an intrinsic/mitochondrial pathway resulting from intracellular events such as ROS release, damaged DNA and permeabilisation of the mitochondrial membrane (Geering and Simon, 2011; Maianski et al., 2003; Zhang et al., 2003a). Depending on their location, apoptotic neutrophils are cleared by resident tissue macrophages or homed to the spleen, liver or bone marrow for clearance by specialised

macrophages such as the Kupffer cells in the liver (Bratton and Henson, 2011; Summers et al., 2010). Interestingly, neutrophil clearance in the bone marrow begins when senescent neutrophils express high levels of CXCR4 which allows homing back to CXCL12 (Martin et al., 2003) The lifespan of neutrophils can be modulated by cytokines such as GM-CSF and TNF-α or by factors secreted by pathogens such as LPS and Pyocyanin, and most recently evidence has started to emerge about the involvement of granule proteases in modulating this function (Benarafa and Simon, 2017; Colotta et al., 1992; Haslett et al., 1985; Lee et al., 2003; Rapoport et al., 1992; Usher et al., 2002). Such a granule protease is cathepsin D that is able to mediate neutrophil apoptosis either through a caspase-dependent or independent pathway (Baumann et al., 2013).

1.1.4.1. The Bcl-2 protein family regulates mitochondrial apoptosis

Programmed cell death occurs in most vertebrate cells via the intrinsic/mitochondrial apoptotic pathway (Adams, 2003; Green and Kroemer, 2004). The intrinsic apoptotic pathway depends on the integrity of the mitochondrial membrane. Interestingly, research has suggested that the sole purpose of the neutrophil mitochondria is to modulate apoptosis and not to be involved in energy generation, the main function of mitochondria in other cell types (Maianski et al., 2003).

The integrity of the mitochondrial membrane is maintained by B cell lymphoma 2 (Bcl-2) family of proteins. Although the name of the family comes from the first protein in the group discovered, neutrophils do not express Bcl-2 (Moulding et al., 2001). This family contains two antagonising groups of proteins, pro- and anti-apoptotic (Martinou and Youle, 2011). From these, the anti-apoptotic proteins found in neutrophils are: myeloid leukemia cell differentiation protein (Mcl-1) and A1, while the pro-apoptotic one includes: Bcl-2 associated x protein (Bax), Bcl-2 like 11 (Bim), Bcl-2 associated antagonist/killer (Bak), Bcl-2 associated death promoter (Bad), Bcl-2 interacting killer (Bik) and BH3 interacting-domain death agonist (Bid) (Geering and Simon, 2011; Santos-Beneit and Mollinedo, 2000; van Raam et al., 2006)

The Bcl-2 family regulate cell death via α-helical BH domains (BH1-4) (Zha et al., 1996). The BH3 domain is used by pro-apoptotic proteins to bind into the hydrophobic cleft formed by

the BH1, BH2 and BH3 domain of the anti-apoptotic group to mediate cell death (Adams and Cory, 1998; Chittenden et al., 1995; Zha et al., 1996). In contrast, the BH1 and BH2 regions of the anti-apoptotic group are required for the heterodimerisation with the pro-apoptotic Bcl-2 family members to inhibit cell apoptosis (Yin et al., 1994).

1.1.4.2. The intrinsic/mitochondria neutrophil apoptosis

In neutrophils, Mcl-1 is probably the best studied Bcl-2 family member and has a key role in the regulation of the intrinsic apoptotic pathway. At high levels, the protein inhibits the pro-apoptotic proteins Bim and Bak, and loss of Mcl-1 in mice results in a profound reduction in neutrophil survival (Dzhagalov et al., 2007; Thomas et al., 2010). Mcl-1 half life in neutrophils is only 3 hours due to the presence of a VTLISFG motif that targets the protein for poly-u-biquitination and degradation, explaining the short neutrophil lifespan (Moulding et al., 2001; Xiao et al., 2014). To initiate intrinsic apoptosis, the Mcl-1 levels decrease allowing Bax and Bak oligomerization and insertion in the mitochondrial membrane (Figure 1.2) (Dzhagalov et al., 2007). This results in cytochrome c release which then associates with apoptotic-protease-activating factor-1 (Apaf-1) to form the apoptosome complex (Murphy et al., 2003; Zou et al., 1997). This complex recruits and activates the apical caspase, caspase-9, which then activates caspase-3, leading to the degradation of cellular components and apoptosis induction (Figure 1.2) (Adams, 2003; Murphy et al., 2003; Zou et al., 1997). Interestingly, caspases have been shown to be involved in regulating Mcl-1 by degrading it in aged neutrophils, to promote neutrophil apoptosis, but not at early time points (Wardle et al., 2011). This suggests that that Mcl-1 promotes neutrophil apoptosis by being both an upstream regulator of the pathway but also by having its turnover regulated by downstream caspases. Other members have shown as well important roles in the intrinsic neutrophil apoptosis pathway. For instance, Bim pro-apoptotic protein knockout in mice resulted in increased neutrophil survival and neutrophilia (Bouillet et al., 1999). Moreover, A1 knockout mice present neutrophils that have accelerate intrinsic apoptosis and diminished survival in response to stimuli as well as inability to trans-migrate through the endothelium (Moulding et al., 2001).

1.1.4.3. The extrinsic apoptotic pathway

As mentioned above, extrinsic apoptosis requires an extracellular signal to activate a transmembrane death receptor such as Fas, TNF-receptor 1 (TNF-R1) and TNF-related apoptosis-inducing ligand receptor 1 or 2 (TRAIL-R1/2) (Guicciardi and Gores, 2009; Park et al., 2007). They are activated by the binding of the trimeric ligands of the TNF cytokine family, TNF-α and Fas ligand (FasL) (Figure 1.2) (Ashkenazi and Dixit, 1998; Park et al., 2007). Upon ligand binding, the death receptor is stabilised and recruits to its C-terminal death domain the Fas-associated death-domain containing protein (FADD) and to FADD N-terminal the death effector domain (DED) to then associate with the procaspase-8 to form the death-inducing signalling complex (DISC) (Galluzzi et al., 2012; Guicciardi and Gores, 2009; Kischkel et al., 1995). In neutrophils, the extrinsic pathway is not enough to initiate downstream signalling involved in cell death and the signal must be amplified via the intrinsic pathway by the activation of Bid through its truncation by procaspase-8 and of consequent processes involved in cell death (Figure 1.2) (Korsmeyer et al., 2000; Scaffidi et al., 1998). The extrinsic pathway does not play a role in neutrophil turnover when neutrophils are in a functional quiescent state but rather when they are dealing with an infection or inflammation (Jonsson et al., 2005; Nadeau et al., 2008).

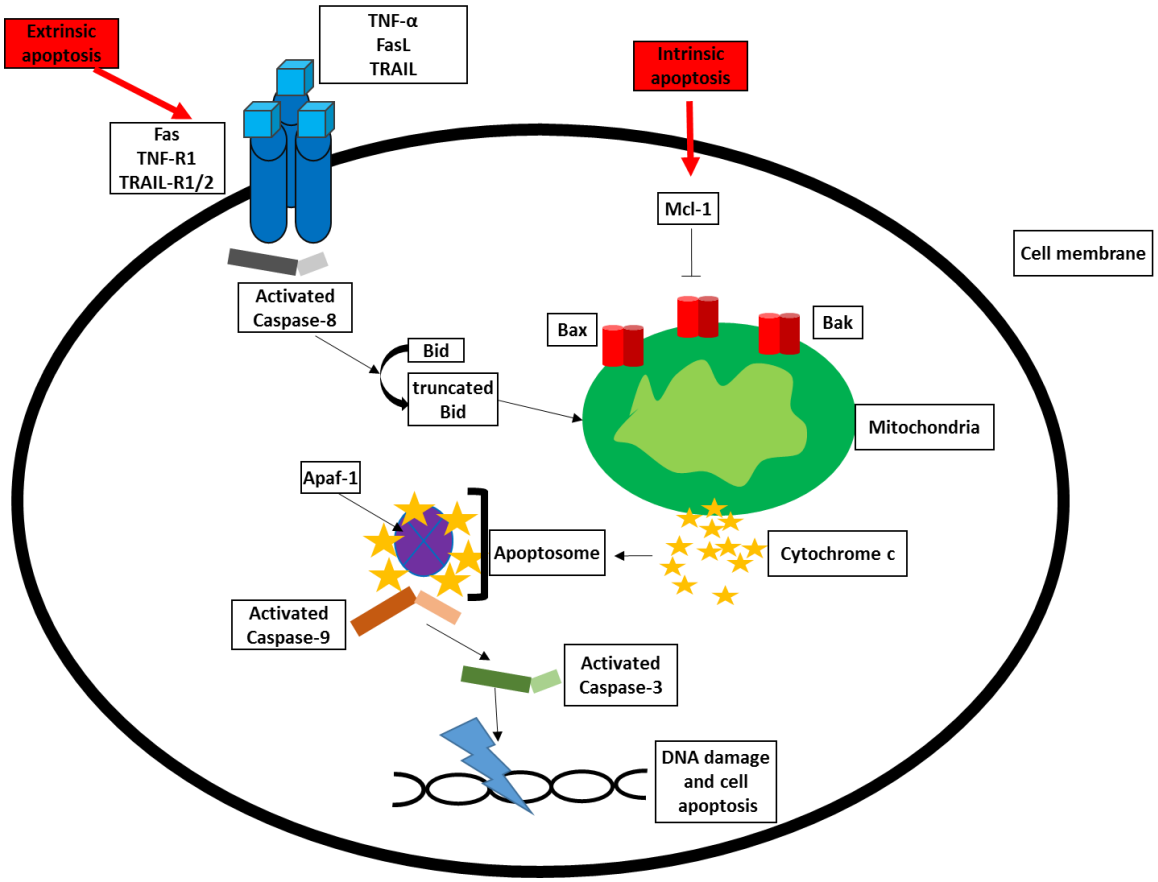

Figure 1.2: Intrinsic and extrinsic neutrophil apoptosis pathways.

The extrinsic apoptotic pathway starts by the recruitment and cleavage of caspase -8 (gray bar) when TNF-α/FasL/TRAIL (blue cube) binds to the Fas/TNF-R1/TRAIL-R1/2 death receptor (dark blue cylinder). This in turn truncates Bid and induces release of cytochrome c (yellow stars) from the mitochondria. The intrinsic apoptotic pathway is activated upon Mcl-1 rapid turnover. As a result, Bax (light red cylinder) and Bak (dark red cylinder) oligomerise and insert into the mitochondria membrane, triggering cytochrome c release. Both pathways then lead to the formation of the apoptosome through the interaction of cytochrome c with Apaf-1 (purple circle with cross). The apoptosome recruits and cleaves caspase-9 (brown), leading to the activation caspsase-3 (green). The activation results in DNA damage, as a result of caspase-3 activated DNase that will fragment the DNA, action which commits neutrophils to irreversible apoptosis.

1.1.5. Neutrophil clearance and inflammation resolution

1.1.5.1. Neutrophil efferocytosis

The clearance of apoptotic cells by macrophages is called efferocytosis. Apoptotic neutrophils express 'find-me' and 'eat-me' signals that are rapidly recognised by tissue macrophages, facilitating their ingestion (Bratton and Henson, 2011; Savill and Haslett, 1995).

'Find-me' signals are diffusible signals that attract macrophages to the site of the apoptotic cell(s). Such signals include ATP, lysophosphatidylcoline or damage associated molecular patters (DAMPs) (Bournazou et al., 2009; Ravichandran, 2010). Neutrophils also have a wide repertoire of cell-surface 'eat-me' signals which are recognised by macrophage receptors and allow docking of the apoptotic cell. Amongst these, the best characterised is phosphatidylserine, found in the inner leaflet of the plasma membrane that becomes exposed on the external leaflet during apoptosis (Fadok et al., 1992). Other factors that can trigger efferocytosis by macrophages are phosphorylcoline, CRs, calreticulin, ICAM-1 epitope changes as well as changes in cell surface charge and glycosation (Bratton and Henson, 2011; Greenlee-Wacker, 2016; Ravichandran, 2010).

Efferocytosis enhances inflammation resolution two-fold. In addition to clearing inflammatory cells it induces an anti-inflammatory macrophage phenotype whereby they secrete anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β), suppress the production of TLRs, IL-6, IL-8 and TNF-α, and promote tissue endothelium and epithelium repair by enhancing the production of vascular endothelial growth factor (VEGF) (Bratton and Henson, 2011; Fadok et al., 1998; Leibovich et al., 2002).

1.1.5.2. Neutrophil reverse migration

There is emerging evidence that neutrophils can be cleared through a process of reverse migration, also known as retrograde chemotaxis (Mathias et al., 2006). This process is characterised by neutrophil migration away from the inflammation site and into the bloodstream during inflammation resolution. Using a rat model, it was shown that more than 70% of neutrophils returned to the circulation rather than undergoing apoptosis

(Hughes et al., 1997). Zebrafish larvae models have shown that neutrophils reverse migrate into the vasculature and factors such as tissue hypoxia can impede this process, thus delaying inflammation resolution (Elks et al., 2011; Hughes et al., 1997; Mathias et al., 2006). The two *in vitro* studies conducted show quite different results. In the first study 0.25% of the neutrophil population could reverse migrate through an epithelial monolayer while in a second, microfluidics analysis up to 90% of neutrophils could reverse migrate for more than 1,000μm from chemoattractants (Buckley et al., 2006; Hamza et al., 2014). Despite the fact that reverse migration has been demonstrated both *in vivo* and *in vitro*, the variation in the number of neutrophils undergoing this process, probably due to the use of different systems, fail to give an accurate quantification of the human cells committing to this.

1.1.6. The role of the neutrophil in inflammatory disease

Neutrophil numbers in tissue have been positively correlated with disease such as chronic obstructive pulmonary diseases (COPD), acute respiratory distress syndrome (ARDS), rheumatoid arthritis, asthma and chronic granulomatous disease (CGD) (Brusselle et al., 2011; Louis and Djukanovic, 2006; Pillinger and Abramson, 1995; Plataki et al., 2006; Quie et al., 1967; Quint and Wedzicha, 2007). In CGD, an X-linked inherited immunodeficiency disease, neutrophils are unable to mount an adequate immune response in infection due to a defect in the NADPH oxidase (Baehner and Nathan, 1967a, b; Kuijpers and Lutter, 2012; Odell and Segal, 1991; Quie et al., 1967).

Neutrophils have also been shown to facilitate the progression of rheumatoid arthritis by destroying joint cartilage and bone as a result of prolonged neutrophil survival at the site of inflammation (Mohr et al., 1981; Ottonello et al., 2002; Pillinger and Abramson, 1995; Wittkowski et al., 2007). What is more, the granule enzymes such as lactoferrin, myeloperoxidase, gelatinase and others, are able to promote an inflammatory phenotype by enhancing neutrophil survival and preventing tissue repair (Wong et al., 2009; Wright et al., 2014). Neutrophilic asthma phenotype is encountered in 50% of all asthmatic patients with the disease progression being modulated by increased IL-8 production that enhances increased neutrophilic chemotaxis to the site of inflammation and augmented neutrophil

survival (Baines et al., 2011; Douwes et al., 2002). What is more, patients that suffer from neutrophilic asthma, unlike healthy individuals, are often colonised more with bacteria such *as S. aureus, M. catarrhalis and Haemophilus influenzae (H. influenzae)*, that have been linked to promoting neutrophilic exacerbations (Green et al., 2014; Redinbo, 2014).

1.1.6.1. Neutrophil implication in COPD pathology

COPD is one of the leading causes of morbidity and mortality worldwide and according to the World Health Organisation (WHO) is predicted to become the third leading cause of death by 2030. The disease is characterised by chronic systemic and airway inflammation and is mainly initiated by cigarette smoking but it may include other genetic and environmental factors (Lowell et al., 1956; Silverman and Speizer, 1996). Interestingly, the most prevalent environmental risk factor is internal air pollution, estimated to affect 3 billion people worldwide, resulted from burning of coal, wood or crop residues for food preparation or home heating (Torres-Duque et al., 2016). Genetic factors for COPD are not well understood but studies of a number of gene polymorphisms have been positively associated with the disease. COPD related gene polymorphisms have been found in the antioxidative system, such as the glutathione S-trasferase (GST) gene, and in the protease inhibitor system, such as the one in the α1-antitripsin gene family, as well as in the inflammation-specific genes, such as the TGF-β1 specific genes (Celedon et al., 2004; de Serres and Blanco, 2012; Shukla et al., 2011). Activated neutrophils colonise the bronchial wall and lumen as well as lung alveolar space (Koenderman et al., 2000; Ludwig et al., 1985; Oudijk et al., 2006; Pesci et al., 1998). Sputum collection from COPD patients has shown increased neutrophil survival and a 30% increase in neutrophil numbers while neutrophil proteases secretion, such as neutrophil elastase, have been correlated with emphysema and mucus hypersecretion in the lungs (Damiano et al., 1986; Nadel, 1991; Perng et al., 2004; Zhang et al., 2012).

To date there is not a full understanding of the mechanisms involved in the dysregulated function of neutrophils in COPD patients. It is known, however, that the collective role of multiple factors such as failure to kill invading pathogens, inability to undergo apoptosis and capacity to release enhanced amounts of proteases can drive disease progression

(Hoenderdos and Condliffe, 2013). The only well-established mutation that has been linked to the development of COPD phenotype is in the α1-antitripsin gene (de Serres and Blanco, 2012; Laurell and Eriksson, 1963). This mutation prevents α1-antitripsin to bind and inhibit neutrophil elastase to protect against tissue damage (Tetley, 1993). Furthermore, bacteria such as *S. aureus* and *H. influenzae* commonly colonise the respiratory tract of COPD patients and have been positively linked to exacerbations (Hoenderdos and Condliffe, 2013; Redinbo, 2014). What is more, people suffering from COPD are at a high risk of sepsis (Almirall et al., 1999). This is most probably because neutrophils from COPD patients show heightened responsiveness to inflammatory mediators such as LPS, TNF-α and GM-CSF (Langereis et al., 2011; Richards et al., 1989).

1.2. Respiratory pathogen *Staphylococcus aureus* and the threat of MRSA

S. aureus is a Gram positive cocci, commonly found on many tissues including skin and respiratory tract tissues. It causes a range of infections from sub-clinical soft tissue infections to the more serious endocarditis and bacteraemia. An NHS report looking at *S. aureus* bacteremia cases between April 2015 and March 2016 showed 11,405 such cases, a 7.1% increase compared to the previous year. Out of these, 7.2% were caused by methicillin resistant *S. aureus* (MRSA) (Accute Trust et al., Official statistics 2016) . Mortality rates for MRSA are three times higher than other *S. aureus* infections (Blot et al., 2002). This is because MRSA is resistant to β-lactam antibiotics as well as other classes of antibiotics, comprising even the last resort antibiotics vancomycin and ciprofloxacin (Blumberg et al., 1991; Roberts et al., 2013; Sieradzki et al., 1999). Initial MRSA cases were only hospital acquired but now community acquired MRSA, genetically distinct from the hospital acquired strains, are more prevalent (Bishara et al., 2012). From the MRSA diagnosed in hospitals, 92.5% are still sensitive to non-lactam based antibiotics. Most alarming, recent statistics estimate that by 2050 there will be 10 million deaths per year due to antimicrobial resistance attributed to *E. coli, Klebsiella pneumoniae* and *S. aureus* (de Kraker et al., 2016). This urges the development of new therapies that do not put high selective pressure on the bacteria and recent therapeutic target strategies have shown great potential for tetraspanins (Ventress et al., 2016).

1.2.1. *S. aureus* evasion of neutrophil defences

The importance of neutrophils in defending against *S. aureus* infections was shown in disease where alterations in the number and function of neutrophils, such as CGD, resulted in the inability of the organism to cope with the infection (Odell and Segal, 1991; Quie et al., 1967). Despite the vast antimicrobial repertoire neutrophils employ in the defense against intruding pathogens, *S. aureus* has evolved many mechanisms to evade neutrophil defenses. These can range from stopping neutrophil recruitment to evading killing by neutrophils, adding to the problem of bacterial resistance.

Initial steps in neutrophil adhesion and rolling to blood vessels endothelium are targeted through two *S. aureus* secreted proteins: staphylococcal superantigen-like 5 (SSL5) and extracellular adherence protein (EAP) that binds to P-selectin and ICAM-1, respectively, on endothelial cells to prevent interaction with their equivalent neutrophil ligands (Figure 1.3) (Bestebroer et al., 2007; Chavakis et al., 2002). Chemokine recognition and activation of neutrophils is also targeted by a number of factors secreted by *S. aureus*. SSLs family members have an important role in the process. SSL5 binds to FPRs and leukotriene B4 receptor to prevent sensing of chemotactic factors while SSL3 binds to TLR2 to inhibit activation of TLR1/TLR2 and TLR2/TLR6 complexes (Figure 1.3) (Bardoel et al., 2012; Bestebroer et al., 2009). Chemotaxis inhibitory protein of *Staphylococcus* (CHIPS) and FPR-1 like inhibitory proteins (FLIPr and FLIPr-like) also bind to FPRs to prevent chemotaxis towards fMLP and phenol soluble modulins (Figure 1.3) (Haas et al., 2005; Prat et al., 2006; Prat et al., 2009). CHIPS also binds to C5aR to prevent interaction with C5a (Haas et al., 2005). Staphopain A is another factor secreted by MRSA to prevent IL-8 binding to its neutrophilic receptor, CXCR2 (Figure 1.3) (Laarman et al., 2012).

S. aureus has also adapted to evade opsonisation. A number of factors are involved in this process. Aureolysin prevents opsonisation by inactivating C3 central complement component while staphylococcal complement inhibitor (SCIN) binds to the C3 convertase to inhibit its enzymatic activity (Figure 1.3) (Laarman et al., 2011; Rooijakkers et al., 2005a). Staphylococcal protein A (SpA) and staphylococcal IgG-binding molecule (Sbi) bind to the Fc region of IgG to prevent phagocytosis (Figure 1.3) (Forsgren and Nordstro.K, 1974). Other

proteins have dual effect such as staphylokinase (SAK) that can cleave both IgG and C3b while SSL7 can bind to IgA and C5 to prevent opsonisation and phagocytosis (Figure 1.3) (Laursen et al., 2010; Rooijakkers et al., 2005b).

Interestingly, the mechanisms that *S. aureus* employs to evade neutrophil killing have also evolved to deactivate both ROS and AMPs. To protect itself from ROS *S. aureus* uses a number of factors including staphyloxanthin, golden pigment, catalases and surface factor promoting resistance to oxidative killing (SOK) (Figure 1.3) (Liu et al., 2005; Malachowa et al., 2011; Mandell, 1975). To protect against lysozyme, the bacteria modifies its peptidoglycan wall by introducing an O-acetylation in the wall while defensins protection is done by reducing its negative membrane charge to repel the cationic peptides or by using SAK to bind α-defensins (Figure 1.3) (Bera et al., 2005; Jin et al., 2004; Peschel et al., 2001). Carotenoid pigments are also widely used to protect against cationic AMP (Figure 1.1) (Mishra et al., 2011).

1.3. Tetraspanin overview

Tetraspanins are a highly conserved superfamily of membrane spanning receptors, consisting of 33 members in mammals (Boucheix and Rubinstein, 2001; Garcia-Espana et al., 2008; Huang et al., 2005). According to a phylogenetic analysis conducted by (Garcia-Espana et al., 2008), the tetraspanin family can be divided into four subfamilies: the uroplakins, the retinal degeneration slow (RDS) subfamilies, the CD (cluster of differentiation) superfamily and the CD63 family. All tetraspanins are membrane spanning proteins involved in a wide variety of processes including cell signalling, adhesion, migration and proliferation (Charrin et al., 2014; Hemler, 2003, 2005; Jiang et al., 2015; Schwander et al., 2003; Termini and Gillette, 2017). Due to the focus of this thesis, the following sections will concentrate on the most studied tetraspanins expressed on immune cells: CD9, CD37, CD53, CD63, CD81, CD82 and CD151.

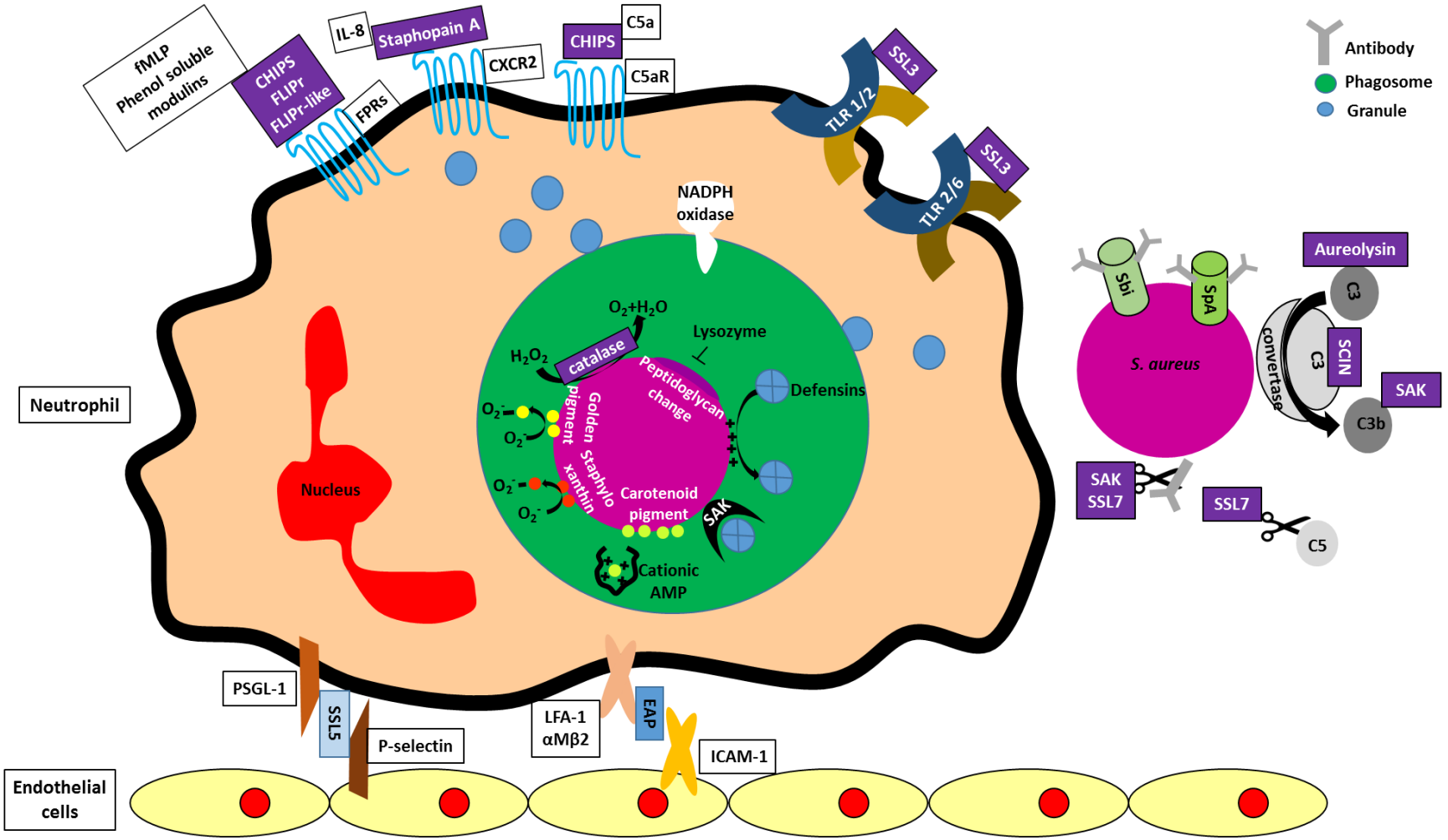
fMLP
Phenol soluble modulins
CHIPS
FLIPr
FLIPr-like
FPRs
IL-8
Staphopain A
CXCR2
CHIPS
C5a
C5aR
TLR 1/2
SSL3
TLR 2/6
SSL3
NADPH oxidase
Neutrophil
Nucleus
O_2+H_2O
Lysozyme
H_2O_2
catalase
Peptidoglycan change
Defensins
O_2^-
O_2^-
Golden pigment
Staphylo xanthin
O_2^-
O_2^-
Carotenoid pigment
SAK
Cationic AMP
PSGL-1
SSL5
P-selectin
LFA-1
αMβ2
EAP
ICAM-1
Endothelial cells
Antibody
Phagosome
Granule
Sbi
SpA
S. aureus
Aureolysin
C3
convertase
C3
SCIN
SAK
C3b
SAK
SSL7
SSL7
C5

Figure 1.3: *S. aureus* evades detection and killing by neutrophils.

Interaction to the endothelial cells is stopped by the secreted proteins SSL5, which prevents interaction of PSGL-1 with P-selectin, and EAP, which prevents interaction of LFA-1 and αMβ2 with ICAM-1. SSL3 secreted protein binds to TLR 1/2 and TLR 2/6 while Staphopain A prevents recognition of CXCR2 by IL-8. CHIPS, FLIPr and FLIPr-like bind to the N-terminus of the FPRs and C5aR receptors, which recognise fMLP, phenol soluble modulins and C5a. *S. aureus* secrets Sbi and SpA proteins on the surface to bind antibodies while SAK and SSL7 cut and deactivate the Fc part of the antibody. Proteins secreted by *S. aureus* also deactivate members of complement cascade: aureolysin deactivates C3, SSL7 cleaves C5, SCIN binds the convertase and SAK cleaves C3b. Protection against killing in the phagosome is mediated by golden pigment (yellow spheres), staphyloxanthin (orange spheres) and catalases which protect against hydrogen peroxide (H_2O_2) and singlet oxygen (O_2^-). Peptidoglycan wall changes allows protection against lysostaphin. Additional mechanism used employ increasing the positive change of the membrane to repel the positively charged defensins or using SAK protein to bind them as well as using carotenoid pigment (yellow-green spheres) to bind the positively charged AMPs.

1.3.1. The biochemistry of tetraspanins

Tetraspanins are small proteins of 200-350 amino acids that protrude 3-5nm from the membrane, with several common structural characteristics: four transmembrane hydrophobic helixes, a small (EC1) and a large (EC2) extracellular domain, a short intracellular loop and amino- and carboxyl intracytoplasmic tails (Figure 1.4) (Boucheix and Rubinstein, 2001; Stipp et al., 2003). Some tetraspanins (CD63, CD151, CD37, CD82) also contain the Yxxω C-terminal tyrosine based internalisation/lysosomal motif, where ω represents a bulky hydrophobic amino acid residue, which allows their endocytosis by adaptor proteins (AP) (Berditchevski, 2001). The crystal structure of tetraspanins took many years to solve due to the requirement of a membrane for the proteins to keep their structures. For this reason, only the structure of the soluble EC2 domain was available (Kitadokoro et al., 2001). However, recently the full crystal structure of CD81 tetraspanin was elucidated. It shows that the tetraspanin adopts a cone like structure with an intramembrane cavity that can be occupied by cholesterol and molecular dynamics simulations suggest that CD81 can alter between a closed and open conformation, where cavity cholesterol binding shifts the state of the tetraspanin into a closed one (Zimmerman et al., 2016). The authors also speculate that the open conformation promotes tetraspanin-partner interactions while the closed conformation may form weaker tetraspanin-partner interactions.

The EC1 and EC2 domains are the most variable regions between tetraspanins and species. The EC2 has a mushroom-like structure where the more conserved 3 α-helical regions (A, B, E) form the “stalk” (Figure 1.4) (Hemler, 2003; Kitadokoro et al., 2001; Stipp et al., 2003). “The head” of the mushroom is the most variable region, containing 2 α-helixes (C, D) as well as the conserved signature motif Cys-Cys-Gly (CCG) and, in some tetraspanins, 2-4 additional Cys residues that form a disulphide bridge network (Figure 1.4). The EC2 domain has been shown to be required to form an association with partner proteins while little is known about EC1 role in cell function, with some evidence showing a role in cell trafficking (Kazarov et al., 2002; Masciopinto et al., 2001; Zhu et al., 2002).

The four transmembrane α-helixes have a pocket formed by the parallel association of the transmembrane helixes 1 and 4 with 2 and 3 (Zimmerman et al., 2016) The transmembrane domains also have polar residues that are involved in hydrogen bond formation with lipid alkyl chains, to stabilise the tetraspanin structure (Figure 1.4) (Gratkowski et al., 2001; Zhou et al., 2001). Furthermore, the tetraspanins have intracellular palmitoylation sites, ranging from 0-6; palmitoylation aids in the orientation of the short intracellular loop and the N and the C intracytoplasmic tails (Figure 1.4) (Yang et al., 2002; Yauch et al., 2000). The palmitoylation sites can form as well hydrophobic interactions with membrane lipids to stabilise the structure of the tetraspanin and confer a level of rigidity to the membrane (Yang et al., 2002; Yauch et al., 2000). Palmitoylation also helps tetraspanins to organise themselves into "tetraspanins enriched microdomains" (TEMs) (Berditchevski et al., 2002; Charrin et al., 2002; Hemler, 2003, 2005). Evidence shows that loss of palmitoylation results in failure of tetraspanins to interact with other partner tetraspanins, leading to impaired cell signalling and disease formation (Charrin et al., 2002; Stuck et al., 2015; Termini and Gillette, 2017; Yang et al., 2002).

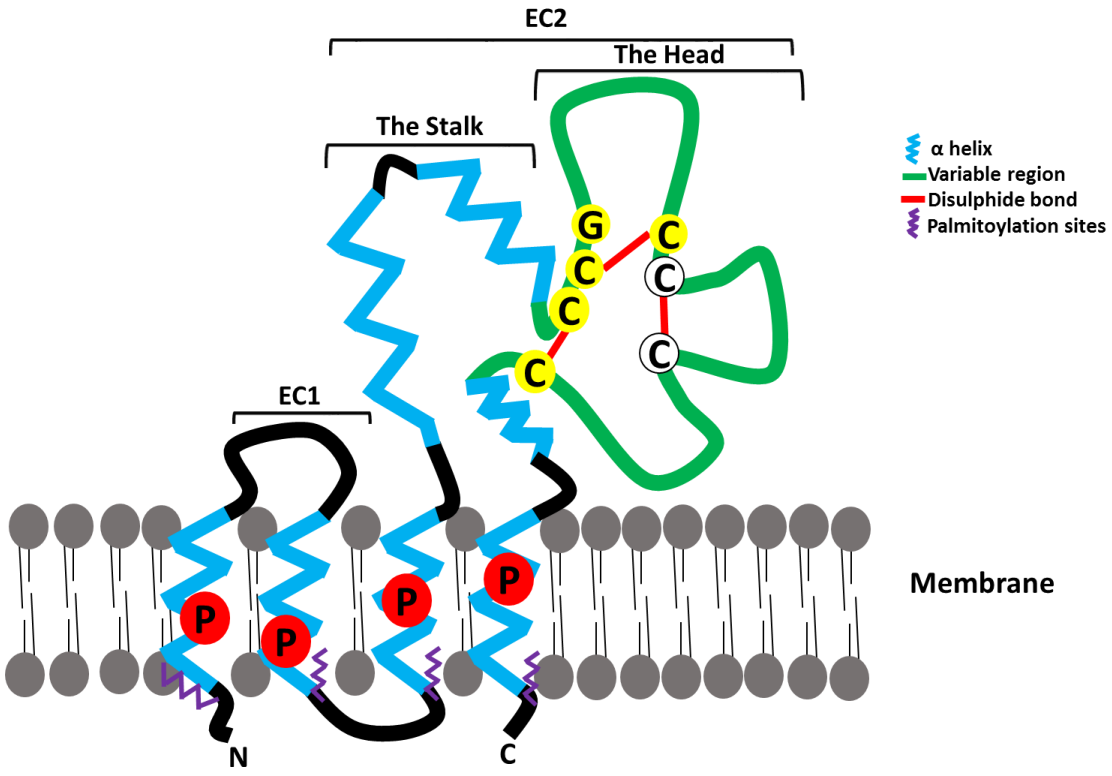

Figure 1.4: The structure of tetraspanins.

The tetraspanin is formed of four transmembrane domains, an EC1 and EC2 domains, an intracellular loop and two, an N- and a C-, intracytoplasmic tails. The EC2 contains a conserved region or "the stalk" comprised of 3 α-helixes and a variable region or "the head". The variable region contains a CCG motif (yellow circles) which form disulfide bridges (red line). Other tetraspanins contain additional 2-4 cysteine residues (white circles) which can form disulfide bridges (only 2 additional cysteine residues are represented). Tetraspanins also have palmitates or intracellular palmitoylation sites. Polar residues are also found in the 4 transmembrane domains (red circles).

1.3.2. Tetraspanin interactions with partner molecules

The CD and CD63 tetraspanin family members can laterally interact via the EC2 or transmembrane domain with members of the integrin family, major histocompatibility complex (MHC), immunoglobulin superfamily and growth factor receptors to induce signal transduction as well as modulating fundamental biological processes by binding to signalling molecules through their cytoplasmic domains (Table 1.1) (Charrin et al., 2009; Hemler, 2003; Yanez-Mo et al., 2009; Yu et al., 2017; Yunta and Lazo, 2003b). The most common interactions of tetraspanins are with integrins. Integrins are membrane proteins involved in cell adhesion and signalling, comprised of heterodimers formed from one of the 18 α subunits and one of the 8 β subunits (Laflamme et al., 1994). The most studied integrin-tetraspanin interactions are with CD151. Direct CD151-integrin interactions have been shown with α3β1, α6β1, α6β4 and α7β1 integrins (Berditchevski et al., 1997a; Serru et al., 1999; Sincock et al., 1999; Sterk et al., 2002; Yauch et al., 2000). This tetraspanin has a unique way of affecting cellular structures. CD151 does not have a direct involvement in adhesion or migration when interacting with integrins but rather affects its adhesion strength by promoting activation of signalling molecules, such as AKT, Src and GTPases, according to the cellular context (Hong et al., 2012b; Lammerding et al., 2003; Nishiuchi et al., 2005; Puklin-Faucher and Sheetz, 2009; Takeda et al., 2007). Loss of this tetraspanin resulted in kidney failure in mice, although this was strain dependent, while additional study showed impaired wound healing in mice lacking CD151 (Cowin et al., 2006; Sachs et al., 2012; Sachs et al., 2006). Additionally, loss of CD151 in tumor cells results in decreased tumor progression or onset (Deng et al., 2012; Hemler, 2014; Sachs et al., 2014). Other tetraspanin-integrin interactions were not as well studied. However, CD81 and CD37 were shown to be required for IgG production by B cells by regulating B cells adhesion to T cells via α4β1 ligands (van Spriel et al., 2012). At the same time, regulation of αIIbβ3 expression on platelets by CD9 is required in order to prevent excessive platelet recruitment at the site of vascular injury (Mangin et al., 2009).

Table 1.1: Main immune tetraspanins interacting partners

Interactions	Tetraspanins	Evidence
Integrins:	**CD9:** α**1**β1, α**2**β1, α3β1, α4β1, α6β1, α5β1, α6β4, α7β1, αIIbβ3 **CD53:** α4β1 **CD81:** α3β1, α4β1, α6β1, α5β1, αVβ5 **CD82:** α3β1, α4β1, α6β1, α5β1, α1β2 **CD151:** α**2**β1, α3β1, α4β1, α6β1, α5β1, αIIbβ3, α6β4, α7β1 **CD63:** α3β1, α4β1, α6β1, α**1**β2, αMβ2, αIIbβ3 **CD37:** β2, α4β1	(Berditchevski, 2001; Berditchevski et al., 1995; Chang and Finnemann, 2007; Hemler, 2005; Mannion et al., 1996; Schmidt et al., 1996; Skubitz et al., 1996; van Spriel et al., 2012; Wee et al., 2015; Yanez-Mo et al., 2011; Yauch et al., 1998)
MHC	**CD9:** - **CD53:** HLA-DR, HLA-DQ **CD81:** HLA-DR, HLA-DQ **CD82:** HLA-DR, HLA-DQ, HLA-DM, HLA-DO **CD151:** - **CD63:** HLA-DR, HLA-DQ, HLA-DM, **CD37:** HLA-DR	(Angelisova et al., 1993; Engering and Pieters, 2001; Hammond et al., 1998; Szollosi et al., 1996)
Growth factor receptors:	**CD9:** Transforming growth factor α(TGF-α), heparin-binding epidermal growth factor-like growth factor (HB-EGF) **CD53:** - **CD81:** - **CD82:** Epidermal growth factor receptor (EGFR) **CD151:** - **CD63:** Vascular endothelial growth factor receptor (VEGFR) **CD37:-**	(Imhof et al., 2008; Lagaudriere-Gesbert et al., 1997; Odintsova et al., 2003; Tugues et al., 2013)

Immunoglobulin superfamily:	**CD9:** FPRP, EWI-2, EWI-F **CD53:** - **CD81:** FPRP, EWI-2, EWI-F **CD82: -** **CD151:** - **CD63:** - **CD37: -**	(Charrin et al., 2003; Montpellier et al., 2011; Stipp et al., 2001a; Stipp et al., 2001b)
Membrane proteins:	**CD9:** CD36, CD19, FcγRII, CD46 **CD53:** CD20 **CD81:** CD9P-1, CD4, CD8, CD21-CD19, CD20, CD46 **CD82:** CD4, CD8, CD19, CD20, CD46 **CD151:** CD46 **CD63:** AP-1, AP-2 **CD37: -**	(Charrin et al., 2013; Imai et al., 1995; Levy et al., 1998; Lozahic et al., 2000; Mazurov et al., 2013; Miao et al., 2001; Pols and Klumperman, 2009; Szollosi et al., 1996; Todd et al., 1996; Worthington et al., 1990)
Signalling proteins:	**CD9:** Protein kinase C (PKC), Phosphatidylinositol 4-kinase (PI4-K), heterotrimetric G proteins **CD53:** PKC **CD81:** PKC, PI4-K, heterotrimetric G proteins, Rac1 **CD82:** PKC **CD151:** PKC, PI4-K **CD63:** PI4-K **CD37: -**	(Berditchevski et al., 1997b; Claas et al., 2001; Le Naour et al., 2006; Little et al., 2004; Tejera et al., 2013; Yauch et al., 1998; Zhang et al., 2001)

1.3.2.1. Tetraspanin trafficking of partner proteins

Tetraspanins containing the tyrosine-based internalisation motif have a primary function in facilitating the trafficking of their partner proteins by either promoting their internalisation or association with late endocytic organelles(Pols and Klumperman, 2009) CD63 is the best studied in this context and is known to have a role in the transport of a number of proteins including the membrane repair regulator protein synaptotagmin 7, the H, K-ATPase pump, as well as CXCR4, stromal cell-derived factor 1/CXCL12 and neutrophil elastase (Duffield et al., 2002; Duffield et al., 2003; Flannery et al., 2010; Kallquist et al., 2008; Yoshida et al., 2008).

There are other tetraspanins involved in protein trafficking. The TspanC8 group, comprised of tetraspanin (TSPAN) 5, TSPAN10, TSPAN14, TSPAN15, TSPAN17 and TSPAN33, is involved in trafficking from the ER to the plasma membrane or late endosomes the metalloproteinase domain-containing protein 10 (ADAM10), a regulator of the embryogenesis regulator protein Notch (Dornier et al., 2012; Haining et al., 2012; Prox et al., 2012). Another example is CD81, which promotes trafficking and organisation into clusters of the co-stimulatory molecule of lymphoid B cells, CD19, along the secretory pathway (Mattila et al., 2013; Shoham et al., 2003). The importance of CD81 in immunity is shown in a patient where CD81 was defective resulting in him having a faulty adaptive immune system (van Zelm et al., 2010). Furthermore, CD82 C-terminal deletion results in failure of epidermal growth factor receptor to be trafficked to the endocytic organelles (Zhang et al., 2003b). CD151 can also regulate integrin α3β1 expression and its silencing in carcinoma cells results in reduced internalisation rates and failure to recruit to tetraspanin enrich microdomains (TEMs) of the integrin (Winterwood et al., 2006).

1.3.2.2. Mechanisms of tetraspanin signalling

There are several proposed tetraspanin signalling mechanisms. The first one suggests that tetraspanins act as signal transducing molecular linkers by bringing into proximity partner molecules such as integrins and members of the immunoglobulin family with the signalling molecules situated in the cytoplasm (Lammerding et al., 2003). The phosphoinositide kinase

family plays a key role in this signalling mechanism. PKC and PI4-K type II interactions with members of the tetraspanin family facilitate cytoskeletal reorganisation and migration, Ca^{2+} dependent signalling, suppression of carcinoma cell motility, angiogenesis and mast cell degranulation (Carloni and Mazzocca, 2009; Liu et al., 2011; Qi et al., 2006; Scharenberg et al., 2007; Yauch et al., 1998). One such example was elegantly demonstrated in neutrophils where CD151 tetraspanin brings in close proximity $\alpha_3\beta_1$ integrin with PI4-K type II to facilitate cell migration (Figure 1.4A) (Yauch et al., 1998).

The second proposed mechanism of action is by clustering partner membrane proteins to promote their increased avidity and affinity for their ligands (Kropshofer et al., 2002). Such an example was shown in $CD4^+$ T cells, where tetraspanins CD9, CD81 and CD82 brought together the MHC type II receptors HLA-DR/DP with the HLA-DM protein, involved in peptide presentation to the MHC class II, and the co-stimulatory molecule CD86 to facilitate T-cell activation by increasing the avidity of MHC (Figure 1.4B) (Kropshofer et al., 2002).

The last signalling mechanism proposed suggests that tetraspanins can act as signalling regulators by sequestering proteins to prevent inadequate cell signalling or responses. One such signalling mechanism was shown using breast cancer cells where CD82 dampened the activity and the dimerisation of epidermal growth factor receptor by redistributing it into the cell surface ganglioside GD1a microdomains (Odintsova et al., 2003).

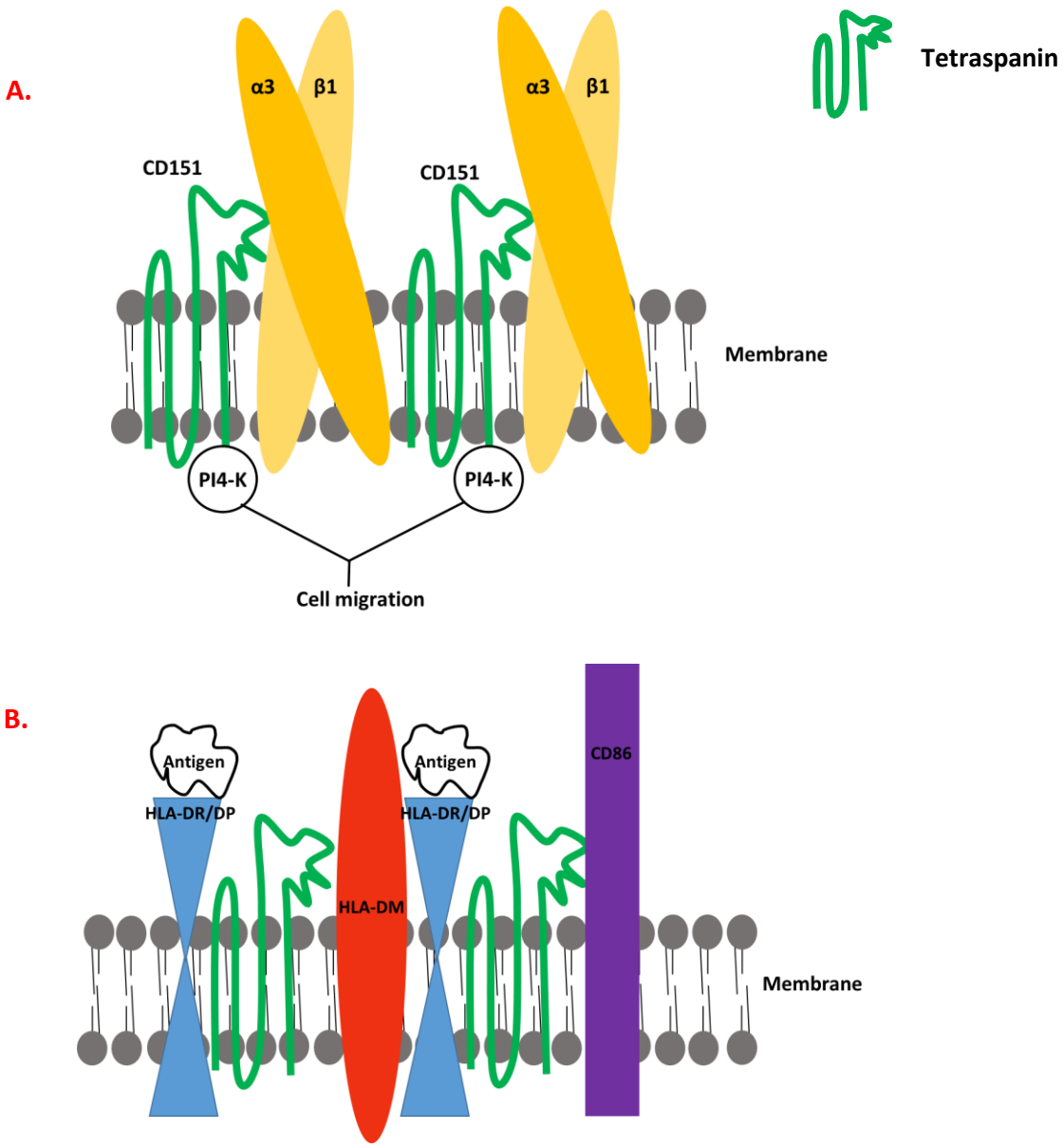

Figure 1.5: Different ways of tetraspanin signalling.

A.) CD151 acting as a transduction linker in neutrophils by interacting via its large extracellular loop with the α domain of the α3β1 integrin and with the cytoplasmic domain with PI4-K resulting in cell migration. B.) Tetraspanin clustering the partner proteins HLA-DR/DP MHC class II complex with the HLA-DM peptide presenting protein and the CD86 co-stimulatory molecule to facilitate T cell activation.

1.3.2.3. Tetraspanin enriched microdomains (TEMs)

Tetraspanins most often promote association and organisation of proteins that are far apart in the membrane into TEMs or a "tetraspanin web", which serves to bring about cell signalling in a more efficient way (Charrin et al., 2003; Hemler, 2008; Levy and Shoham, 2005a; Zuidscherwoude et al., 2015). The interactions found in TEMs are diverse. Initially demonstrated by co-immunoprecipitation studies, they have been classed into direct/primary interactions, between tetraspanins and nontetraspanin partners such as α3β1 integrin-CD151, while others are indirect and include secondary, between members of the tetraspanin family, and tertiary interactions, formed by indirect association of tetraspanins with additional proteins (Berditchevski, 2001; Hemler, 2003; Levy and Shoham, 2005a; Yauch et al., 1998). There are two different proposed models of TEM assembly, indicating a controversy in the field. The first proposed model, is more widely accepted, and has already been proven by several groups. This models shows that multiple tetraspanins and partner proteins co-localise in the membrane to bring about molecular changes and induce different functions (Figure 1.5A) (Andre et al., 2006; Andreu and Yanez-Mo, 2015; Levy and Shoham, 2005b; Nydegger et al., 2006). The second one recently came from (Zuidscherwoude et al., 2015). Using dual colour stimulated emission depletion it was demonstrated that CD37, CD53, CD81 and CD82 group in small clusters, with their associated binding partners, no bigger than 10 tetraspanins (Figure 1.5B). As opposed to previous dogma, the membrane cluster form associations that are less than 120nm wide. These clusters can then co-localise with other clusters when they move through the membrane.

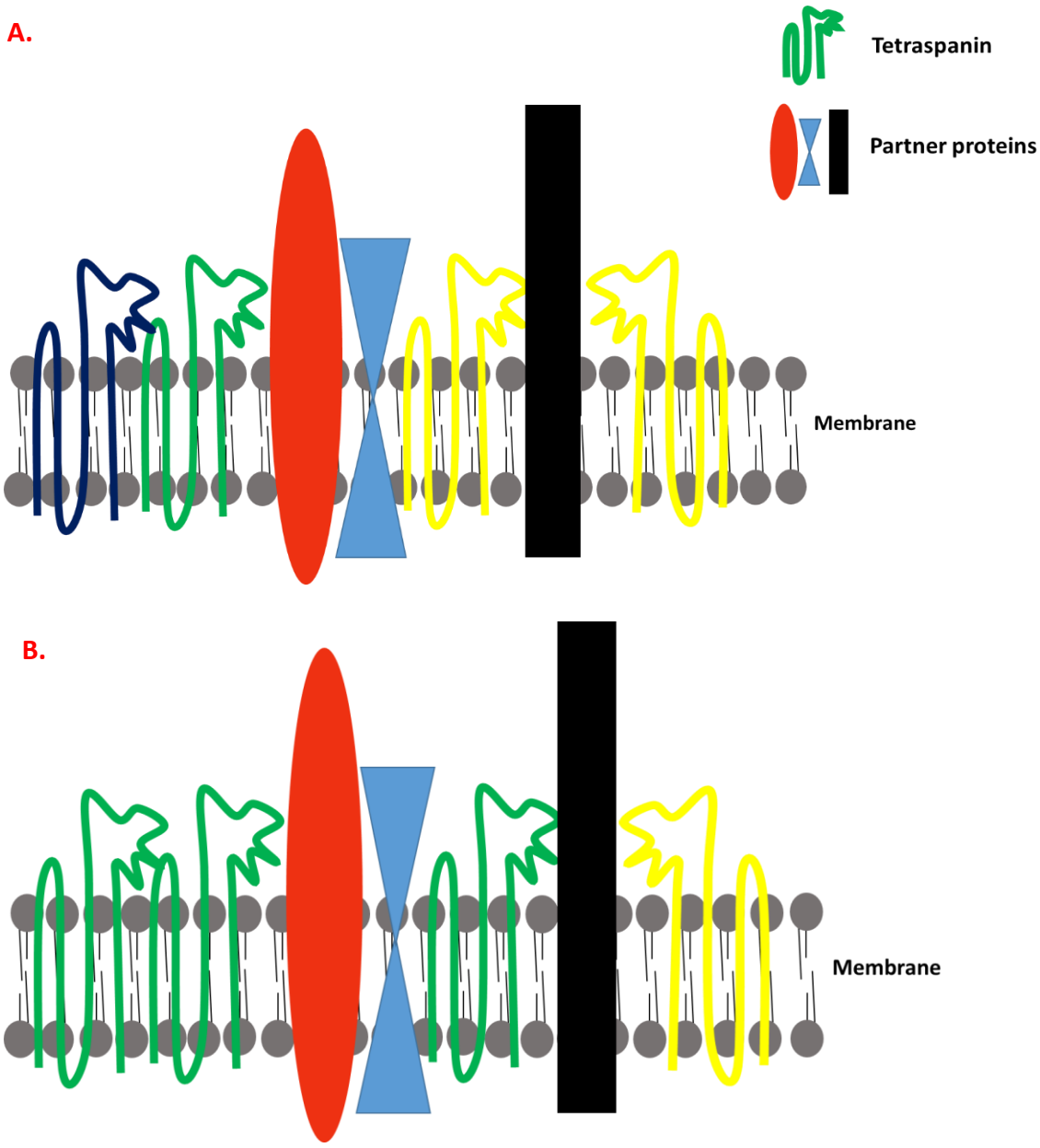

Figure 1.6: The two models of TEMs.

A) Tetraspanins and interacting partner proteins group together to make a mixed domain structure. B) TEMs formed from the interaction of mostly one tetraspanin type and its partner proteins.

1.3.3. Tetraspanins and immunity

The function of tetraspanins is quite diverse in the innate and adaptive immune system ranging from the regulation of immune system activation, level of cytokine/chemokine release as well as prompt response in inflammation resolution (Jones et al., 2011; van Spriel, 2011; Veenbergen and van Spriel, 2011).

Looking at the adaptive immune system, dendritic cells present opposite roles for tetraspanins when knockout models were used, probably due to the tetraspanin different functions. For instance, while CD9 has a role in clustering MHC class II dimers to enhance stimulation of T cell receptors, CD37 regulates peptide/MHC presentation and CD151 has a key role in co-stimulation of T-cells (Sheng et al., 2009; Unternaehrer et al., 2007). CD9 abolition results in reduced dendritic function while, in contrast, CD37 and CD151 removal results in T cell hyper-stimulation (Sheng et al., 2009; Unternaehrer et al., 2007). More recently, CD37 and CD82 have also been shown to have opposing functions in dendritic cells, with CD37 being found in unactivated cells and promoting migration, while CD82 promotes antigen presentation and activation of T cells (Jones et al., 2016). In B cells, CD81 has an important role in activation and transport of CD19 and mutation in CD81 results in antibody production deficiency (Tsitsikov et al., 1997; van Zelm et al., 2010). CD81 was also shown to have key role in regulating natural killer cell recruitment in response to cytokines/chemokine stimulation (Kramer et al., 2009).

With respect to the innate immune system, macrophage CD9 negatively regulates the expression of CD14 on the cell membrane in response to LPS to prevent a heightened cell response to the LPS stimuli (Suzuki et al., 2009; Takeda et al., 2015). In eosinophils, CD63 migration to cell surface is positively correlated to cell activation and degranulation (Mahmudi-Azer et al., 2002). In fact, CD63 surface expression is widely used as a marker of cell activation including platelets, basophils and, most importantly, neutrophils (Azorsa et al., 1991; Gamboa et al., 2003; Lopez et al., 1995). Furthermore, CD37 presence positively promotes β2-dependent neutrophil cell migration in mice while CD63-αMβ2 association promotes neutrophil migration in humans (Skubitz et al., 1996; Wee et al., 2015).

1.3.3.1. Bacterial infections and tetraspanins

There are a few studies that show that bacteria are able to exploit tetraspanins in order to adhere to and infect cells, contributing to their pathogenesis. Although not one of the immune tetraspanins, Uroplakin 1a is found on bladder cells and is known to be exploited by the uropathogenic *E. coli* to adhere to urothelial cells (Zhou et al., 2001). Regarding immune tetraspanins, CD9 increased cell expression renders mouse L cells much more susceptible to diphtheria toxin, an important virulence factor for *Cornyebacterium diphteriae* (Iwamoto et al., 1994). In dendritic cells, CD82 is known to be recruited to fungi and bacteria containing phagosomes, prior to their fusion with the lysosomes, but its role is in this process is yet to be elucidated (Artavanis-Tsakonas et al., 2011). Studies conducted here in Sheffield on epithelial cell lines have shown that CD9, CD63 and CD151 have a collective role in the adhesion of *Neisseria meningitidis*, via its type IV pili, as well as *S. aureus, Neisseria lactamica, E. coli and S. pneumoniae*, by presumably organising adhesion molecules via TEMs (Green et al., 2011). The group also demonstrated the therapeutic aspect of the tetraspanins. By using recombinant peptides representing regions of the EC2, the adhesion of *S. aureus* to keratinocytes via CD9 was greatly inhibited (Ventress et al., 2016). Furthermore, CD81 is required for the entry of the opportunistic pathogen *Listeria monocytogenes* into epithelial cells (Tham et al., 2010). Recently, it was also demonstrated that metalloproteinase ADAM10 bound and internalised less *S. aureus* pore-forming α hemolysin toxin, when cells were deficient in TSPAN14 (Virreira Winter et al., 2016).

Tetraspanins can also act as initial binding partners for viruses, fungi and even protozoa. Detailed descriptions of the interactions and their consequences for the organism have been reviewed in several papers (Monk and Partridge, 2012).

1.3.3.2. Tetraspanin association with disease

Since their discovery, tetraspanins have been linked to disease as a result of their increased expression, abolition or due to various gene mutations. Probably the most studied implications of tetraspanins are in the context of cancer, described in depth in a couple of recent published reviews (Hemler, 2014; Yang et al., 2016). Furthermore, tetraspanins

mutations have been linked to inherited diseases such as X-linked mental retardation and familial vitreoretinopathy exudative (Abidi et al., 2002; Yang et al., 2011).

CD151 has been shown to have a key role in inflammatory disease progression. It is essential for normal function of alveolar epithelial cells, its loss resulting in pulmonary fibrosis in mice (Tsujino et al., 2012). Similarly, CD151 truncation results in renal failure, deafness and skin blistering due to failure in basement membrane assembly (Crew et al., 2004). In addition, mutations in the EC2 region of CD81 has also been linked with severe immune deficiency due to the inability of B cells to mount an adequate antibody response (van Zelm et al., 2010). Most importantly, CD9 was shown to have a preventive role in COPD due to the ability of CD9 rich TEMs to negatively regulate CD14 expression on macrophage membrane (Takeda et al., 2008; Takeda et al., 2015).

1.3.4. Known roles of tetraspanins in neutrophil functions

To date, there is limited knowledge on the functions of tetraspanins in human neutrophils. There is evidence that neutrophils express CD9, CD151, CD53, CD63, CD37 and CD82 (Meyer-Wentrup et al., 2007; Tarrant et al., 2003; Tohami et al., 2004). There is, however, some controversy in the literature concerning tetraspanin CD81 and its expression by neutrophils. The general consensus is that neutrophils do not express CD81 but Tohami et al. showed that a small subpopulation of neutrophils do express CD81 at high levels (Andria et al., 1991; Tarrant et al., 2003; Tohami et al., 2004).

Tetraspanins have a key role in neutrophil migration. For instance, fMLP stimulation promotes CD63-αMβ2 integrin-serine/threonine kinases recruitment, such as Lyn, Hck and Src, which results in the activation of an integrin specific adhesion and migration (Skubitz et al., 1996; Skubitz et al., 2000; Toothill et al., 1990). CD151 was also shown to modulate this function by forming a stable complex with α3β1 and PI4-K type II in human neutrophils that promotes cell activation (Yauch et al., 1998). According to recent mice *in vivo* studies, CD37 can positively modulate neutrophil migration by regulating the cell surface expression of β2 integrin (Wee et al., 2015). Although a β2-CD37 association was not detected, CD37 presence promoted actin polymerization, neutrophil cell spreading and cell polarization, its

loss resulting in increased β2 internalization (Wee et al., 2015). This suggests that neutrophil migration possibly requires a tetraspanin-integrin interaction. At the same time it is possible that different tetraspanins-integrin interactions mediate different steps in the neutrophil migration process.

Roles for tetraspanins have been shown in neutrophil interaction and defense against pathogens. For instance, *Leishmania* containing vacuoles can fuse with neutrophil $CD63^{+}$ azurophilic granules, in a process orchestrated by the pathogen to ensure its survival (Mollinedo et al., 2010). CD63 is also known to be involved in specific HL-60 azurophilic granule targeting and trafficking to the phagolysosome of neutrophil elastase (Kallquist et al., 2008). Furthermore, neutrophils from patients infected with *S. pneumoniae* and uropathogenic *E. coli* showed decreased CD81 and CD82 expression (Tohami et al., 2004). In contrast, tetraspanin CD53 may also act as pathogenic risk factor in the immunodeficiency syndromes characterised by known recurrent infections and unidentified pathophysiology (Mollinedo et al., 1997). This suggests perhaps that tetraspanins functions depend on the bacterial pathogen the cell encounters.

There is limited knowledge about tetraspanins and neutrophil cell survival. Biochemical techniques indicate that human apoptotic neutrophils express more CD63 and CD53 compared to healthy cells, suggesting either that it plays a role in the apoptotic process or is merely happening in parallel (Beinert et al., 2000; Mollinedo et al., 1998).

Similarly, involvement of tetraspanins in inflammatory disease is poorly understood. For instance, CD9 is overexpressed in periodontal disease, indicating a potential role for this tetraspanin in the pathology of this inflammatory disease (Bisson-Boutelliez et al., 2001). More is known about CD63. A polymorphism at the CD63 locus has been associated with decreased CD63 expression in neutrophils from COPD patients, but not healthy individuals, while Lopez et al. findings suggest that this tetraspanin can act as a marker for rheumatoid arthritis (Lopez et al., 1995; Zhang et al., 2007). Considering that COPD and rheumatoid arthritis patients have increased neutrophil survival, the above data could indicate a potential role for CD63 in neutrophil survival in inflammatory disease (Ottonello et al., 2002; Plataki et al., 2006; Pletz et al., 2004).

1.4. Hypothesis and aims

Increased neutrophil lifespan and poor bacterial clearance by neutrophils are common causes of COPD disease pathology and progression. Available treatment options only treat the symptoms but not the cause of the disease.

Tetraspanins are members of a large protein family that have roles in many cellular functions including cell survival, adherence, fusion and membrane trafficking but they can also be exploited by bacteria and other pathogens as gateway for infection. Tetraspanins functions in neutrophils have been poorly investigated. I therefore **hypothesise** that tetraspanins play a role in neutrophil survival and bacterial interactions.

Specific aims of this project include:

1. Identify the specific tetraspanin(s) involved in human neutrophil apoptosis and phagocytosis using anti-tetraspanin antibodies (Chapter 3-4).
2. Investigate the role of tetraspanin(s) in neutrophil survival in the context of COPD (Chapter 3).
3. To understand tetraspanin(s) role in neutrophil interaction with different Gram positive/ negative respiratory pathogens using techniques such as light and fluorescent microscopy and gentamicin protection assay (Chapter 4).

Chapter 2: Materials and methods

2.1. Ethics

The experiments conducted using blood taken from healthy human volunteers followed the ethics protocol as agreed by the South Sheffield Regional Ethics Committee, study number STH13927. COPD patients or their equivalent healthy controls (HC) used in the study were under the approval of the Yorkshire and the Humber Regional Ethics Committee-STH15949 study. All volunteers gave informed consent and donated a maximum of 500mls over a 6 month period. Any stored human tissue sample (protein, RNA, DNA) were to be destroyed in 5 years. The use and storage of human neutrophils was recorded in a user log book.

2.2. Reagents

Sterile, preservative free monoclonal antibodies directed against tetraspanins CD9, CD37, CD63 and fragment antigen-binding (Fab) fragment directed against CD63, CD151 and the Immunoglobulin G 1(IgG1) isotype control (JC1) were provided by Dr. Lynda J. Partridge, Department of Molecular Biology and Biotechnology, University of Sheffield, United Kingdom (Table 2.1). AHN-16.1 anti-CD63 antibody was a gift from Dr. Keith Skubitz, Department of Medicine, University of Minnesota Medical School, Minneapolis, USA and anti-CD63 antibody 6H1 clone was a gift from Dr. Martin Hemler, Harvard Medical School, Boston, USA (Table 2.1). LEAF^{TM} (Low Endotoxin, Azide-Free) purified anti-human CD151 (PETA-3) blocking antibody and LEAF^{TM} purified mouse IgG1, κ isotype control were purchased (Table 2.1). Allophycocyanin (APC) labeled anti-human CD151 and Mouse IgG1 K isotype control were obtained from eBioscience, San Diego, USA. Pyocyanin was a gift from Dr. Graham Taylor, Imperial College London (Usher et al., 2002). *S. aureus* SH1000 (wild type-WT), USA300 (JE2), *S. aureus* SH1000 protein A (SpA) mutant, SRCO11 and SCRCO12 SH1000 background fibronectin mutants, as well as SH1000 chromosomal (chr)-green fluorescent protein (GFP) strains were provided by Prof. Simon Foster, Department of Molecular Biology and Biotechnology, University of Sheffield, United Kingdom. *S. pneumonia* D39 strain and *Nontypeable Haemophilus Influenzae* (NTHi) NCTC 1269 strain were obtained from American Type Culture Collection, Manassas, USA and were a gift from Dr. Martin Bewley, Department of Infection, Immunity and Cardiovascular disease, Royal

Hallamshire Hospital, Sheffield, UK. The purchase origin of other reagents was from various companies and listed in the sections bellow when referring to them.

2.3. Plasma/Percoll purification of neutrophils from whole blood

2.3.1. Leukocyte isolation and purification from whole blood

Venous blood was taken from healthy volunteers, age-matched HC or COPD patients (see 'ethics' Section 2.1 above) and transferred to a 50ml falcon tube containing 3.8% sodium citrate (Martindale pharmaceuticals, Essex, UK) at 1ml sodium citrate per 9ml of blood. The tube was gently rolled to mix. The tube lid was parafilmed and the blood centrifuged at 350 g for 20 minutes at room temperature (RT) to allow the separation of blood cells from platelet rich plasma (PRP). The PRP was carefully aspirated, to avoid cell pellet disruption, and transferred to a 50ml falcon tube. Platelet poor plasma (PPP) was obtained by centrifuging the PRP at 800 g for 20 minutes at RT. The PPP was subsequently transferred to a fresh 50ml falcon tube.

2.3.2. Dextran sedimentation of red blood cells

A volume of 6ml of pre warmed 6% dextran T-500 (3g of dextran powder T-500 was added to 50 ml of 0.9% saline) (Sigma-Aldrich, Poole, UK) was added to the blood cell rich lower layer and topped up to 50ml with pre-warmed 0.9% saline (Baxter, Newbury, UK). The liquid was then mixed by gentle rolling. The droplets on the lid and the bubbles on the surface were removed with a Pasteur pipette to prevent disruption of the sedimentation process. The lid was left loosened and the red cells were allowed to sediment, at RT, until a clear interface was visible (approximately 25 minutes). The white cell rich upper layer was then transferred to a clean 50ml falcon tube and centrifuged at 320 g for 6 minutes to pellet the cells.

Target	Clone	Isotype	Source	Storage buffer	Purity
CD9	602.29	IgG1	Andrews et al., 1981	PBS	>95%
CD37	WR17	IgG1	Moore et al., 1987	PBS	>95%
CD63	H5C6	IgG1	Azorsa et al., 1991	PBS	>95%
	AHN-16.1	IgG1	Skubitz et al., 1996	PBS	>95%
	6H1	IgG1	Berditchevski et al., 1995	PBS	>95%
CD63 Fab fragment	H5C6	IgG1	Obtained in house Higginbottom et al., 2000	PBS	>95% for old and new stock
CD151	14A2	IgG1	Fitter et al., 1995	PBS	>95%
	50-6-blocking	IgG1	Biolegend, Cambridge, UK	PBS	Purity not specified by the manufacturer Endotoxin level<0.1 EU/µg
β1	P4C10-blocking	IgG1	Novus Biologicals, Abington, UK	PBS containing 0.02% Sodium Azide	Purity not specified by the manufacturer
Control	JC1	IgG1	Muranova et al., 2004	PBS	>95%
	LEAF purified	IgG1	Biolegend, Cambridge, UK	PBS	Purity not specified by the manufacturer Endotoxin level<0.1 EU/µg

Table 2.1: Description of the antibodies used in this thesis

2.3.3. Plasma/Percoll-gradient cell isolation

During the centrifugation stage, the plasma/Percoll (Sigma-Aldrich, Poole, UK) gradient was prepared using accurate measurements of 90% Percoll stock solution (9ml of 100% Percoll was made up with 1ml of 0.9% saline) and PPP. Two densities of PPP/Percoll were made: 51% Percoll (1.02ml of 90% Percoll and 0.98ml of PPP) and 42% Percoll (0.84ml of 90% Percoll and 1.16ml of PPP). The two Percoll densities were overlaid in a 15ml polystyrene tube as follows: 51% Percoll was placed at the bottom of the tube, followed by the 42% Percoll. The supernatant from the cell pellet from section 2.3.2 was discarded and the cell pellet was gently resuspended in PPP to a final volume of 2ml and carefully overlaid on the top of the Percoll gradient (Figure 2.1 left hand panel). The gradient was centrifuged at 320 g for 11 minutes at RT without a brake. Following centrifugation, three layers were observed: red blood cell (RBC) pellet at the bottom of the tube, followed by a middle band containing the granulocyte layer (neutrophils/eosinophils/basophils) and an upper band containing peripheral blood mononuclear cells (PBMCs) (Figure 2.1 right hand panel).

2.3.4. Harvesting the gradient

Using a Pasteur pipette, the PBMC and the granulocyte layers were transferred to 50ml falcon tubes containing 10ml PPP. Cell washing was done by topping them with 40mls hanks buffer saline solution (HBSS) without Ca^{2+} or Mg^{2+} (Gibco life technology, Paisley, UK). A cell count was performed using a haemocytometer chamber. A cells pellet was then obtained by centrifugation at 320 g for 6 minutes. Neutrophils were afterwards resuspended in pre-warmed complete media (Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich, Poole, UK) + 10% fetal bovine serum (FBS) (Autogen Bioclear, Wilshire, UK) + 1% Penicillin/Streptomycin (Pen/Strep) (Life Technologies, Paisley, UK)) or further purified as per section 2.3.5.

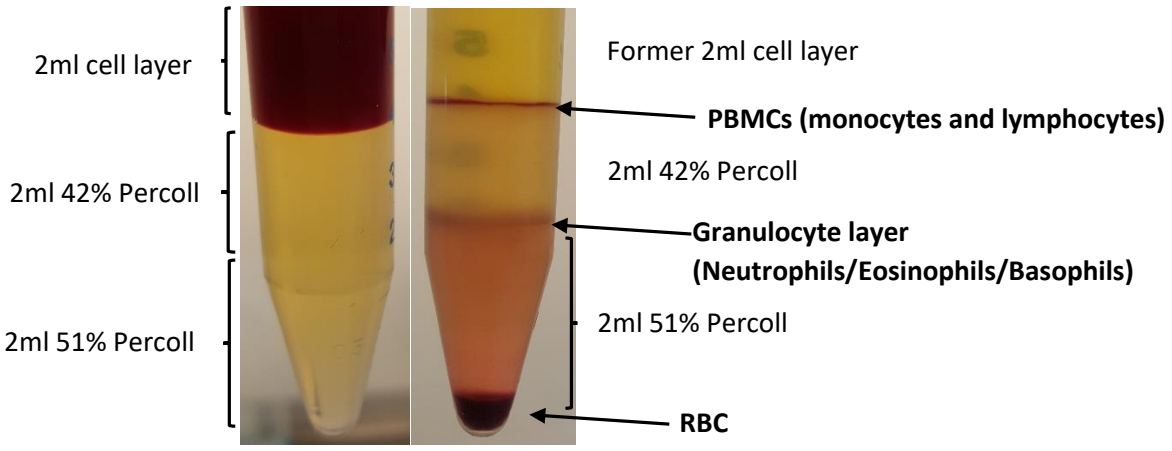

Figure 2.1: Separation of the cells on the plasma/Percoll gradient

The left hand panel shows the construction of the 3 gradient layers. The right hand panel shows appearance of the gradient following centrifugation; a red cell pellet, a granulocyte intermediate layer containing neutrophils, basophils and eosinophils and a PBMC upper layer comprised of monocytes and lymphocytes.

2.3.5. Neutrophil ultrapurification using negative magnetic selection

Granulocytes isolated from the plasma/Percoll gradient were purified further by negative magnetic selection. A StemSep Human Custom Enrichment Kit and a magnetic MACS column removed contaminating cells (eosinophils, PBMCs, RBCs) by antibody labelling of contaminating cells. Resuspension of the cells was done initially at $1x10^8$ cells/ml in neutrophil column buffer (HBSS without Ca^{2+} and Mg^{2+} and 2% FBS). A 15 minute incubation at RT with 70µl antibody cocktail (Stemcell technologies, Meylan, France) per 1ml cell suspension (Sabroe et al., 2004) with intermittent gentle mixing was then conducted. The antibody cocktail contains antibodies directed against CD2, CD3 (T lymphocytes), CD19 (B lymphocytes), CD36 (eosinophils), CD56 (natural killer cells) and glycophorin A (RBCs). A further 15 minute incubation was carried out in the presence of 50µl/ml magnetic colloid beads (Stemcell technologies, Meylan, France) with gentle mixing. During the second incubation, the negative magnetic selection column (Miltenyi Biotec, Surrey, UK) was assembled and primed with 10ml neutrophil column buffer. The cells were subsequently added to the column. Contaminating cells were bound to the column via the colloid beads allowing collection of neutrophils into a clean tube. To enrich the neutrophil population, the column was washed further with 10ml of column buffer. The number of neutrophils was then counted using a haemocytometer chamber, followed by cell centrifugation at 320 g for 6 minutes and their resuspension in complete RPMI 1640 at $5x10^6$ cells/ml.

2.4. Bacterial growth and storage

2.4.1. *S. aureus* strains

A range of *S. aureus* strains and mutants, indicative of the functions targeted, were used for the work detailed below. The low virulence SH1000 laboratory strain was obtained in the laboratory of Professor Simon J. Foster by restoring the rsbU deletion in the RN6390 lineage (Horsburgh et al., 2002). The features of this strains make it a good laboratory tool since it behaves as a clinical isolate. *S. aureus* SpA, SRCO11, SRCO12 and chromosomal-gfp mutant strains were obtained in the laboratory of Professor Simon J. Foster on the background of SH1000. The SpA mutant is protein A deficient (Chan and Foster, 1998). Protein A allows *S. aureus* binding to the Fc immunoglobulin region in humans to avoid recognition and

phagocytosis by neutrophils (Dossett et al., 1969). SRCO11 and SRCO12 are mutants that lack the ability to bind to the integrin binding extracellular matrix glycoprotein, fibronectin. SRCO11 lacks fibronectin binding protein A (fnbA) and fnbB while SRCO12 lacks fnbA, fnbB as well as the iron-regulated surface determinant protein A precursor (isdA) (Buck and Horwitz, 1987; Clarke et al., 2004; Jonsson et al., 1991). The *S. aureus* SH1000 chr-GFP strain has a kanamycin resistant gfp gene, stably incorporated in the genome and therefore constitutively expresses it (data not published yet). The JE2 strain was obtained from the USA300, the most common community-associated MRSA strain, by the double removal of macrolide resistance and a cryptic plasmid (Kennedy et al., 2008).

2.4.1.1. Growth and storage of *S. aureus* strains

A -80°C *S. aureus* master bead stock of WT, SH1000 chr-GFP, SRCO11 and SRCO12 were obtained from the laboratory of Prof. Simon Foster. Before usage, the bacteria were thawed at RT for 30 seconds. One bead from each stock was placed on brain heart infusion (BHI) agar (Sigma-Aldrich, Poole, UK) plate and left to melt for 1 minute. To select for SH1000 chr-GFP, 50µg/ml kanamycin (Gibco life technologies, Paisley, UK) was added to the agar and selection for SRCO11 and SRCO12 was done by using 5µg/ml erythromycin (Sigma-Aldrich, Poole, UK). No antibiotics were required for the growth of WT strain. The plates were incubated overnight at 37°C/ 5%CO_2. The following day, a single colony was inoculated into 5ml of BHI broth (Sigma-Aldrich, Poole, UK) with or without the antibiotics, depending on the strain, at the concentrations defined above. The cultures were then grown overnight on a shaker at 37°C/ 5%CO_2. In order to achieve bacteria in exponential growth phase, the overnight culture was subcultured into fresh BHI broth (plus the antibiotics concentrations specified for each strain) by placing the bacteria in fresh BHI broth at an OD_{600} of 0.05. The bacteria was then grown to 1X 10^9 colony forming unit (CFU)/ml (OD_{600} of 2.2). At this stage the bacterial cells were ready to be used in experiments or stock storage was done as follows. Equal amounts of bacterial broth with 50% glycerol diluted in water were mixed, aliquoted out and stored at -80°C. The CFU/ml of bacteria was confirmed by conducting a Miles-Misra test as described by (Miles et al., 1938). In neutrophil experiments conducted in this thesis, 6x10 fold dilutions were made for each condition and 3x10µl drops from the

last 3 dilutions were plated on BHI agar plates followed by overnight incubation at 37°C/ 5%CO_2. The CFU/ml was determined the subsequent day by using the formula:

CFU/ml= (colonies at the dilution where they are distinguishable/30)*1000* dilution factor.

S. aureus JE2 and *S. aureus* SpA strains were grown by Miss. Dingyi Yang, Infection, Immunity and Cardiovascular Disease, Medical School, University of Sheffield, UK.

2.4.1.2. *S. aureus* strain heat inactivation

Heat inactivation/ killing (HK) of a bacteria is conducted to remove its ability to kill cells. For light microscopy, western blotting and flow cytometry experiments, overnight cultures of *S. aureus* WT, SpA mutant and *S. aureus* JE2 strains were incubated in the heating block for 10 minutes at 100°C to inactivate them. For fluorescent microscopy, *S. aureus* WT subculture was incubated for 30 minutes at 70°C, as indicated by the group who published initially with the Alexa Fluor 647 and pHrodo stained *S. aureus* (Ellett et al., 2015).

2.4.2. Growth and storage of *S. pneumoniae* and NTHi

The -80°C *S. pneumoniae* or NTHi master stock obtained from following the manufacturers' protocol, were thawed for 30 seconds at RT before usage. A bead was placed on the blood agar plate (made in house by mixing BHI agar and overlaying horse blood (Thermo Fisher Scientific, Loughborough, UK)) for *S. pneumoniae* and one on chocolate agar (E&O Laboratories Limited, Bonny Bridge, Scotland) for NTHi. NTHi cannot synthesise V factor, found inside red blood cells, therefore it must be released by cell lysis, which in turn give the plates the appearance of chocolate. The colonies were then streaked out and incubated overnight at 37°C/ 5%CO_2. The following day, 10-20 colonies of *S. pneumoniae* were placed in BHI broth + 20% FBS and placed on the orbital shaker for approximately 4.5 hours, or until the OD_{600} reached 0.6. To grow NTHi, the BHI broth + 20% FBS was supplemented with 2µg/ml of nicotinamide adenine dinucleotide (NAD) (Sigma-Aldrich, Poole, UK) and 10µg/ml of Hemin from stock (Sigma-Aldrich, Poole, UK) (see Appendix I) and placed on the orbital shaker until OD_{600} reached 0.6. The broth was then aliquoted out and the samples stored in the -80°C freezer. The CFU/ml of bacteria was determined by conducting a Miles-Misra test (see section 2.4.1.2.).

Heat inactivation of overnight culture of *S. pneumoniae* was done by placing the vial for 10 minutes in the heat block at 100°C.

2.4.2.1. *S. pneumoniae* opsonisation

The method for opsonising *S. pneumoniae* D39 was first described by (Gordon et al., 2000). To opsonise *S. pneumoniae*, bacterial stocks (see section 2.4.2.) were thawed at RT and washed 3 times in 1ml of PBS (Gibco life technologies, Paisley, UK) at 11,000 g for 5 minutes. The bacteria were subsequently incubated for 30 minutes at 37°C/ 5%CO_2 on an orbital shaker with 500µl of RPMI 1640 (without any serum) + 10% serum containing anti-serotype antibodies from immunised people. To obtain the serum, blood was taken from healthy human volunteers immunised 3-6 months prior to this with Pneumovax vaccine. The plasma was separated from the blood by centrifugation, heat inactivated and tested for the anti-serotype antibodies using flow cytometry or ELISA. The plasma from 3-4 donors was pooled together, aliquoted out and frozen at -80 until needed. The serum separation from blood, and determination of antibody levels was performed by our in house technician, Mrs. Catherine J. Cooke. The opsonised *S. pneumoniae* were then washed 3 times with PBS (Lonza, Veveris, Belgium) by centrifuging the bacteria for 3 minutes at 3,400 g and resuspended in RPMI 1640 + 10% FBS at 1x 10^9 CFU/ml. The low centrifugation speed for washing opsonised *S. pneumoniae* was used to prevent the dissociation of the opsonins from the target.

2.5. Light microscopy

2.5.1. Preparation of cytospins

In order to assess purity, apoptosis and phagocytosis, neutrophils were cytocentrifuged onto glass slides as monolayers, allowing the observation of subcellular features by oil immersion microscopy. To do this, a volume of 100µl containing neutrophils at 5X10^6/ml, incubated under various conditions (see individual experimental detail), were transferred to clip-slide-filter-chambers in duplicates. The cells were then centrifuged at 300 g for 3 minutes using a Shandon Cytocentrifuge (Shandon Cytospin 3, Thermo Scientific, Hemel Hempstead, UK). Allowing a few moments to air dry, cells were then fixed on the slides with a drop of 100% methanol. Staining (based on hematoxylin and eosin) was carried out by

placing the slides in Diff-Quik stain I (orange dye) (Gentaur, London, UK) for 1 minute, to stain cell organelles, followed by Diff-Quick stain II (blue dye) (Thermo Fisher Scientific, Loughborough, UK) for 2-3 minutes, to stain nucleus and cell membrane. The slides were allowed to fully dry before being coverslipped. To coverslip, a drop of distrene-80 plasticiser xylene (DPX) (Fisher Scientific, Loughborough, UK) was placed on the slides followed by addition of a glass coverslip on top. The slides were visualised using Zeiss Axioplan microscope with 100X oil immersion lens.

2.5.1.1. Assessment of neutrophil purity, apoptosis and phagocytosis

Healthy and apoptotic neutrophils have characteristically distinct cellular morphologies (Haslett et al., 1989). Healthy neutrophils have a textured, multi-lobed, nucleus connected by fine chromatin bridges, small granules and a lilac/pink coloured cytoplasm (Figure 2.2A). In contrast, apoptotic neutrophils present two main characteristics: some have a very dense, round nucleus due to chromatin condensation, while others appear as “ghost cells” due to nuclear components leaking out (Figure 2.2B). Other cells were also seen under the microscope: eosinophils had a textured, horseshoe-like nucleus with distinct large, salmon pink granules (Figure 2.2C); RBC were seen as grey circles (Figure 2.2D) and monocytes and lymphocytes (PBMCs) were seen as having a large textured nucleus and blue cytoplasm (Figure 2.2E-F). Bacteria under the microscope appeared as small purple spheres (*S. aureus*) (Figure 2.2G) or rods (*S. pneumoniae*) (Figure 2.2H).

Neutrophil apoptosis was assessed by counting a total of 300 (apoptotic and viable) cells on each of the duplicate slides. Purity slides were prepared from freshly isolated neutrophils at 0 hours, and 500 cells (red cells excluded) were counted on each of duplicate slides based on their morphology. Phagocytosis was assessed by calculating the phagocytic index (PI) using the formula specified below. This formula takes into account not only the percentage of neutrophils that phagocytose bacteria, but also the number of bacteria each cell has phagocytosed (Sano et al., 2003).

Phagocytic index:

((total number of engulfed bacteria/total number of cells)*(number of cells containing bacteria/total number of cells))*100

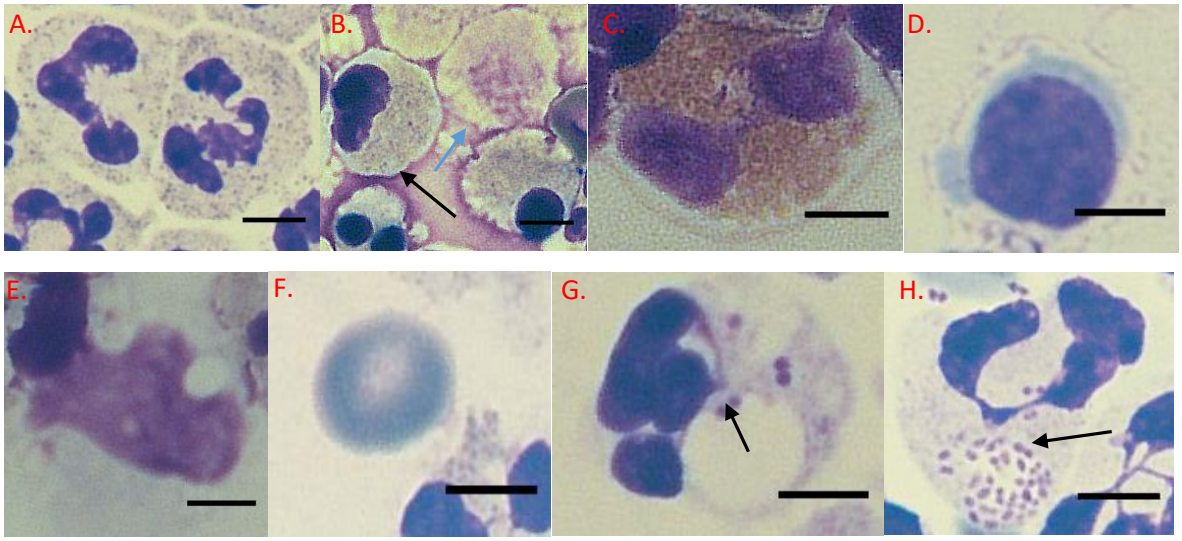

Figure 2.2: Cell and bacteria morphology under the light microscope

Light microscopy visualisation of cells and bacteria: A) healthy neutrophils with multi-lobed nucleus; B) Apoptotic neutrophils that underwent chromatin condensation (black arrow) or where cytoplasmic cell content leaked out to form 'ghost cells' (blue arrow); C) Eosinophils with horseshoe-like nucleus and orange cytoplasm; D) Lymphocyte with round nucleus and blue cytoplasm; E) Monocyte with horseshoe-like nucleus and blue cytoplasm; F) Anucleated Red blood cell; G) Neutrophil containing *S. aureus* in the clear phagolysosome (black arrow); H) Neutrophil with the engulfed, rod-like *S. pneumoniae* (black arrow). The image blurriness was corrected using Fiji program with the 'Enhance contrast' feature. Scale bar, 5µm.

2.5.1.2. Analysis of neutrophil survival in the presence of anti-tetraspanin antibodies

Neutrophils obtained from Section 2.3 were resuspended in complete media at $5X10^6$ cells/ml, as previously determined by our group and others to be an optimal density. Neutrophils were added to 96 well flexible plates (Costar, High Wycombe, UK). Monoclonal antibodies directed against CD9, CD37, CD63 and CD151 or fragment antigen binding (Fab) fragments directed against CD63 were obtained in the laboratory of Dr. Lynda Partridge via purification from hybridoma supernatants using protein G Sepharose. The obtain of Fab fragments from whole antibodies was done using pepsin digestion and was previously described by the Partridge group (Higginbottom et al., 2000). They were individually added to the wells following the concentrations on Table 2.2. Each antibody concentration will be specified for each experiment in Chapter 3. The JC1 isotype control was used in all experiments as a reference. The following provides key information for experiment design. All wells were supplemented with complete media followed by incubation for 4 or 20 hours at 37°C/ 5%CO_2, as specified for each experiment. Comparison between plasma/Percoll-pure and ultrapure/ column purified neutrophils was done by using only anti-CD63 antibodies (20µg/ml) and JC1 isotype control (20µg/ml). The neutrophils from age matched HC and COPD patients were incubated for 4 and 20 hours with media, JC1 isotype control (20 µg/ml) or anti-CD63 antibodies (20µg/ml). In a single dataset, neutrophils were incubated for 4 and 20 hours with media, JC1 isotype control (20 µg/ml) and anti-CD63 antibodies clones AHN-16.1 and 6H1 (20µg/ml), in addition to the H5C6 anti-CD63 antibody clone used in all neutrophil survival experiments. For all the above experiments, the 4 hour time point was used since that is when neutrophil apoptosis is starting to be seen while the 20 hour time point was selected because neutrophil apoptosis starts to plateau out at that time (Sabroe et al., 2004).

In key survival experiments, neutrophils were pre-incubated for 1 hour with JC1 isotype control or anti-CD63 antibodies at 10µg/ml followed by survival stimulatory/inhibitory compounds to modulate neutrophil apoptosis. Neutrophil incubation was then done for 20 hours with LPS-10ng/ml (Enzo life sciences, Exeter, UK) and GM-CSF-50units/ml (Gibco life technology, Paisley, UK) or for 5 hours with Pyocyanin (Pyo) (50µM). The 5 hour time point incubation with pyo was selected due to previous evidence in the literature of significant

neutrophil apoptosis reduction when the cells were incubated for 5 hours with the apoptosis acceleration stimuli (Usher et al., 2002). Light microscopy assessment and cytospins preparation was done as per section 2.5.1. and 2.5.1.1.

2.5.1.3. Investigation of CD63-GST peptides LPS contamination

Tetraspanin peptides are small constructs that target the EC2 tetraspanin domain and have a GST tag for purification, having been shown recently to have valuable therapeutic implications (Higginbottom et al., 2003; Parthasarathy et al., 2009; Ventress et al., 2016). Considering that these constructs were obtained in and *E. coli* system, they were checked for endotoxin contamination. Neutrophils obtained from Section 2.3 were resuspended in complete media at $5X10^6$ and added to 96 well plates. The cells were then pre-incubated with LPS from *Rhodobacter sphaeroides* (LPS-RS) (10µg/ml) (InvivoGen, Toulouse, France), a potent endotoxin inhibitor, for 1 hour followed by a 20 hour incubation with LPS (10ng/ml), GST (10µg/ml) and GST-CD63 (10µg/ml). Light microscopy assessment and cytospin preparation was done as per section 2.5.1. and 2.5.1.1.

2.5.1.4. Modulation of neutrophil survival using tissue inhibitor metalloproteinase-1

Tissue inhibitor metalloproteinase-1 (TIMP-1) is a protein identified via yeast two-hybrid system to interact with CD63 (Jung et al., 2006). Neutrophils were resuspended in complete media at $5X10^6$ cells/ml. Neutrophils (50µl/well) were incubated in a 96 well plate with 0.05, 0.1, 0.5 or 1 µg/ml of recombinant human TIMP-1 protein (Insight Biotechnology Limited, Wembley, UK) for 20 hours. The recombinant human TIMP-1 protein had 95% purity and the endotoxin level <0.1ng/µl. Concentrations were chosen considering the manufacturer's half minimal inhibition value of 0.05µg/ml. Light microscopy assessment and cytospin preparation was done as per section 2.5.1. and 2.5.1.1.

2.5.1.5. Neutrophil phagocytosis of live and HK bacteria in the presence of anti-tetraspanin antibodies

Neutrophils have long been known to clear bacterial infections, however we are still unaware of all the proteins involved in the process of bacterial interaction, phagocytosis

and killing (Holmes et al., 1967). Tetraspanins have been shown in Chapter 1 to be involved in bacterial interactions, as a result their roles in neutrophil phagocytosis was investigated. Neutrophils (50µl/well at $5x10^6$ cells/ml) obtained from section 2.3. were resuspended in complete media (for HK bacteria), or media without antibiotics (for live bacteria) and added to 96 well flexiwell plates. Monoclonal antibodies, directed against CD9, CD37, CD63 and JC1 isotype control were used at a final concentration of 10µg/ml while anti-CD151 antibodies were used at 0.5, 1, 5, 10, 20 µg/ml. Biolegend anti-CD151 and matched isotype control antibodies were used at 10 µg/ml. The concentrations the antibodies were used at will be specified for each experiment in Chapter 4. Cells were pre-treated with media or antibodies described above for one hour at 37°C/ 5%CO_2. Following this, neutrophils were incubated with different multiplicity of infection (MOI) of HK and live *S. aureus* and opsonised HK or live *S. pneumoniae* (see Sections 2.4.1.2., 2.4.2. and 2.4.2.1. for bacterial HK and *S. pneumoniae* opsonisation) at different time points as described in Table 2.3. The incubation time and MOI of bacteria will be specified for each experiment in Chapter 4. In a single experiment, neutrophils were incubated with latex beads (Sigma-Aldrich, Poole, UK) at MOI10 for 2 hours. The number of latex beads particles was calculated using the formula $(6.03x10^{10}xS)/ (3.3xd^3)$, where S is % solids and d is the bead diameter. This information is batch specific and written on the bottle. Light microscopy assessment and cytospin preparation was done as per section 2.5.1. and 2.5.1.1.

In key experiments, anti-β1 integrin blocking antibodies (see Table2.1) were used to test integrin-tetraspanin interactions. The P4C10 antibody clone was used since it has been previously shown to act as a blocking antibody (Byron et al., 2009). To test this, neutrophils were incubated for 1 hour with media, JC1 isotype control, anti-CD151 antibodies, anti-β1 integrin antibodies at 5µg/ml or anti-CD151 antibodies + anti-β1 integrin antibodies (10µg/ml+ 5µg/ml). Live *S. aureus* WT at MOI10 was then added to the cells for 45 minutes.

Antibodies	Concentrations				
	0.5µg/ml	1µg/ml	5µg/ml	10µg/ml	20µg/ml
JC1 isotype	-	-	-	+	+
CD9	-	-	-	+	-
CD37	-	-	-	+	-
CD151	-	-	-	+	-
CD63-H5C6	+	+	+	+	+
CD63-H5C6-Fab	-	-	+	+	+
CD63- AHN16.1	-	-	-	-	+
CD63-6H1	-	-	-	-	+

Table 2.2: Tetraspanin antibody concentrations used in the survival experiments.

Anti-tetraspanin antibodies were used at different concentrations for survival experiments. The '+' symbol mark the concentration the antibody is used at in the thesis while the '-' symbol indicates the antibody was not used at that concentration.

Bacterial strains	MOI					
	1	2.5	5	10	20	40
HK *S. aureus* WT	120 minutes	120 minutes	120 minutes	30, 60 or 120 minutes	15, 30, 60, 120, 180, 240 minutes	-
HK *S. aureus* JE2	-	-	-	120 minutes	-	-
HK *S. aureus* SpA	-	-	-	120 minutes	120 minutes	-
HK *S. pneumoniae*	-	-	120 minutes	30,60 or 120 minutes	120 minutes	120 minutes
Live *S. pneumoniae*	-	-	-	60 minutes	-	-
Live *S. aureus* WT	-	-	45 or 75 minutes	-	-	-
Live *S. aureus* SRCO11/SRCO12	-	-	45 minutes	-	-	-

Table 2.3.: *S. aureus* and *S. pneumoniae* MOI and incubation time used in the phagocytosis experiments.

Neutrophils were incubated for different amounts of time with different MOI of *S. aureus* and *S. pnemoniae*. The table shows the MOI of each strain of *S. aureus* and *S. pneumoniae* against the amount of time they were incubated for. The '-' symbol indicates that the MOI was not used for that bacteria.

2.6. Flow cytometry

2.6.1. Determination of CD151 and CD63 expression by flow cytometry

2.6.1.1. Conjugation of tetraspanin antibodies with Alexa Fluor 488 antibody labelling kit

The conjugation of JC1 isotype control, anti-CD63 H5C6 antibody clone and anti-CD151 antibody was done as per manufacturer's instructions and using the reagents provided in the Alexa Fluor 488 antibody labelling kit (Life technologies, Paisley, UK). In brief, a 1M solution of sodium bicarbonate was made by adding 1ml of dH_2O to the provided vial and vortexing until the powder was completely dissolved. The antibody concentration was then adjusted to 1mg/ml with sterile PBS by making a dilution from the individual antibody stock concentration. To prepare the antibodies for the column, $1/10^{th}$ volume of 1M sodium bicarbonate solution was added to each antibody mixture. One hundred microliters of antibody solution was then transferred to individual reaction vials containing Alexa Fluor 488 dye and incubated for 1 hour with periodical mixing by inversion. A spin column was meanwhile prepared for each antibody by adding 1.5ml of resuspended purification resin per columns followed by centrifugation at 1,100 g for 3 minutes to remove the excess liquid. The 100µl of conjugated antibody was added to individual columns by placing drop by drop the liquid into the centre of the column. After the liquid was absorbed into the resin, the columns were centrifuged at 1,100 g for 5 minutes to remove the excess dye. The antibodies were collected into the provided collection tubes and stored at -20°C until required.

2.6.1.2. Determination of CD151 and CD63 expression on neutrophils by flow cytometry

Neutrophils were resuspended in complete RPMI at $5x10^6$ cells/ml and 90µl were added in each well in a 96 well plate and topped up to 100µl with complete medium. Neutrophils were incubated with media for 0, 4 and 20 hours at 37°C/ 5%CO_2. A positive control in which neutrophils were incubated for 30 minutes at 37°C/ 5%CO_2 with HK *S. aureus* at MOI10 was also set up. *S. aureus* was used as positive control since it induces degranulation and activation of neutrophils (Faurschou and Borregaard, 2003). Following incubation, neutrophils were centrifuged at 300 g for 2 minutes. The pellets were then washed in 200µl of PBS by centrifugation at 300 g for 2 minutes. Depending on the experimental requirements, cells were resuspended in 20µl of PBS and then stained at RT in the dark with

different antibodies which will be specified for each experiment in Chapters 3 and 4. These are as follows: Alexa Fluor 488-labeled antibodies (JC1, anti-CD63 and anti-CD151) (see section 2.6.1.1.) incubation was done for 30 minute using 10µg/ml of the antibodies; APC labeled isotype and anti-CD151 antibody incubation was done for 30 minutes using 5µl of the commercially available antibodies; when unlabeled primary antibodies were used initially, neutrophils were pre-blocked for 15 minutes with 10% goat serum (Biolegend, Cambridge, UK) followed by a 20 minute incubation with primary JC1 isotype control or anti-CD151 antibodies at 10µg/ml followed by a further 20 minute incubation with an equal volume, to the primary antibody, of secondary fluorescein 5-isothiocynate (FITC) labelled anti-mouse IgG antibody (Sigma-Aldrich, Poole UK). The labeled cells were then added to fluorescent activated cell sorting (FACS) tubes containing 300µl of PBS. The tubes were then analysed in duplicate using the FACScalibur flow cytometer (BD Biosciences-Pharmingen) and Cell Quest Pro program. The neutrophils were gated on the forward scatter (FSC)/ side scatter (SSC) parameter based on their size and granularity and any contaminating cells were excluded (Figure 2.3). Gain of FITC and Alexa Fluor 488 fluorescence was measured by a right shift on the FL1-H (Ex=490nm, Em=525nm) parameter while the binding of APC-labeled antibodies was measured on the FL4-H (Ex=650nm, Em=660nm) parameter. Analysis of data was conducted using Flowjo analysis software version Vx0.7 (Tree Star, Inc).

2.6.1.3. Determination of anti-CD63 Fab fragment binding to A549 cells

To validate the anti-CD63 Fab fragments binding to CD63, the A549 adenocarcinomic human alveolar basal epithelial cell line, expressing stably CD63 on plasma membrane, was used (Funakoshi et al., 2003). A549 cells were dissociated from an 80% confluent flask (obtained from general laboratory stocks) by incubation for 20 minutes with 2ml of non-enzymatic cell dissociation solution (Millipore, Watford, UK). The reaction was stopped by adding 6ml of Dulbecco's modified eagle medium (DMEM) (Lonza, Veveris, Belgium). A 5 minute centrifugation at 200 g was subsequently done, followed by cell resuspension in 10ml of PBS. A haemocytometer chamber was then employed to count the number of cells. To conduct the experiment, the cells were centrifuged as previously and $1x10^6$ cells/condition were resuspended in 50µl PBS. Blocking of Fc receptors on cell surface was then conducted for 15 minutes with 10% goat serum followed by 1 hour incubation on ice

with JC1 isotype control or anti-CD63 Fab fragments at 10μg/ml. A further 20 minute incubation with 1μl of FITC labeled anti-mouse antibody was conducted on ice. Unstained and a second antibody only negative controls were used. The conditions were analysed using the LSRII flow cytometry machine (BD Biosciences). Separation of cell/debris was done on the FSC/SSC and gain of FITC fluorescence was analysed using the Blue 530/30 channel (Ex=490nm, Em=525nm). Analysis of data was carried out using Flowjo analysis software version Vx0.7.

2.6.1.4. Determination of anti-CD151 antibody binding to HEC-1-B cells

To check anti-CD151 antibodies binding, the human endometrial adenocarcinoma cell line HEC-1-B that expresses CD151 was used (Green et al., 2011). To conduct the experiment, an 80% confluent flask was obtained from the laboratory of Prof. Peter Monk- Medical School, Sheffield, UK- and 2 ml of non-enzymatic cell dissociation solution was added for 20 minutes. The reaction was stopped by adding 6ml of DMEM. A 5 minute centrifugation of the cells at 400 g was then conducted, followed by their resuspension in 10ml PBS. Counting of the cells was done using a haemocytometer chamber. After a subsequent centrifugation for 5 minutes at 400 g, $1x10^6$ cells/condition were resuspended in 10μl of PBS. Blocking of extracellular Fc receptors was initially conducted for 15 minutes with 10% goat serum. A 30 minute staining on ice was initially done with 10μg/ml of Alexa Fluor 488 labeled JC1 isotype control and anti-CD151 antibodies; and with primary, unstained anti-CD151 antibodies (10μg/ml) or with 5μl of commercially obtained APC labeled isotype and anti-CD151 antibodies. Where the primary, unstained anti-CD151 antibody was used, a further 20 minute incubation with equal volume, to the primary antibody, of FITC labeled anti-mouse antibody was done. An unstained and a secondary antibody only controls were used. Separation of cells/debris was done based on their FSC/SSC parameters. Gain of FITC and Alexa Fluor 488 fluorescence was measured by a right shift on the FL1-H (Ex=490nm, Em=525nm) parameter while the binding of APC-labeled antibodies was measured on the FL4-H (Ex=650nm, Em=660nm) parameter. Analysis of data was carried out using Flowjo analysis software.

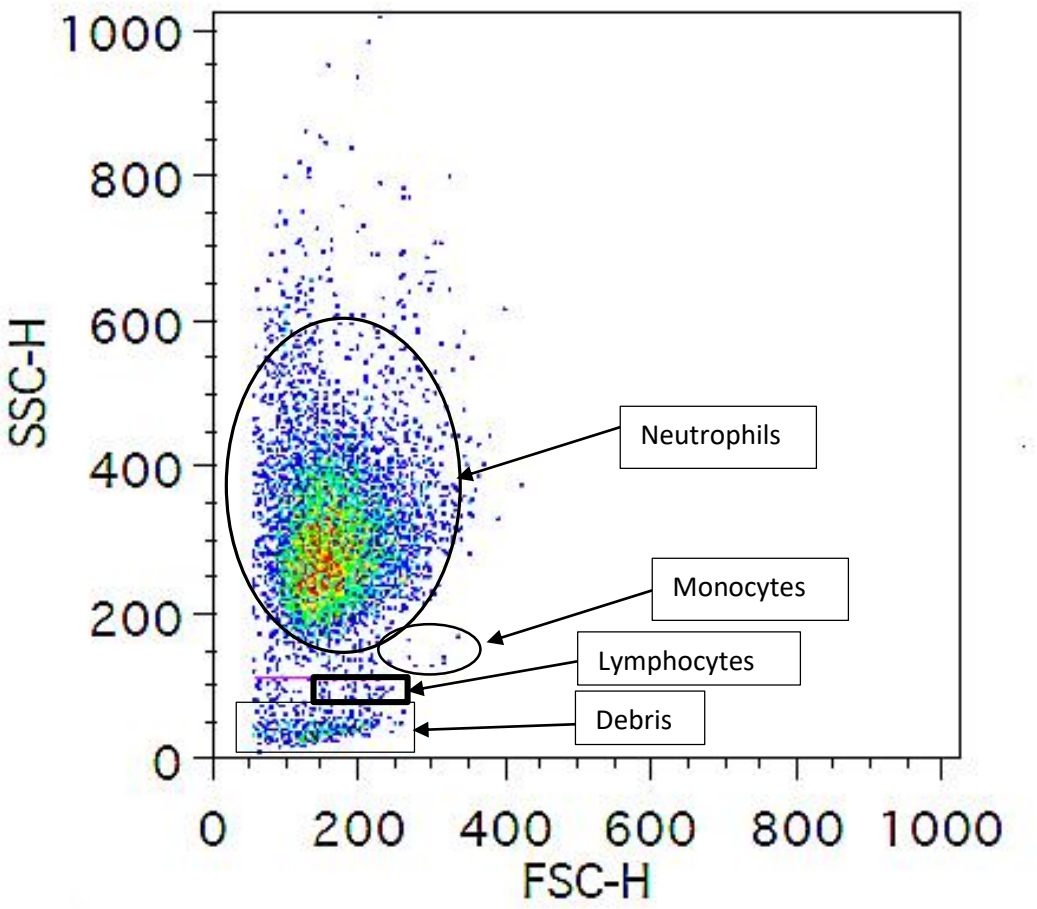

Figure 2.3: Distribution of cells on a flow cytometry dot plot.

Diagram shows FSC parameter (indicator of cell size) and SSC parameter (indicator of cell density/granularity) profile of a plasma/Percoll-pure granulocyte preparation. Distinct populations of neutrophils can be seen on the FSC/SSC parameter. Neutrophils appear more granular than monocytes. A small population of monocytes and lymphocytes can be seen, as well as some debris. Due to the high purity in our neutrophil population the monocytes cannot be seen clearly.

2.6.2. Determination of neutrophil death using Annexin V/ToPro-3 staining

Neutrophils obtained from plasma/Percoll gradient centrifugation (see section 2.3.) were resuspended in complete media at $5x10^6$ cells/ml and 50µl neutrophils/well were added to a 96 well plate. Following the addition of 20µg/ml of JC1 isotype control or anti-CD63 antibodies, the wells were topped to 100µl with complete media and incubated for 20 hours at 37°C/ 5%CO_2. After incubation, the cells were centrifuged at 300 g for 2 minutes, the supernatant removed and the pellet resuspended in 50µl of 1x Annexin V buffer (BD Biosciences, Oxford, UK). Annexin V-phycoerythrin (PE) (2.5µl/reaction, BD Biosciences, Oxford, UK) was then added to reaction tubes and incubated for 20 minutes in the dark at RT. Annexin V binds to phosphatidylserine groups that become permanently exposed on the outer membrane as cells undergo apoptosis (Babiychuk et al., 2008; Cantinieaux et al., 2004; Solito et al., 1998). The cells were subsequently added to FACS tubes containing 300µl of 1x Annexin binding buffer with ToPro-3 (Life technologies, Paisley, UK) at 1:10,000 dilution. A negative control of cells incubated with neither Annexin V nor ToPro-3 was used and was kept at RT for the duration of staining protocol. The samples were then analysed using LSRII flow cytometer using the Blue575/26 laser to detect PE fluorescence (Ex max-496nm, Em max-578nm) and Red 660/20 to detect ToPro-3 fluorescence (Ex max-642nm, Em max-661nm). Analysis of data was carried out using Flowjo analysis software.

2.6.3. Determination of neutrophil lysis by *S. aureus* using flow cytometry

Neutrophils obtained from plasma/Percoll gradient centrifugation (see section 2.3.) were resuspended in RPMI+ 10% FBS at $5x10^6$ cells/ml. A volume of 90µl/well was added per condition to a 96 well plate. A 1 hour incubation in the presence of 10µg/ml JC1 control or anti-CD151 antibodies at 37°C/ 5%CO_2 was initially conducted, followed by a 2.5 hours incubation with live *S. aureus* WT MOI5 at 37°C/5% CO_2. After a 2 minute centrifugation at 300 g, the supernatants were discarded and the pellet resuspended in 200µl of PBS and transferred to a clean 96 well plate. A positive control was used where 200µl neutrophils resuspended in PBS were incubated at -80°C for 8 minutes, followed by their thawing at RT and lysing by vigorous vortexing. ToPro-3 was then added to the wells at 1:10,000 dilution and the samples were immediately run using the Attune flow cytometer (Applied

biosystems, Loughborough, UK). The cells were separated on FSC/SSC and gain of ToPro-3 fluorescence (Ex -642nm, Em -661nm) was visualised on the RL1-A channel. Analysis of data was carried out using Flowjo analysis software.

2.6.4. Extracellular bacterial fluorescence quenching using trypan blue

Trypan blue (TB) is a dye that is commonly used in research to quench fluorescence of external particles due to its light absorbing properties in the green spectrum (Nuutila and Lilius, 2005). Freshly isolated neutrophils were resuspended in RPMI-1640+ 10% FBS at 5x 10^6 cells and 90µl/well of these were added for each condition to a 96 well plate. Neutrophils were then incubated for 1 hour in the presence of media at either 37°C/ 5%CO_2 or 4°C and with JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml) only at 37°C/ 5%CO_2. Media treated neutrophils were then incubated for a further 45 minutes with live *S. aureus* (WT chr-GFP) MOI 5, 10 or 20 at both 37°C/ 5%CO_2 and 4°C. Antibody treated neutrophils were further incubated for 45 minutes with live *S. aureus* (WT chr-GFP) at an MOI10 at 37°C/ 5%CO_2. *S. aureus* (WT chr-GFP) has a GFP that can be detected in the green portion of the spectrum at 509nm. A 2 minute centrifugation of the cells at 300g was subsequently conducted, followed by supernatants removal and pellet resuspension in 125-150µl of PBS and addition to FACS tubes. For media only incubated neutrophils, TB solution 0.4% (Sigma-Aldrich, Poole, UK) was then added to tubes at either 1:4 or 1:2 dilutions for 1 or 15 minutes while for antibody incubated cells a 1:2 dilution of the dye for 15 minutes was used. ToPro3 (1:10,000 dilution in PBS) was used as a control to determine membrane leakage and was added to cells just before their running on the flow cytometer. The 4°C incubated samples were kept on ice until they needed to be run on the flow cytometer. Detection of GFP (Ex -488nm, Em -509nm) fluorescence gain was seen as a right shift in the FL1-H channel of FACSCAlibur flow cytometer while ToPro-3 (Ex -642nm, Em -661nm) fluorescence gain was indicated on the FL-4 channel. Analysis of data was carried out using Flowjo analysis software.

2.6.5. Determine HK *S. aureus* WT staining with Alexa Fluor 647 and pHrodo

PHrodo is known for some years as a dye that can be used to measure phagocytosis since it can be activated at low pH while Alexa Fluor 488 has recently been used to co-conjugate

bacteria with pHrodo to study bacterial interaction and localisation in cells (Aziz et al., 2013; Ellett et al., 2015). To investigate fluorescent microscopy results outcome (see section 2.9), the intensity and uniformity of HK *S. aureus* WT conjugation with Alexa Fluor 647 (Life technologies, Paisley, UK) and red pHrodo (Life technologies, Paisley, UK) was checked. Conjugated HK *S. aureus* WT with both Alexa Fluor 647 and red pHrodo obtained as per Section 2.9.1 were analysed for their fluorescence using LSRII flow cytometer. Unstained HK *S. aureus* WT was used as a negative control. The bacteria were separated on a FSC/SSC plot using a logarithmic scale to account for the bacterial size of 1-2µm. Red pHrodo (Ex -560nm, Em -585nm) staining was assessed by a right fluorescence shift on the Blue 610/20 channel while a right shift on the Red 660/20 axis represented staining with the Alexa Fluor 647 (Ex -650nm, Em -565nm) dye. Analysis of data was carried out using Flowjo analysis software.

2.7. Western Blot

2.7.1. Preparation of neutrophil and bacteria whole cell lysates

Neutrophils were centrifuged at 300 g for 2 minutes while HK *S. aureus* WT and SpA were centrifuged at 16,100 g for 3 minutes. Followed by washing with 200µl ice cold PBS, the cells were lysed by adding 10 µl lysis buffer (see Appendix II) per 10^6 cells, vortexing them vigorously and placing them on ice for 1 minute. Equal amounts of hot 2X sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) (see Appendix II) loading dye was added followed by heating to 95°C for 10 minutes to denature and linearise protein. Samples were frozen at -20°C until used (average storage 1-2 months).

2.7.2. SDS-PAGE gel preparation and electrophoresis

To make SDS-PAGE gels, glass moulds and plates were cleaned with 70% IMS, and assembled into clamps (Bio-Rad, Berkeley, CA). The resolving gel (12%) was made using the reagents specified in Appendix II. N,N,N',N'-Tetramethylethylenediamine (TEMED) was added last to the mixture and immediately preceding pouring, since in conjunction with ammonium persulfate (APS), it catalyses acrylamide polymerisation to form a gel matrix for protein sieving. The gels were poured into each apparatus. Butan-1-ol/isopropanol was placed on top, to prevent evaporation, and the gels were let to set at RT for 10-20 minutes. The stacking gel was made using the reagents in Appendix II. After the butan-1-

ol/isopropanol was rinsed off with distilled water, the stacking gel was layered on top of the resolving gel. The combs were subsequently inserted in each apparatus, preventing bubble formation, and allowed to set for 10-15 minutes. The gels were transferred to the gel running apparatus (Protean 2 gel assembly, Bio-Rad, Berkeley, CA) and immersed in 1X Running buffer (10X Running buffer made as described in Appendix II and diluted with distilled water). The combs were removed and 40µl of neutrophil cell lysates, *S. aureus* cell lysates equivalent of an MOI10, 10µl PLB-985 (obtained by Dr. Pranvera Sadiku) as well as 5µl of CSL-BBL prestained protein ladder (11-245kDa) (Geneflow, Lichfield, UK) or 7µl of ColorPLus Prestained Protein Ladder (11-245kDa) (New England Biolabs, Hitchin, UK) were loaded to individual lanes. The ladder ensured sizing of the sample protein. The gels were run at 100 volts, using the Power Pac 300 (Bio-Rad, Berkeley, CA), until the samples entered the resolving gel, after which the voltage was increased to 150 volts until the blue dye front reached the very bottom of the gel.

2.7.3. Wet transfer of the protein

A Geneflow wet transfer kit was used for protein transfer. The Transfer buffer (see Appendix II) was pre-cooled at 4°C. Just before complete protein electroporation, 4 filter cards, 2 fibre pads and one Polyvinylidene difluoride (PVDF) (Merckmillipore, Watford, UK) membrane were soaked for each blot in Transfer buffer. The PVDF membrane was previously activated by soaking for 5 minutes in 100% methanol. This allows the membrane to switch from hydrophobic to hydrophilic, helping the membrane to expose its full protein binding capacity. Following electrophoresis, the stacking gel was removed and the cassette holder was assembled as follows: black side cassette, fiber pad, 2 filter cards, PVDF membrane, gel, 2 filter cards, fiber pad and red side cassette. The assembled cassette was placed in the tank so that the red side faced the black side of the tank and its black side faced the red side of the tank. The tank was immersed on ice during protein transfer to prevent overheating of the transfer assembly. Membrane transfer was then conducted at 120V for 70 minutes.

2.7.4. Antibody incubation

Following the transfer, the PVDF membrane was removed and placed in a plastic container. The membrane was blocked for 2 hours in 5% milk powder (Sigma-Aldrich, Poole, UK)/ 1x

TBS (Tris-buffered saline) (see Appendix II). The primary antibodies (see Appendix II) were diluted in 4ml 5% Milk/ 1xTBS-0.1% Tween-20 (see Appendix II) and incubated with the membrane in a 50ml Falcon tube on a roller overnight at 4°C. The blot was rinsed three times in 1xTBS-0.1% Tween-20 for 10 minutes. The use of the Tween-20 detergent in low concentrations allows the dissociation of the nonspecifically bound antibodies. The secondary anti-rabbit or anti-mouse antibodies were diluted in 5mls of 5% milk powder/ TBS-0.1% Tween-20 as described per Appendix II and incubated for 1 hour at RT on the shaker. The blot was rinsed three times in TBS-0.1% Tween-20 for 10 minutes.

2.7.5. Blot developing

During the final wash, blot developing was started by mixing equal parts of solution A and B from the EZ-ECL imaging kit (Bio Rad Laboratories, Hercules, California), followed by a 5 minute incubation in the dark. The mixture was added to the membrane for a further 2 minutes. The membrane was transferred to a plastic pocket and all the air bubbles and excess liquid removed. Depending on the antibody, imaging of the blot was done for 1-50 minutes, taking intermediate images, using the Bio-Rad ChemiDoc imaging machine.

2.7.6. Membrane stripping and reprobing with p38 loading control antibody

Membrane stripping is done to remove the antibodies used to visualise a target and probe for another target, usually a protein loading control. To strip a membrane, 2X10 minutes wash with distilled water was conducted followed by a 15 minutes wash with 0.2M sodium hydroxide (NaOH) (Alfa Aesar, Heysham, UK) and 2X10 minutes with distilled water. A 2 hour blocking in 5% milk powder/ 1xTBS was conducted after which the membrane was reprobed for the unphosphorylated p38 housekeeping protein as per section 2.7.4, using the dilution from the antibody table in the Appendix II. Unphosphorylated p38 was previously used by our group as control due to its steady expression in neutrophils (Walmsley et al., 2011). Blot developing was subsequently done using the protocol described at section 2.7.5.

2.8. Bacterial viability assays

2.8.1. Neutrophil infection

Neutrophils obtained from plasma/Percoll-gradient centrifugation (see section 2.3) were resuspended at $5x10^6$ cells/ml in RPMI 1640 + 10% FBS and 90µl/well was added to a 96 well plate. Neutrophils were initially incubated for 1 hour with 10µg/ml of JC1 isotype control or anti-CD151 antibodies. Meanwhile, bacterial stock of NTHi obtained from section 2.4.2.1 was thawed at RT and centrifuged at 11,000 g for 5 minutes and the *S. aureus* WT stock obtained from section 2.4.1. was thawed on ice and centrifuged at 16,100 g for 3 minutes. Thawing was done differently due to the different preservation protocols used for the two bacteria while the centrifugation methods were used following the in house protocols. The supernatants were discarded and the NTHi was washed 3 times in 1ml PBS at 11,000 g for 5 minutes while *S. aureus* WT was washed only once in 1ml PBS at 16,100 g for 3 minutes. The bacteria were then resuspended in RPMI 1640 + 10% FBS at $1x10^9$ CFU/ml. *S. aureus* WT was incubated with neutrophils for 45 minutes at an MOI5 while neutrophil incubation with NTHi was done for 1 hour at an MOI10 at 37°C/5% CO_2.

2.8.1.1. Determination of viable intracellular bacteria using gentamicin protection assay

This method allows quantification of internalised viable bacteria after killing extracellular bacteria using antibiotics that cannot penetrate the eukaryotic cell (Vaudaux and Waldvogel, 1979). Gentamicin (20µg/ml for *S. aureus* WT and 60µg/ml for NTHi) (Sanofi, Surrey, UK) was added for 30 minutes or 75 minutes (*S. aureus* only) to the infected neutrophils from section 2.8.1.1. Gentamicin is a compound that stops protein synthesis by binding to the 30S eukaryotic, ribosomal subunit in both Gram positive and Gram negative bacteria (Yoshizawa et al., 1998). A subsequent centrifugation of the cells for 2 minutes at 300 g was conducted. Following supernatant discard, neutrophils were incubated for 10 minutes with 100µl of 1% saponin (Sigma-Aldrich, Poole, UK), a detergent known to associate with cholesterol and permeabilise the eukaryotic cell membrane by forming pores in it (Bangham et al., 1962). A volume of 900µl of sterile PBS was then added to each tube. Additional six 10 fold dilutions were conducted and plated by placing 3x10µl drops/condition on BHI agar (*S. aureus*) or chocolate agar (NTHi) plates using the Miles-

Misra dilution techniques (Miles et al., 1938). Plates were incubated overnight at 37°C/ 5%CO_2 .The colonies were counted the following day and the CFU/ml calculated as per section 2.4.1.1.

2.8.2. Monocyte derived macrophages (MDMs) viability assay with *S. aureus*

2.8.2.1. Differentiation of PBMCs into MDMs

In vitro differentiated MDMs have been shown to be a good model for alveolar macrophages (Daigneault et al., 2010). PBMCs obtained from plasma/Percoll purification (see section 2.3.) were resuspended in RPMI 1640+ 1% Pen/Strep at 4x10^6 cells/ml. In a 24 well-plate (Costar, High Wycombe, UK) 500µl of the resuspended cells were added to each well and incubated at 37°C/ 5%CO_2 for an hour. By starving the cells of FBS for an hour, the monocytes adhere to the bottom of the plate. Following this, the non-adherent cells (predominantly lymphocytes) were removed by vigorous pipetting and 500µl of RPMI+ 1%Pen/Strep+ 10% FBS was added to each well. The media was changed the day after and then every other day for 14 days. The number of differentiated, adherent MDMs after the 2 weeks was typically 2x10^5/well.

2.8.2.2. Infection of MDMs with *S. aureus*

MDMs were incubated for 1 hour with RPMI 1640+ 10% FBS containing 10µg/ml JC1 isotype control or anti-CD151 antibodies. In the meantime, a stock vial of *S. aureus* WT was thawed on ice followed by cell centrifugation at 16,100 g for 3 minutes. The supernatants were discarded and the *S. aureus* WT washed once with sterile PBS. The bacteria were resuspended in RPMI 1640+ 10% FBS at 1x10^9 CFU/ml. An MOI5 of *S. aureus* WT was then added to each well. The plates were placed on ice for 1 hour to allow the *S. aureus* WT to adhere to the MDMs followed by a further 1 hour incubation at 37°C/ 5%CO_2.

2.8.2.3. Determination of viable intracellular bacteria in MDMs using gentamicin protection assay

Following the *S. aureus* WT incubation, the cells were washed 3 times with 1ml ice cold sterile PBS. A volume of 500µl fresh RPMI 1640+ 10% FBS containing 60µg/ml gentamycin was added to each well and the plate placed at 37°C/ 5%CO_2 for 30 minutes. This step

allowed the killing of the extracellular and surface bound bacteria. The wells were washed 3 times with sterile PBS. For a second time point, half of the cells were incubated for a further 90 minutes in 500µl fresh RPMI 1640+ 10% FBS containing 6µg/ml gentamycin followed by 3 times PBS wash. The lower gentamycin concentration was chosen to avoid bacterial growth in the wells. After each time point, MDMs plasma membrane was permeabilised by adding 250µl of 2% saponin for 15 minutes. The wells were subsequently topped to 1ml with sterile PBS. MDMs were further permeabilised by scraping the bottom of the well with a pipette tip and vigorous pipetting. Three 5 fold dilutions were conducted and 3x 10µl drops/condition were placed on BHI agar plates. Plates were incubated overnight at 37°C/ 5%CO_2. Next day colony count and CFU/ml calculation was conducted as per section 2.4.1.1.

2.9. Fluorescent microscopy

Fluorescent microscopy is a useful tool that enables visualisation of protein interactions and co-localisation using fluorophores that emit different fluorescence. For the experiment, Alexa Fluor 647 and pHrodo red succinimidyl esters were chosen. The succinimidyl ester feature of the dyes allows binding to the amine groups to form highly stable amide bonds (Brinkley, 1992). The succinimidyl ester is susceptible to hydrolysis, however if the conjugation reaction is done under alkaline conditions, this setback can be overcome (Lomant and Fairbanks, 1976). PHrodo is a dye that can be activated at acidic pH, therefore it can be useful tool for studying bacterial phagocytosis due to its activation upon bacterial entrapment in the phagolysosome (Aziz et al., 2013; Segal, 2005). Alexa Fluor 647 is a highly stable dye, from acid to basic pH, that has been recently used to co-stain bacteria with pHrodo to study bacterial interaction and localisation in cells (Ellett et al., 2015). The fact that pHrodo (Ex -560nm, Em -585nm) and Alexa Fluor 647 (Ex -650nm, Em -665nm) dyes have non-overlapping excitation and emission spectra, allows their simultaneous use without interference.

2.9.1 Fluorescent labelling of *S. aureus* with Alexa Fluor 647 and pHrodo

Both HK and live *S. aureus* WT were used for this protocol. *S. aureus* was centrifuged at 6,200g, 4°C for 10 minutes and resuspended in sterile PBS at pH9 to achieve 2.5x10^3CFU/nl.

To a clean 1.5ml eppendorf tube, 200µl of the resuspended *S. aureus* WT was added followed by Alexa Fluor 647 succinimidyl ester (10mg/ml) addition at 1:200 dilution and/or pHrodo succinimidyl ester (10mM) at 1:1000 dilution. The tube was incubated in the dark for 30 minutes at 37°C/ 5%CO_2 with gentle shaking. The mixture was subsequently washed by adding 1ml of PBS pH8 or PBS pH8+ 1% bovine serum albumin (BSA) (Melford Biolaboratories, Ipswich, UK)/ 1% FBS and centrifuging at 16,100 g for 3 minutes. The bacteria was afterwards washed using 1ml of 25mM Tris pH8.5 or 25mM Tris pH8.5+ 1% BSA/1% FBS by centrifugation at 16,100 g for 3 minutes. A last wash with 1ml of PBS pH8 or PBS pH8+ 1% BSA/1% FBS was conducted by centrifugation at 16,100 g for 3 minutes. The buffers were used at alkaline pH to allow adequate staining of the *S. aureus* WT, while BSA/FBS were added to prevent bacterial loss during centrifugation. The use of the buffers will be specified for each of the fluorescent microscopy experiment in Chapter 4. After supernatant removal, the bacteria were resuspended in 100µl of PBS pH 7.4.

2.9.2. Neutrophil incubation with Alexa Fluor 647 and pHrodo stained *S. aureus*

Neutrophils obtained from healthy volunteers as per section 2.3. were resuspended at $5x10^6$ in complete RPMI 1640 (HK *S. aureus* WT) or RPMI 1640+ 10% FBS (live *S. aureus* WT). The cells (90µl/well) were incubated in 96 well plates with media or 10 µg/ml of JC1 isotype control or anti-CD151 antibodies for 1 hour at 37°C/ 5%CO_2. Following this, neutrophils were either incubated for 45 minutes at 37°C/ 5%CO_2 with MOI10 of HK *S. aureus* WT or for 30 minutes at 37°C/ 5%CO_2 with MOI5 of live *S. aureus* WT that were previously stained with Alexa Fluor 647 and/or pHrodo.

2.9.3. Neutrophil membrane labelling

Following *S. aureus* incubation, neutrophil membrane labelling was done using PKH67 green fluorescent cell linker kit (Sigma-Aldrich, Poole, UK). Staining of the membrane could affect cell properties to phagocytose bacteria, therefore the staining protocol was carried out (as indicated by the manufacturer) following phagocytosis. In brief, duplicate wells from each condition specified in Section 2.9.2 were pulled together and centrifuged at 400 g for 5 minutes. The supernatant were then discarded and pellet washed once using RPMI 1640 without FBS or Pen/Strep. The supernatant was carefully aspirated making sure that no

more than 25µl of liquid remained. The cells were resuspended in 1ml/ $2x10^7$ cells of Diluent C. Following this, 4µl of PKH67 ethanolic dye solution was resuspended in 1ml of Diluent C and an equal volume of this solution was added to the Diluent C resuspended cells. A prompt mixture by pipetting was conducted, to allow even staining, followed by incubation at RT for 5 minutes. The reaction was stopped with an equal volume of FBS and incubated for 1 minute. Centrifugation was subsequently conducted at 400 g for 10minutes. To remove excess dye, the cells were washed twice in 1.5mls sterile PBS by 400 g centrifuging for 5minutes. Cells were resuspended in 200µl of complete RPMI 1640.

2.9.4. Preparation of fluorescent microscopy slides

Cytospins of labeled neutrophils were made following the protocol on the Section 2.5.1., with the exception that cells were cytocentrifuged on a glass coverslip of 0.13mm that was placed between the glass slide and the filter card. The thin coverslip was used to enable better resolution during microscopy. Cell fixing was done by adding 50µl of 4% paraformaldehyde (PFA) (Sigma-Aldrich, Poole, UK) (see Appendix III) for 15 minutes. PFA was used instead of methanol because it preserves the physiology of the cells. The coverslip was then washed 3x 5minutes in 50µl of PBS. The stock solution of 50mg/ml of DAPI (4,6-diamidino-2-phenyl-indolehydrochloride, Biolegend, Cambridge, UK) was diluted 1:10,000 in PBS and 50µl was added to each slide for 15 minutes to stain neutrophil nucleus. DAPI is a dye that intercalates into DNA, therefore staining the nucleus of cells (Manzini et al., 1985). A 3x 5 minutes wash with 50µl/coverslip of PBS was conducted. The cover slips were afterwards inverted using tweezers and fixed on glass slides by being placed directly over 10µl of the nonfluorescing mounting medium fluoromount G (SouthernBiotech, Birmingham, USA). Tweezers were used to push out air bubbles trapped under the coverslip by pushing gently on it. Great care was taken not to move the coverslip, to prevent cell loss and smearing. The slides were dried in the dark, in the fume cupboard and then kept at 4°C for up to 1 month. The slides were imaged using the Olympus upright epifluorescence system BX63 (Olympos, Essex, UK).

2.9.4.1. Count of pHrodo stained *S. aureus* internalised by neutrophils

To analyse the fluorescent microscopy data, Fiji software (free software, Rasband, 1997) was used. Dr. Darren Robinson, Department of Biomedical Science, University of Sheffield, UK has designed the specific coding program (Figure 2.4A) to be run via Fiji software to count the pHrodo stained *S. aureus* WT inside the neutrophils. Before the program could be run, individual neutrophil borders were delimitated by hand (Figure 2.4B). The multi-lobed neutrophil nucleus shape does not allow the program to delimitate the cells accurately, confusing it with multiple cells (Figure 2.4B). Cells that did not fit completely in the frame or had a nuclear structure that was not specific to the neutrophil one were excluded from the count. The first 3 commands in the software were performed manually due to varying nature of the samples. Initially, the 'Unsharp Mask' command was used to correct the blurriness of the image (Figure 2.4C). The 'Enhance contrast' function was removed because the samples were bright enough to be picked up by the program. Following this, the 'Find maxima' function was used. This function allows the noise to value ratio to be adjusted so that an accurate number of bacteria can be picked by the program (Figure 2.4D). The output box in the 'Find maxima' function was set to 'point selection' to allow the display of multiple points with a point per each maxima. Once these commands were set, they were excluded from the program using two forward dashes (Figure 2.4E). The threshold was then set by the program. Based on these setting, the program created a mask by using the 'Create Mask' function (Figure 2.4F). This function allowed the counting of the bacteria inside the region of interest (ROI), which in this instance was the neutrophil. The 'for' function (for (i=0, i<n, i++)) is a common function used in programming that allowed bacterial counting in each neutrophil by giving a number to the neutrophil of 0 to n. A summary of the analysis was generated at the end containing the number of bacteria in each neutrophil (Figure 2.4F-summary panel). The program was run for each individual image. A number of 100 cells were counted per each conditions.

A.

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File Edit Language Templates Run Tools Tabs

*Untitled.ijm.ijm

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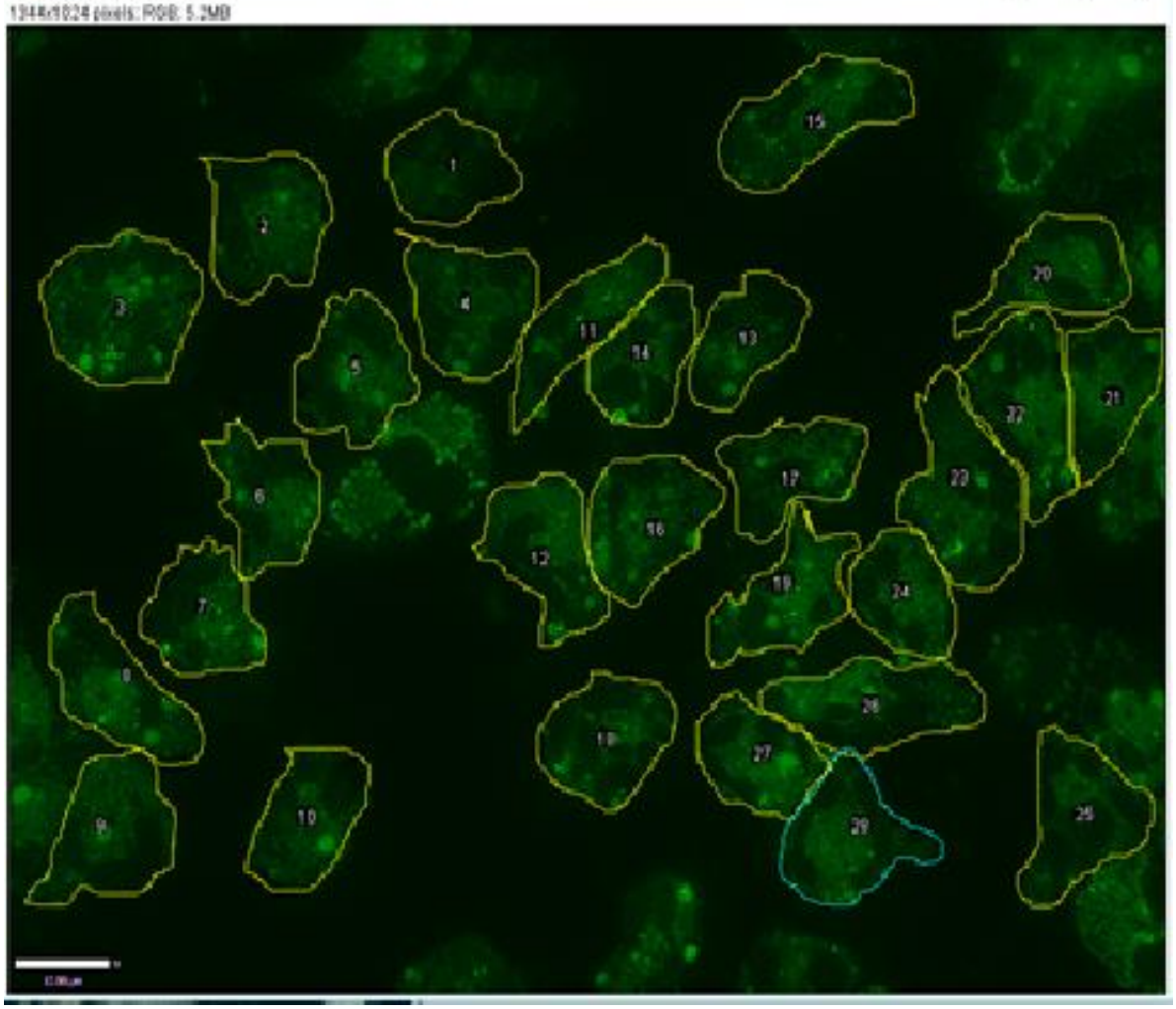
2  run("Enhance Contrast...", "saturated=0.3");
3  run("Find Maxima...", "noise=20 output=[Point Selection]")
4  setThreshold(255, 255);
5  run("Create Mask");
6  n=roiManager("count");
7      for(i=0;i<n;i++){
8          roiManager("select", i);
9          run("Analyze Particles...", "size=0 circularity=0.00 display summarize");
10     }
11
```

B.



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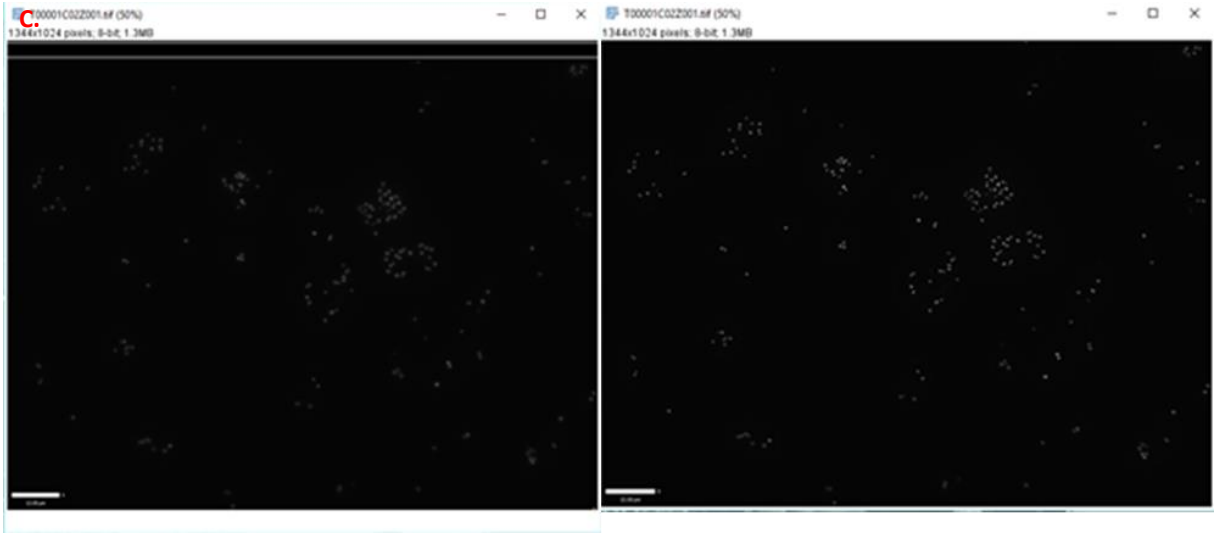
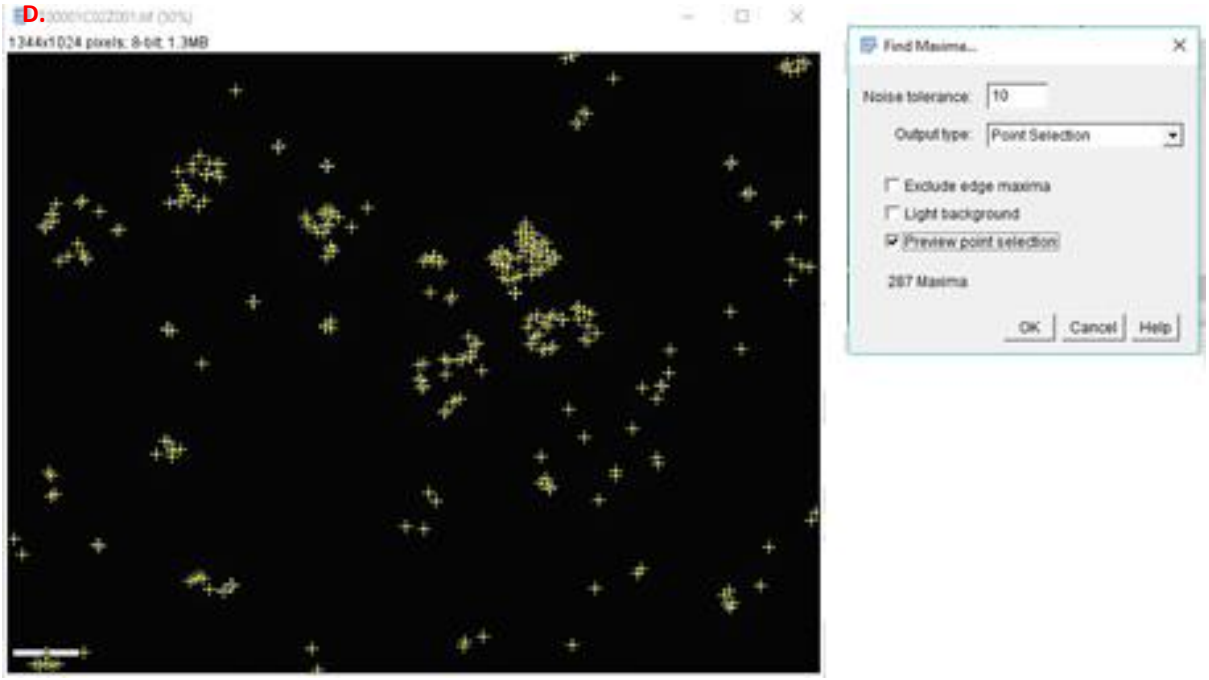


D.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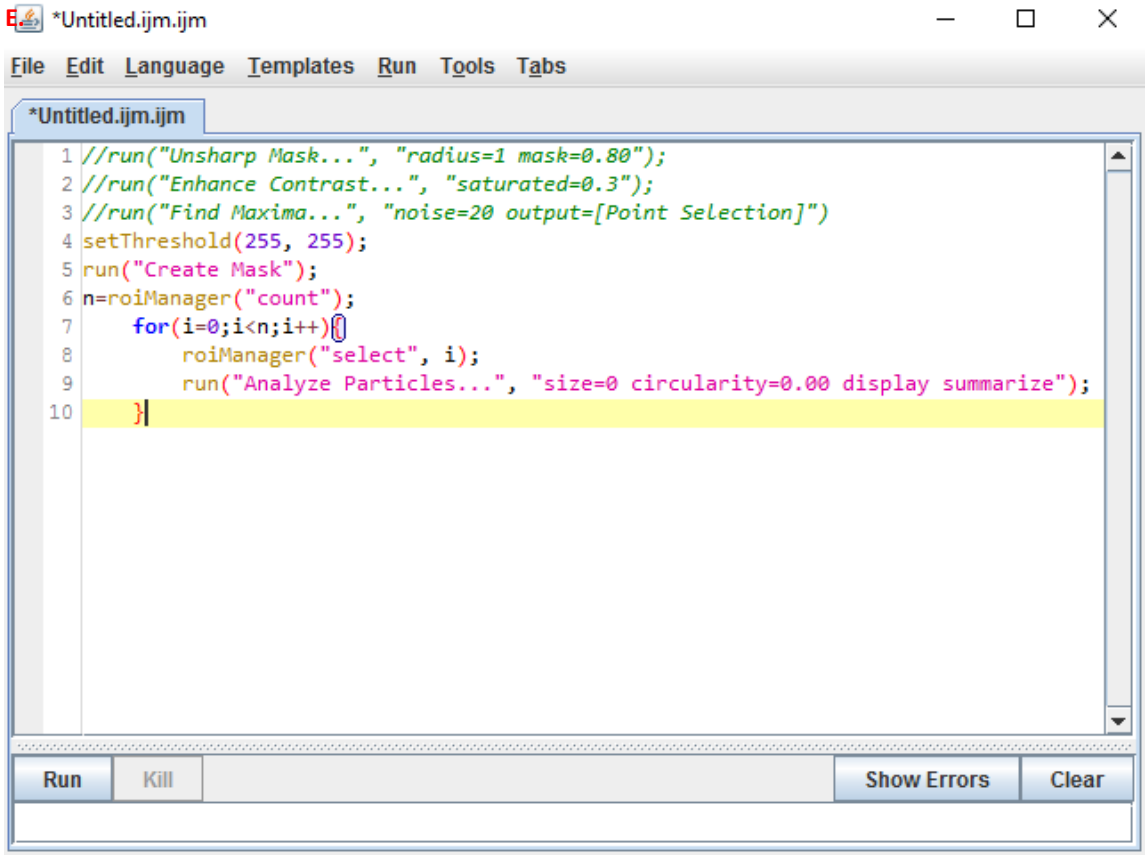
Find Maxima...
Noise tolerance:
10
Output type:
Point Selection
Exclude edge maxima
Light background
Preview point selection
287 Maxima
OK
Cancel
Help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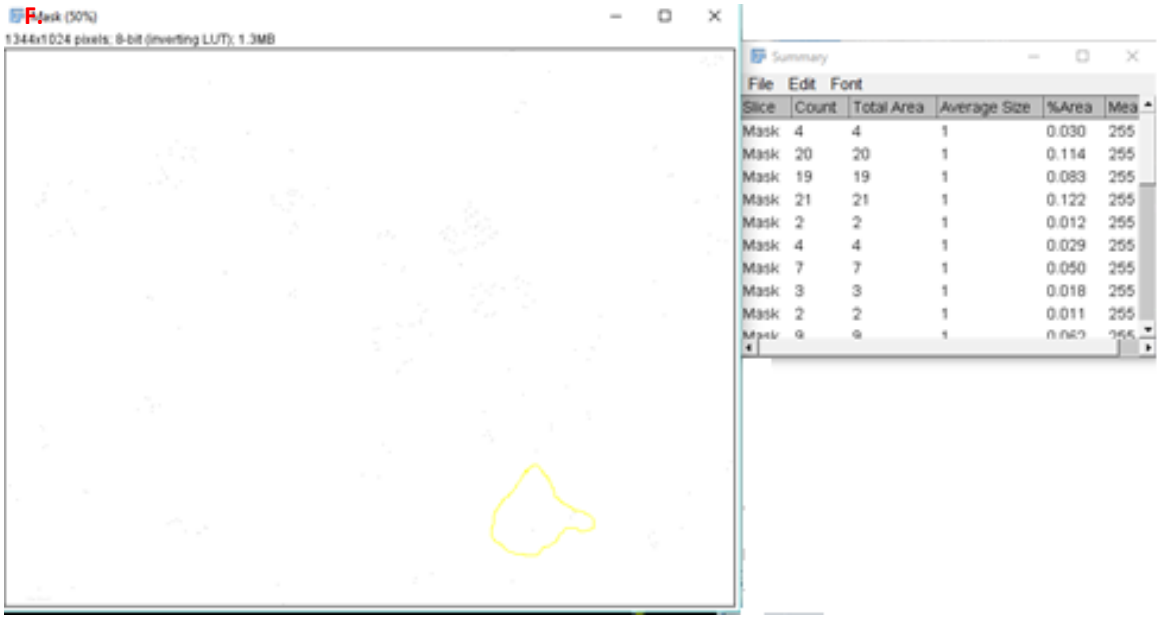
*Untitled.ijm.ijm
1 //run("Unsharp Mask...", "radius=1 mask=0.80");
2 //run("Enhance Contrast...", "saturated=0.3");
3 //run("Find Maxima...", "noise=20 output=[Point Selection]")
4 setThreshold(255, 255);
5 run("Create Mask");
6 n=roiManager("count");
7 for(i=0;i<n;i++){
8 roiManager("select", i);
9 run("Analyze Particles...", "size=0 circularity=0.00 display summarize");
10 }
Run
Kill
Show Errors
Clear


1344x1024 pixels; 8-bit (inverting LUT); 1.3MB
Summary
File Edit Font
Slice Count Total Area Average Size %Area Mea
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Mask 20 20 1 0.114 255
Mask 19 19 1 0.083 255
Mask 21 21 1 0.122 255
Mask 2 2 1 0.012 255
Mask 4 4 1 0.029 255
Mask 7 7 1 0.050 255
Mask 3 3 1 0.018 255
Mask 2 2 1 0.011 255

Figure 2.4: Fiji program print screens of the steps involved in the counting of pHrodo stained *S. aureus* WT within neutrophils

A) The step by step coding program that determines the number of pHrodo stained *S. aureus* WT inside neutrophils. B) Manual individual neutrophil border delimitation. C) The 'unsharp mask' function is used to reduce the blurriness of the image (left-before, right-after). D) The 'Find maxima' function was used to mark each bacteria with a yellow cross by adjusting the noise tolerance. The output type will have 'point selection' command to allow selection of individual points. E) The green lines in the program represent manually excluded steps. F) Output after the program was run. Yellow line represents a Mask for each neutrophil. This will change from cell to cell as the program counts the bacteria in each one. Only the last cell analysed can be viewed. A summary is produced giving the number of bacteria in each cell, the average bacteria size, the % area occupied by the bacteria, and the mean intensity.

2.10. Statistical analysis of the data

Statistical analysis was performed using GraphPad Prism version 6 (San Diego, California). Data sets were analysed using paired student's t-test when there were only two groups or One way ANOVA with Bonferroni's post-test with repeated measure or Dunnett's test when there were three or more groups. The Bonferroni correction with repeated measures accounts for the variability in the donors by allowing the control in each donor to be compared to its equivalent condition. The Dunnett's test was used when the Bonferroni test could not be used due to uneven repeats between the groups compared. When statistical analysis was conducted on responsive and nonresponsive donors, the test used was One-way ANOVA with non-parametric distribution and Dunn's post-test to account for the fact that the samples din not assume a uniform, Gaussian distribution. Statistical significance is represented by *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Chapter 3: The expression and role of the tetraspanin, CD63, during neutrophil apoptosis

3.1. Introduction

3.1.1. Neutrophil survival

Neutrophils are released from the bone marrow into the blood stream as terminally differentiated cells (Haslett et al., 1989; Nathan, 2006). Representing 45-70% of the total leukocyte pool, these cells can survive in the circulation for up to 8 hours or, if they migrate in the tissue, their lifespan can increase to 1-2 days, after which they die by programmed cell death (apoptosis) (Bainton et al., 1971; Cronkite and Fliedner, 1964). Apoptotic cells are then cleared in the liver, spleen, bone marrow and inflamed tissue by phagocytosis by the resident macrophages (Savill et al., 1989). Neutrophils are produced at a rate of 10^8 cells/min, therefore regulation of neutrophil apoptosis is of great importance in maintaining homeostasis (Haslett, 1999).

3.1.2. Neutrophil involvement in inflammatory disease

Failure of neutrophils to undergo apoptosis and clearance by macrophages can result in abnormal healing and chronic inflammation (Ariel and Timor, 2013; Nathan and Ding, 2010). This is due to the formation of a prolonged inflammatory feedback loop where neutrophils release tissue damaging ROS and proteolytic granule contents that induce the release of DAMPs, which are involved in attracting additional immune and inflammatory cells (Nathan, 2006; Nauseef and Borregaard, 2014; Soehnlein et al., 2009). Neutrophilia has been positively correlated with diseases such as COPD, rheumatoid arthritis and asthma (Brusselle et al., 2011; Louis and Djukanovic, 2006; Pillinger and Abramson, 1995; Plataki et al., 2006; Quint and Wedzicha, 2007). These diseases pose a major economic burden and are a major cause of morbidity and mortality, since the treatments presently available only alleviate the symptoms rather than targeting the underlying cellular inflammation. In the event of the sustained current trends, 400 million asthmatics are predicted to exist worldwide by 2025 and, according to the WHO, COPD will become the third leading cause of death worldwide by 2030 (Bahadori et al., 2009). Design of effective treatments that

actually treat the disease rather than the symptoms is, therefore, of paramount importance. However, considering the fragile, infection prone state of these patients, designing a neutrophil-targeted treatment has been slow due to potential immunosuppressive effects.

3.1.3. The role of tetraspanins in neutrophil survival

The 33 highly conserved tetraspanin members have wide-ranging functions including cell adherence and fusion, cell motility, protein membrane trafficking, endocytosis and bacterial adherence as well as cell survival (Charrin et al., 2014). Most cell survival implications are positively correlated with disease progression. For instance, tetraspanin-2 promotes diabetes mellitus progression by contributing to regulation of glucotoxic apoptosis in human pancreatic cells, while CD151 induces survival of A549 lung adenocarcinoma cells and CD53 activates the AKT pathway responsible for radioresistant tumor survival (Hwang et al., 2016; Li et al., 2015; Yunta and Lazo, 2003a). In the context of immunity, CD37 has a dual role in B cell survival by being able to activate opposite pathways involved in apoptosis and cell survival (Lapalombella et al., 2012; van Spriel et al., 2012).

The role of tetraspanins in neutrophils, and more precisely in neutrophil survival, is poorly understood. Tetraspanin CD53 and CD63 expression has been linked to the onset of neutrophil apoptosis as a consequence of their increased expression with age (Beinert et al., 2000). In addition, tetraspanin expression is also linked to neutrophil-dominant inflammatory disease. For instance, a polymorphism at the CD63 locus was shown to result in decreased expression of CD63 at mRNA and plasma membrane protein level in neutrophils from COPD patients (Zhang et al., 2007). CD63 was also demonstrated to be a potential suitable marker for rheumatoid arthritis due to its up regulation on neutrophil membrane, while CD9 expression was shown to be increased in periodontal disease (Bisson-Boutelliez et al., 2001; Lopez et al., 1995). Other evidence of tetraspanin involvement in neutrophil functions show that CD63 has a role in modulating neutrophil adhesion to endothelial cells by associating with tyrosine kinase activity to regulate this process (Skubitz et al., 1996; Skubitz et al., 2000).

These studies indicate a potential association between the tetraspanin family and neutrophil survival as well as neutrophil-dominant inflammatory disease. Therefore, my **hypothesis** is that tetraspanins play a role in neutrophil survival. The first aim of the project is to test my hypothesis by determining if tetraspanin(s) are involved in the survival process by targeting them using anti-tetraspanin antibodies. Secondly, the aim is to investigate the potential correlation between neutrophil survival and tetraspanin(s) expression. Furthermore, I aim to confirm key findings in neutrophils isolated from patients with COPD.

3.2. Results

3.2.1. Neutrophil purity from plasma/Percoll-pure and ultrapurified neutrophils via magnetic selection

Data from our group and others show that neutrophil purity from plasma/Percoll purification is typically >95%, while neutrophils further purified using magnetic selection (ultrapurified) resulted in purities above 99% due to elimination of PBMCs (Haslett et al., 1985; Sabroe et al., 2002). PBMC contamination has been shown to alter neutrophil survival by producing neutrophil-activating molecules such as cytokines and lipid mediators, and therefore achieving a highly pure neutrophil population is important when studying neutrophil function (Sabroe et al., 2004). Neutrophil purity from the experiments was therefore analysed to see if it is in accordance with the published literature. Cytospins of freshly isolated neutrophils from both plasma/Percoll purification and ultrapurification were analysed via light microscopy (Figure 3.1). Plasma/Percoll purification resulted in neutrophil mean±SD purity of 92.45±3.54 (Figure 3.1A) while ultrapurification resulted in neutrophils with a mean±SD purity of 98.92±1.15 (Figure 3.1B). The average value from plasma/Percoll purification is lower than expected because some donors were eosinophilic and eosinophils are not separated from neutrophils via Percoll gradients. PBMCs were not present at levels >2%. The data from these experiments would be considered accurate since previous literature shows that eosinophils do not influence neutrophil survival (Sabroe et al., 2004).

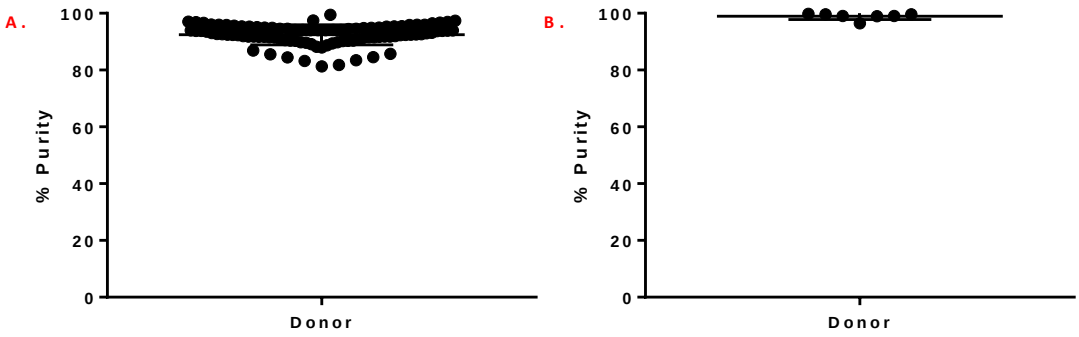


Figure 3.1: Neutrophil purity following plasma/Percoll and negative magnetic selection purification.

Neutrophils were isolated from whole venous blood of healthy volunteers via plasma/Percoll gradient centrifugation (A) or further ultrapurified using negative magnetic selection (B). Purity was assessed by using light microscopy and calculating the percent neutrophils from 500 cells. The mean±SD purity of plasma/Percoll pure neutrophils was 92.45±3.54 (n=98, A) while ultrapurification resulted in neutrophils with purity of 98.92±1.15 (n=7, B).

3.2.2. Neutrophil apoptosis increases over time

Previous results from our group and others show that neutrophil apoptosis increases over time, plateauing at around 20 hours (De Souza et al., 2002; Mecklenburgh et al., 2002; Sabroe et al., 2004). Current results show that incubation of neutrophils from 0-26 hours results in an increase in apoptosis with time. This can be seen in the photomicrographs where a predominantly viable population with multi-segmented nuclei, can be seen at 0 hours while at 20 hours there are mainly apoptotic neutrophils (Figure 3.2 A-B). Neutrophil apoptosis reaches a mean±SD of 68.65±6.73 at 20 hours while at 26 hours apoptosis reaches a mean±SD of 70.46±2.91 (Figure 3.2C). These changes can be distinguished due to differences in neutrophil morphology between apoptotic and viable neutrophils, as described in Section 2.5.1.1. The results show that the plasma/Percoll purified neutrophils obtained in our experiments undergo apoptosis *in vitro* over time in accordance with time courses in the published literature.

3.2.3. Anti-CD63 antibodies inhibit neutrophil apoptosis

To test the hypothesis that tetraspanins play a role in neutrophil survival, neutrophils were incubated for 20 hours with anti-tetraspanin antibodies directed against CD9, CD37, CD63 and CD151 at 10µg/ml. These particular tetraspanins are found more commonly on immune cells and this is the reason for selection in this project (Jones et al., 2011). The concentration was chosen because it was found by the group of Dr. Lynda Partridge to give saturating binding with cells that express these tetraspanins. Media and an IgG1 isotype antibody (JC1, 10µg/ml) were used as controls. Photomicrographs of media (Figure 3.3A) and JC1 isotype control (Figure 3.3B) show higher numbers of apoptotic neutrophils in culture compared to the anti-CD63 antibody treated cells (Figure 3.3C). There is a significant reduction in neutrophil apoptosis in the presence of anti-CD63 antibodies, however the anti-CD9, CD37 and CD151 antibodies had no effect on neutrophil apoptosis (Figure 3.3D). Different individuals show a high variability in the donor response in the presence of anti-CD63 antibodies, as shown in the table in Figure 3.3E. Neutrophil apoptosis in some donors was not affected by anti-CD63 antibodies while others donors showed a profound inhibition.

Overall, the data indicated a potential role for CD63 in neutrophil survival and this hypothesis was tested in further experiments.

3.2.4. Anti-CD63 antibodies inhibit neutrophil apoptosis at 10 and 20μg/ml

Considering the effect of anti-CD63 antibodies on neutrophil apoptosis, their working concentration was further refined to determine the optimal use in the current study. To do this, neutrophils were incubated for 20 hours with low (0.5-1μg/ml) and high (5-20μg/ml) concentration ranges of anti-CD63 antibodies (Figure 3.4). While the low concentrations of anti-CD63 antibodies had no effect on reducing neutrophil apoptosis (Figure 3.4A), higher concentrations of 10 and 20μg/ml significantly reduced neutrophil apoptosis (Figure 3.4B). To ensure optimal inhibition, anti-CD63 antibody was used at 20μg/ml in all further experiments.

3.2.5. Alternative anti-CD63 antibody clones inhibit neutrophil apoptosis

Since a number of factors can influence the efficacy of antibodies, such as affinity, epitope and isotype class, a panel of different anti-CD63 antibody clones were used in the neutrophil survival assay. In Figures 3.3 and 3.4 the anti-CD63 antibody was clone H5C6. Two additional clones were used, AHN-16.1 and 6H1. These clones were used by other groups to study tetraspanin-integrin interaction, AHN-16.1 being also used to show a link between CD63, tyrosine kinase activity and αMβ2 in promoting neutrophil migration (Berditchevski et al., 1995; Skubitz et al., 1996). Neutrophils were incubated for 4 and 20 hours with H5C6, AHN-16.1 and 6H1 anti-CD63 antibody clones and JC1 isotype control at 20μg/ml (Figure 3.5). Although at 4 hours there was visible reduction in apoptosis with AHN-16.1, significance in reducing neutrophil survival was reached only with H5C6 (Figure 3.5A). At 20 hours, apoptosis was significantly inhibited with AHN-16.1 (Figure 3.5B). Although at 20 hours AHN-16.1 resulted in a more profound inhibition of apoptosis than H5C6, all further neutrophil apoptosis modulation experiments will be conducted using H5C6, since this antibody clone is effective and readily available via Dr Lynda Partridge.

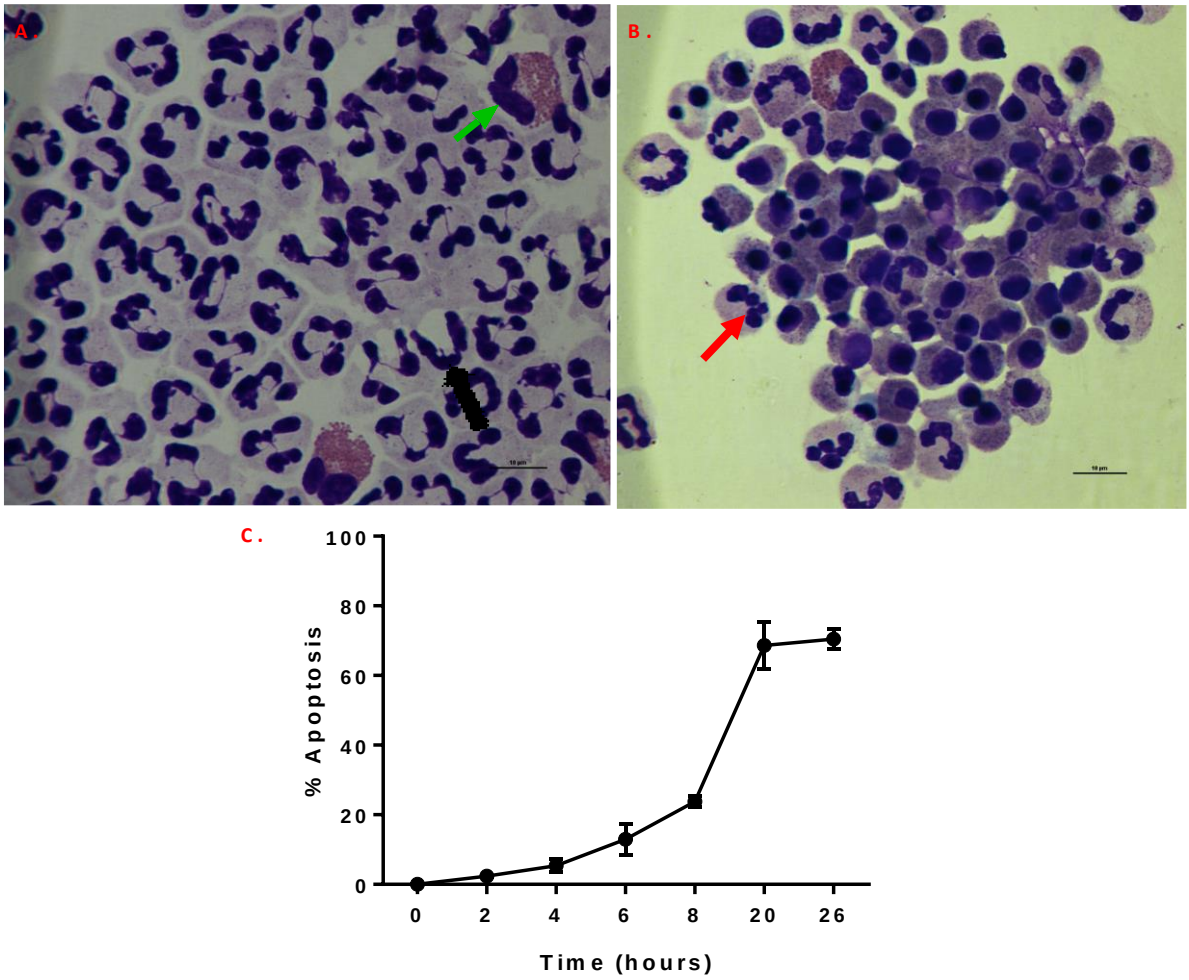
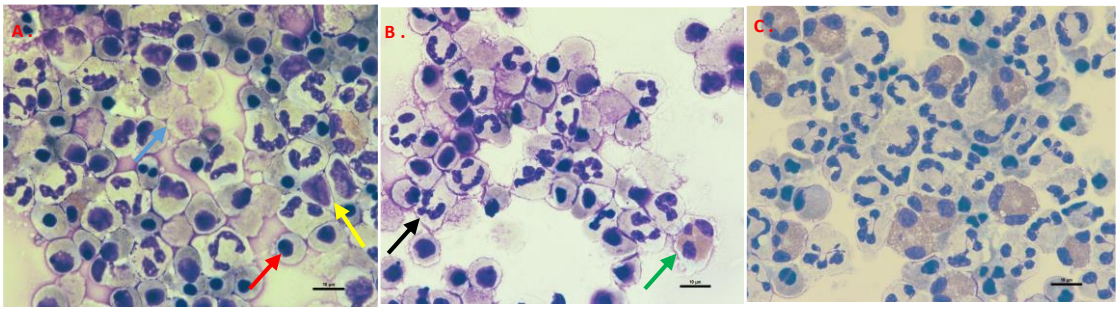
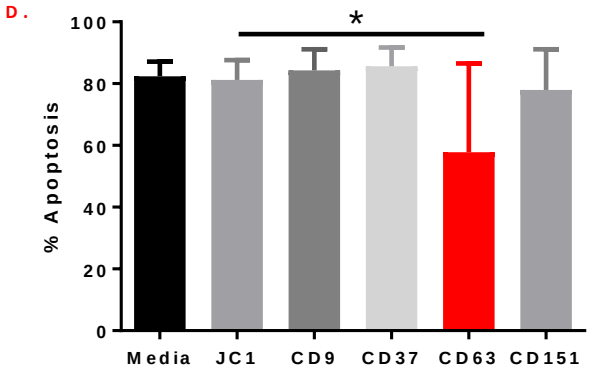


Figure 3.2: Constitutive neutrophil apoptosis time course.

Freshly isolated neutrophils from venous blood of healthy volunteers were cultured for 0, 2, 4, 6, 8, 20 and 26 hours. Apoptosis was assessed by light microscopy. (A-B) Representative photomicrographs of neutrophils incubated for 0 hours (A.) and 20 hours (B.). Apoptotic neutrophils - red arrow, viable neutrophils -black arrow, eosinophils - green arrow. Scale bar 10µm. C.) Time course from 3 independent donors expressed as mean±SD.





E.

Donor	Media	JC1	CD9	CD37	CD63	CD151
D1	79.72	73.38	77.79	79.98	29.72	81.06
D2	77.85	78.13	83.71	84.65	76.59	63.33
D3	89.73	90.21	91.43	92.16	78.44	91.33
D4	80.45	80.10			23.10	64.94
D5	84.26	84.28			80.95	89.04
Mean±SD	82.40±4.71	81.22±6.37	84.31±6.84	85.59±6.14	57.76±28.76	77.94±13.18

Figure 3.3: The effect of different anti-tetraspanin antibodies on neutrophil apoptosis. Freshly isolated neutrophils from venous blood of healthy volunteers were cultured for 20 hours with media or 10µg/ml of anti-tetraspanin antibodies directed against CD9, CD37, CD63, CD151 or JC1 IgG1 isotype control. Survival was assessed by light microscopy. (A-C) Photomicrographs of neutrophils incubated with media (A), JC1 isotype control (B) and anti-CD63 antibodies (C). Apoptotic cells -red arrow, 'ghost cells' - blue arrows, viable neutrophils - black arrow. Contaminating cells are eosinophils (green arrow) and lymphocytes (yellow arrow). Scale bar 10µm. (D) Quantification of the data from 3-5 independent experiments expressed as Mean±SD. Statistical analysis carried out by one-way ANOVA with Dunnett's post-test (*$p<0.05$). (E.) Table of data points for each condition, used in chart D.

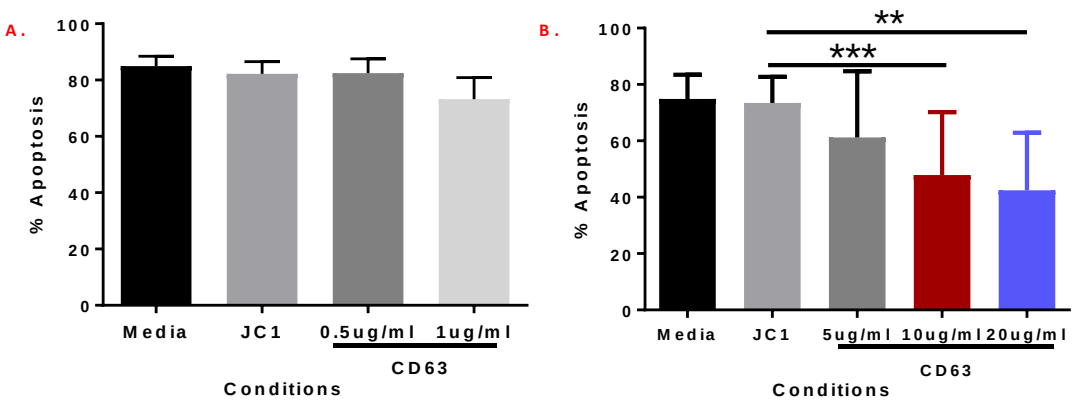


Figure 3.4: Anti-CD63 antibodies inhibit neutrophil apoptosis at higher concentrations. Freshly isolated neutrophils from venous blood of healthy volunteers were cultured for 20 hours with media, JC1 isotype control (10µg/ml) and low concentrations (0.5-1µg/ml) (A) or high concentrations (5-20µg/ml) (B) of anti-CD63 antibodies. Survival was assessed by light microscopy. Data representative of at least 3 (A) or 7 (B) independent experiments is expressed as mean±SD. Statistical significant differences are highlighted as **p<0.01 and *** p<0.001 (ANOVA, Bonferroni's repeated measures post-test).

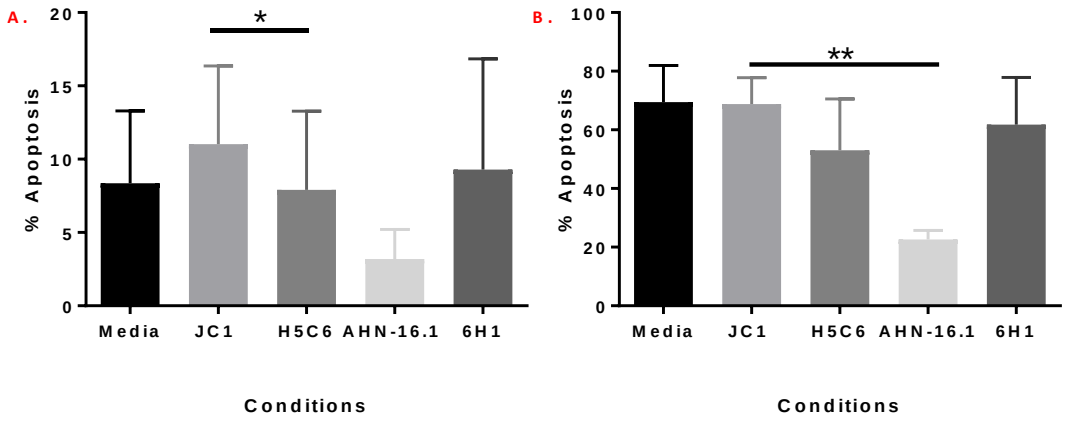


Figure 3.5: The effect of different anti-CD63 antibody clones on neutrophil apoptosis.

Neutrophil isolated from venous blood of healthy volunteers were incubated for 4 (A) or 20 hours (B) with different anti-CD63 antibody clones (H5C6, AHN-16.1, 6H1) (20µg/ml) and JC1 isotype control (20µg/ml). Apoptosis was assessed by light microscopy. Data from 4 different donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's repeated measures post-test (*$p<0.05$ and **$p<0.01$).

3.2.6. Flow cytometry confirmation of the survival effects of anti-CD63 antibodies

Although assessing neutrophil apoptosis by light microscopy is considered the gold standard, it is important to validate these findings with a biochemical measurement of apoptosis. Annexin V/ToPro3 staining and assessment by flow cytometry is a more objective measurement. Phosphatidylserine (PS) is irreversibly exposed on the neutrophil surface during apoptosis and can be quantified by binding to the fluorescent probe, Annexin V (Nusbaum et al., 2005). ToPro3 is a vital dye that intercalates between double stranded DNA when cells become permeable and therefore necrotic (Babiychuk et al., 2008; Cantinieaux et al., 2004; Solito et al., 1998; Vanhooijdonk et al., 1994). Neutrophils incubated for 20 hours with media, JC1 isotype control (20µg/ml) or anti-CD63 antibodies (20µg/ml) were stained with Annexin V-PE and ToPro3 (Figure 3.6). A shift of the cells into the top left quadrant represents Annexin V-PE$^+$ cells, visualised using Blue 575/26 channel, while a shift into the bottom right quadrant is indicative of ToPro3$^+$ events, detected on the Red 660/20 channel (Figure 3.6A). The top right quadrant represents Annexin V-PE$^+$/ToPro3$^+$ dual stained cells. Quantification of percentage apoptotic cells showed that neutrophil apoptosis is significantly inhibited in the presence of anti-CD63 antibodies (Figure 3.6B). These data support the previous results that CD63 plays a role in neutrophil survival.

3.2.7. Anti-CD63 antibody-mediated neutrophil survival is not affected by low levels of contaminating PBMCs

Evidence from the literature showed that PBMC contamination of neutrophil preparations can alter neutrophil survival in *in vitro* cultures (Lee et al., 1993; Sabroe et al., 2004). Therefore, the next hypothesis was to test if neutrophil survival in the presence of anti-CD63 antibodies is mediated by contaminating cells. To test this, neutrophils from the same donor were purified by plasma/Percoll centrifugation or further ultrapurified using negative magnetic selection to remove PBMCs. The cells were subsequently incubated for 20 hours with media, JC1 isotype (20µg/ml) control or anti-CD63 antibodies (20µg/ml). Apoptosis of both plasma/Percoll-pure (Figure 3.7A), mean±SD purity of 98.26±1.05, and ultrapure neutrophils (Figure 3.7B), mean±SD purity of 99.26±0.9, was significantly inhibited in the

presence of anti-CD63 antibodies. This data therefore indicates that low levels of PBMCs contaminations do not affect neutrophil survival in the presence of anti-CD63 antibodies.

3.2.8. H5C6 anti-CD63 antibody clone detects and binds to CD63

Neutrophils are known to express CD63 in the lysososmal granules which can be mobilised to the cell surface (Ageberg and Lindmark, 2003; Metzelaar et al., 1991). In order to validate that the H5C6 antibody was indeed binding to CD63, immunoblotting was used. Neutrophil whole cell lysates from 3 independent donors were subjected to SDS-PAGE and immunostained with the H5C6 clone anti-CD63 antibody or JC1 isotype control. The staining confirmed CD63 expression in all 3 donors (Figure 3.8), due to the presence of bands between 45-60kDa and 30-35kDa. CD63 is a highly glycosylated protein capable of yielding immunoreactive products of different sizes, depending on the amount of glycosylation, ranging from 27-60kDa (Ageberg and Lindmark, 2003). As a control, the same neutrophil lysates were probed with JC1 isotype control antibodies, and this showed a very faint band at 30-35kDa (Figure 3.8), suggesting possible nonspecific binding of the isotype control. No bands between 35-60kDa were detected with the isotype control.

Antibody binding was further verified by flow cytometry. Binding of anti-CD63 antibodies on the neutrophil surface in cells incubated with media or HK *S. aureus* (known to cause degranulation of neutrophils and externalisation of CD63) was then investigated. Freshly isolated neutrophils were incubated for 15 minutes with media or HK *S. aureus* at MOI10, followed by a 30 minute incubation with Alexa Fluor 488 conjugated JC1 isotype control (10µg/ml) or anti-CD63 antibodies (10µg/ml). FSC/SSC dot plots showed a right and upwards shift of neutrophils in the presence of HK *S. aureus*, indicative of both neutrophil phagocytosis of bacteria and activation (Figure 3.9A). FL-1H histograms also showed a right shift in $CD63^+$ fluorescence in HK *S. aureus* treated neutrophils when compared to JC1 control but no right shift in $CD63^+$ fluorescence is seen in neutrophil only incubated cells when compared to the control (Figure 3.9B). Overall, these data confirms CD63 expression by neutrophils from whole cell protein extracts but does not confirm surface expression of CD63 on unstimulated neutrophils.

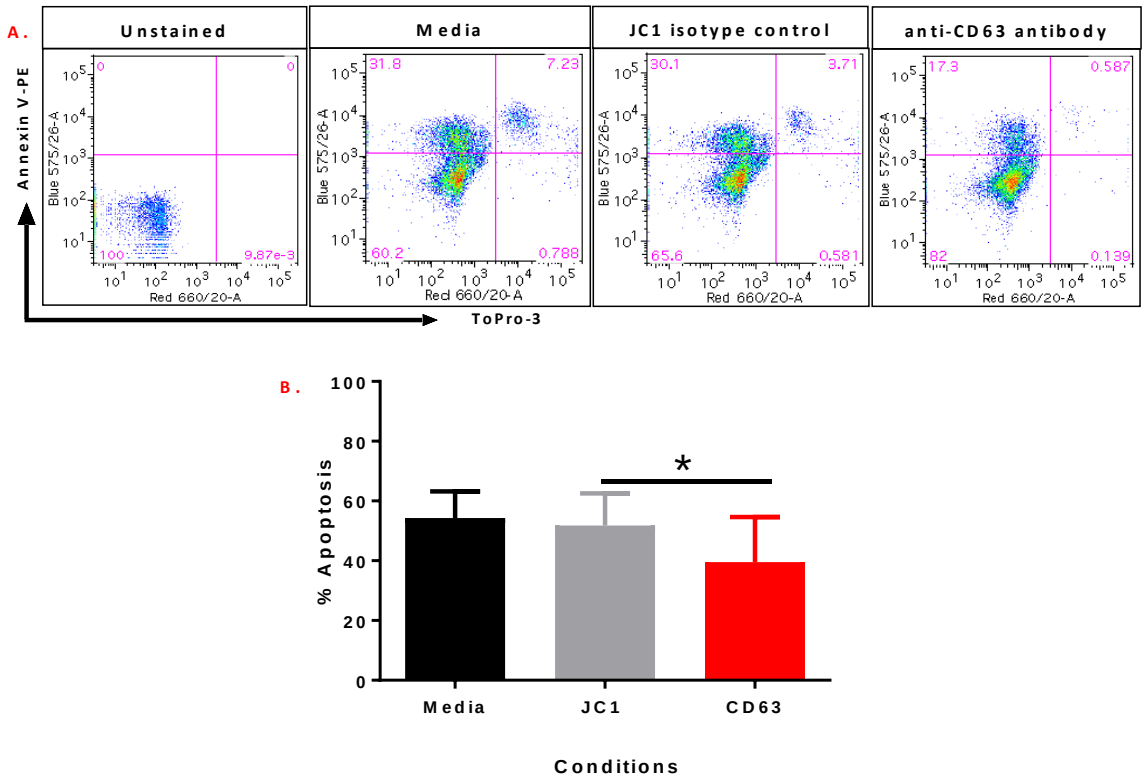


Figure 3.6: Flow cytometry evidence of anti-CD63 antibodies inhibition of neutrophil apoptosis.

Neutrophils from venous blood of healthy volunteers were isolated and cultured for 20 hours in the presence of media, JC1 isotype control (20µg/ml) or anti-CD63 antibodies (20µg/ml). A) Representative plots of unstained neutrophils and media, JC1 isotype control and anti-CD63 antibody treated cells. Neutrophil apoptosis and necrosis was assessed using Annexin V-Pe and ToPro3 stain, respectively. Gain of far red fluorescence (Red 660/20) indicates ToPro3 positivity while gain of blue fluorescence (Blue 575/26) represents Annexin V-PE positivity staining. B) Quantification (top two quadrants) of neutrophil apoptosis from 7 independent experiments is shown as mean±SD. Statistical significant differences are highlighted as *$p<0.05$ (ANOVA, Bonferroni's repeated measures post-test).

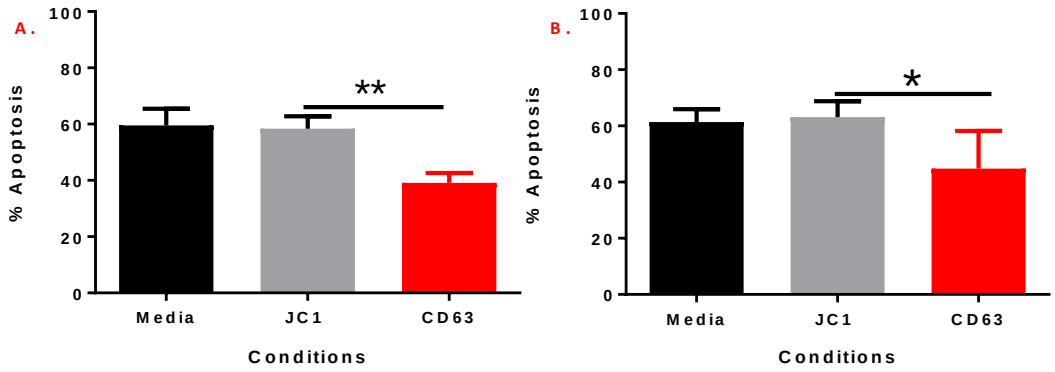


Figure 3.7: Anti-CD63 antibodies inhibit apoptosis of plasma/Percoll-pure and ultrapure neutrophils.

Freshly isolated neutrophils from venous blood of healthy volunteers were purified via plasma/Percoll gradient centrifugation (A) or further ultrapurified using a negative magnetic selection column (B). The cells were subsequently incubated for 20 hours with media, JC1 isotype (20µg/ml) control or anti-CD63 antibodies (20µg/ml). Survival was assessed by light microscopy. Data from 4 different donors is expressed as mean±SD. Statistical significant differences are highlighted as *$p<0.05$ and **$p<0.01$ (one-way ANOVA, Bonferroni- repeated measures post-test).

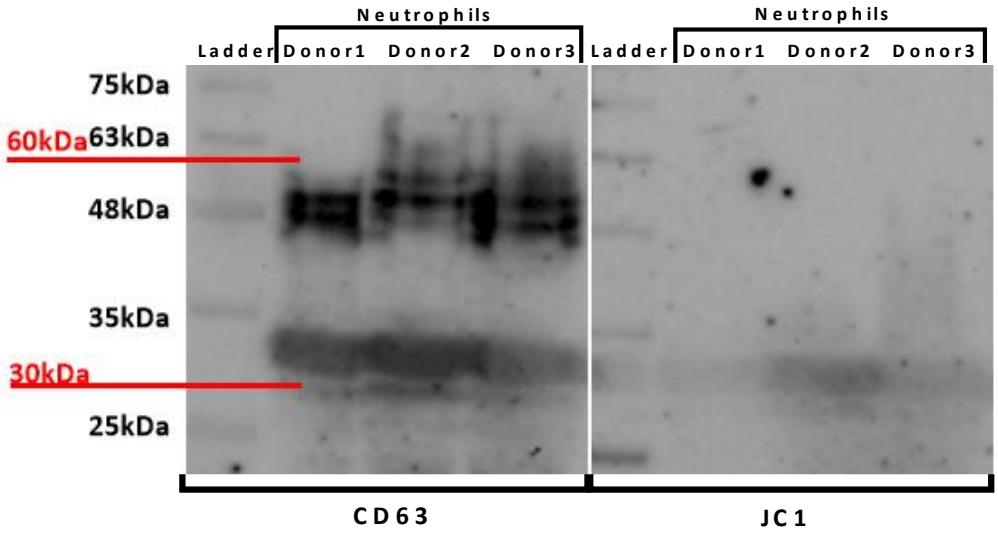


Figure 3.8: Evidence of CD63 expression by neutrophils.

Whole cell lysates from freshly isolated neutrophils from three independent donors were run on a 12% SDS-PAGE gel. Protein lysates from $2X10^6$ neutrophils were loaded in each lane. Identical blots were probed for CD63 (left) or for JC1 isotype control (right) (1:600). On the CD63 blot there are two bands: one between 60-45kDa and one between 30-35kDa. On the JC1 blot there is a faint band between 30-35kDa. Red line at 60kDa and 30kDa represents the expected molecular weight range of CD63.

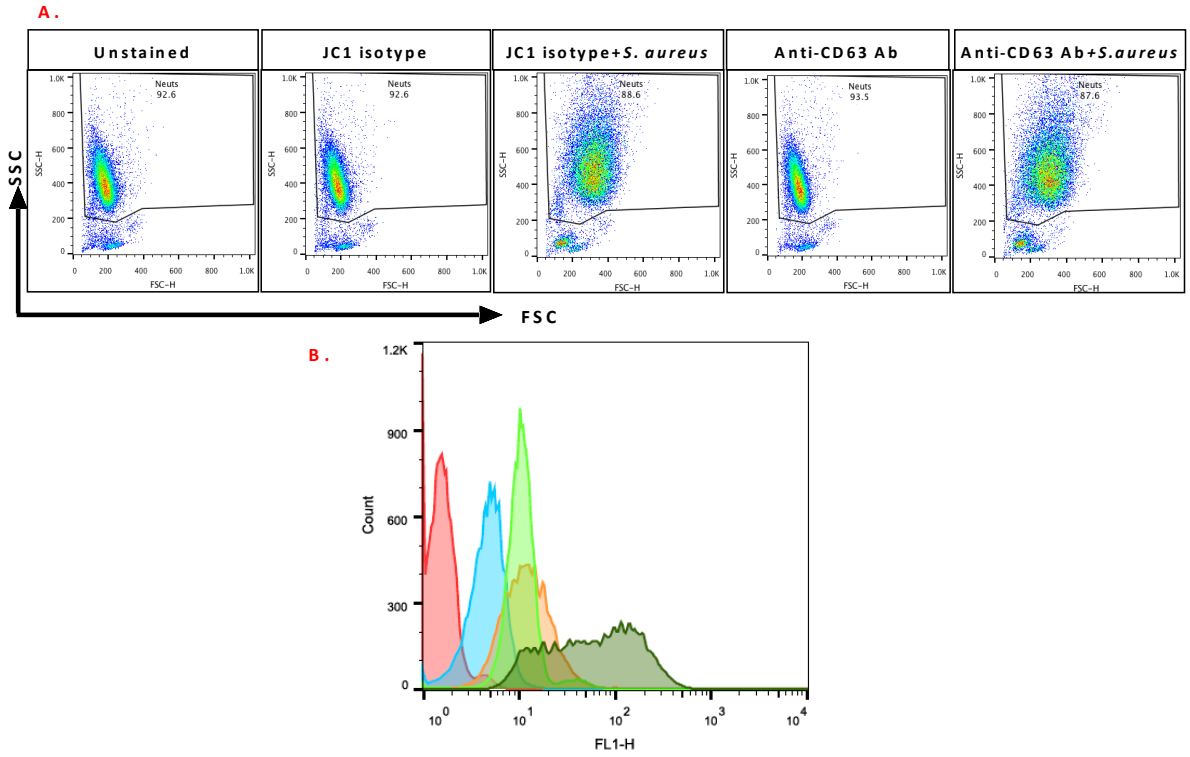


Figure 3.9: Evidence of CD63 expression by neutrophils on their surface upon incubation with HK *S. aureus*.

A) Representative FSC/SSC dot plots of freshly isolated neutrophils from venous blood of healthy volunteers incubated for 15 minutes with media or HK *S. aureus* at MOI10 and probed for Alexa Fluor 488 conjugated JC1 isotype control (10µg/ml) or anti-CD63 antibodies (10µg/ml) for 30 minutes. (B) Histogram overlay of neutrophils from a single experiment: unstained-red, JC1 isotype control-orange, JC1+ *S. aureus*-blue, anti-CD63 antibody- light green, anti-CD63 antibody+ *S. aureus*- dark green. FACScalibur was used to detect a right shift in fluorescence on the FL1 parameter.

3.2.9. LPS contamination of GST-CD63 fusion proteins

GST-CD63 constructs that correspond to the EC2 domain of CD63 were used previously by the Partridge group to confirm the involvement of tetraspanins in bacterial adhesion (Green et al., 2011; Hassuna et al., 2009). Glutathione S-transferases (GSTs) are molecular tags that aid in the purification process of a recombinant protein by improving their solubility and stability (Harper and Speicher, 2011; Ketley et al., 1975). GST fused proteins have been previously shown to have activity and were used by Dr. Lynda Partridge group as a tool for investigating cell functions (Green et al., 2011; Ho et al., 2006; Parthasarathy et al., 2009; Wong et al., 2001). These constructs are obtained in a bacterial expression system, therefore contamination with LPS is highly likely. Neutrophils are highly responsive to endotoxin, where concentrations of 1ng/ml can influence their functions including lifespan (Sabroe et al., 2002). To control for this, the TLR4 antagonist LPS-RS was used, which works in this context by preventing LPS-induced survival (Coats et al., 2005). In a single experiment, neutrophils were pre-incubated for 1 hour with LPS-RS (10µg/ml), followed by a 20 hour incubation with LPS (10ng/ml), GST (10µg/ml) and GST-CD63 (10µg/ml). Results confirm that LPS inhibits neutrophil apoptosis which is preventable by LPS-RS (Figure 3.10). Both GST and GST-CD63 treatments resulted in a dramatic decrease in neutrophil apoptosis. Co-incubation of GST and GST-CD63 fusion proteins with LPS-RS resulted in a moderate but visible increase in the apoptosis levels. The experiment therefore indicates that the GST and GST-CD63 proteins have moderate endotoxin levels that can influence result outcome. For this reason, the constructs will not be used in future experiments.

3.2.10. Anti-CD63 Fab fragments significantly inhibit neutrophil apoptosis

Antibodies are large divalent molecular structures that can cause changes by cross-linking protein partners causing indirect effects. Consequently, the hypothesis that anti-CD63 antibodies influence neutrophil survival indirectly by cross-linking partner molecules was tested by using Fab fragments. These fragments are monovalent structures and the equivalent of 1/3 of the antibody size. They represent only the region of the antibody that can bind the target directly, as a result they are less likely to cross-link and any effects are more likely to be due to direct binding. Neutrophils were incubated for 20 hours with media,

JC1 isotype control (20µg/ml), anti-CD63 antibodies (20µg/ml) or anti-CD63 Fab fragments (5-20µg/ml). Apoptosis was significantly inhibited in the presence of anti-CD63 antibodies but no effect was seen with any concentration of CD63-Fab fragments (Figure 3.11).

These data may suggest that the full antibody is required for functional effect because of the need for the antibody to cross-link with tetraspanin binding partners. Failure of Fab fragments to modulate neutrophil apoptosis also led to the consideration that CD63-Fab fragments may have lost their activity in storage. To test this, their target binding capacity was investigated using the A549 adenocarcinomic human alveolar basal epithelial cell line, that stably expresses CD63 (Funakoshi et al., 2003). Cells from the A549 epithelial cell line ($1x10^6$/ condition) were incubated for 1 hour with primary JC1 isotype control (10µg/ml) antibody or anti-CD63 Fab fragments (10µg/ml) followed by a 20 minute incubation with 1µl of secondary FITC labeled anti-mouse antibody (Figure 3.12). The full JC1 isotype control, in contrast to a Fab fragment, was used to highlight any unintended, non-specific and off-target effects of the antibody. FSC/SSC dot plots showed a gated cell population which was separated from debris (Figure 3.12A). Blue 530/30 histogram showed little to no right shift in $CD63^+$ Blue 530/30 in CD63-Fab incubated neutrophils when compared to JC1 or secondary antibody only staining controls (Figure 3.12B). This is indicative of a failure of CD63-Fab to bind to CD63, potentially because of breakdown during storage.

For this reason, a newer batch of CD63-Fab fragments was obtained. Fab fragments at 10 and 20µg/ml showed a significant, concentration dependent inhibition in apoptosis when compared to JC1 control (Figure 3.13).

The binding specificity of the new anti-CD63-Fab to CD63 was checked using the $CD63^+$ A549 cell line, as previously. The experiment was conducted by Mr. Anesu R. Matamba using the in house flow cytometry protocol of Dr. Lynda J. Partridge. FSC/SSC dot plots showed cell separation from debris (Figure 3.14A). FL-1H histogram showed a right shift in $CD63^+$ FL1 fluorescence with both anti-CD63-Fab fragments (dark green) and anti-CD63 antibody (light green) stained neutrophils when compared to JC1 (orange) or secondary only (blue) antibody controls (Figure 3.14B). This confirms binding of the CD63 Fab fragments to CD63 exposed A549 cell surface.

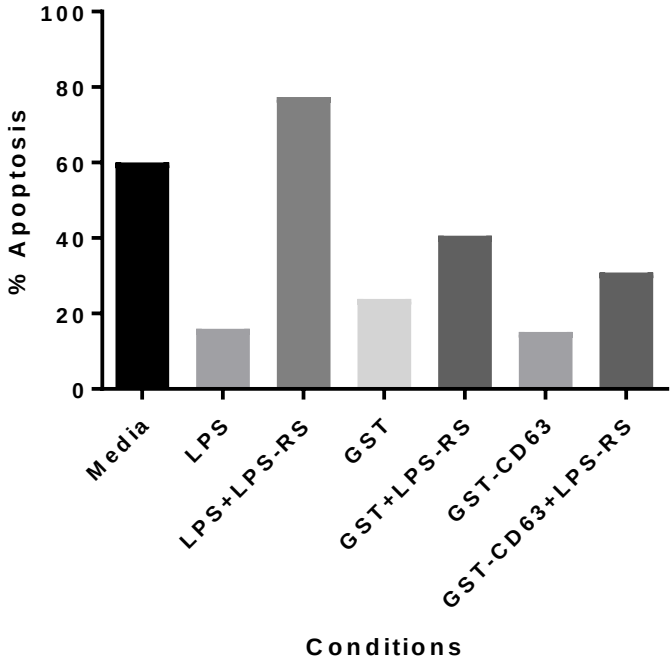


Figure 3.10: Evidence for endotoxin contamination of GST and GST-CD63 protein constructs.

Freshly isolated neutrophils from venous blood of healthy volunteers were pre-incubated with LPS-RS (10µg/ml) for 1 hour followed by a 20 hour incubation with LPS (10ng/ml), GST (10µg/ml) and GST-CD63 (10µg/ml). Neutrophil apoptosis was assessed by light microscopy. Data obtained from a single experiment.

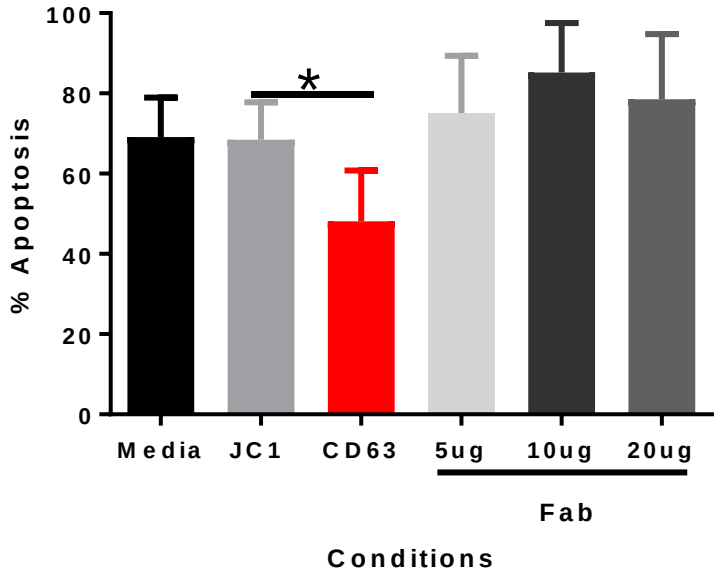


Figure 3.11: Anti-CD63 Fab fragments have no effect on neutrophil apoptosis.

Neutrophils obtained from venous blood of healthy volunteers were incubated for 20 hours with media, JC1 isotype control (20µg/ml), anti-CD63 antibodies (20µg/ml) or increasing concentrations of anti-CD63 Fab fragments old stock (5-20µg/ml). Neutrophil apoptosis was assessed by light microscopy. Quantification of the data from 4 different experiments is expressed as mean±SD. Statistical significant differences are highlighted as *$p<0.05$ (one-way ANOVA, Bonferroni- repeated measures post-test).

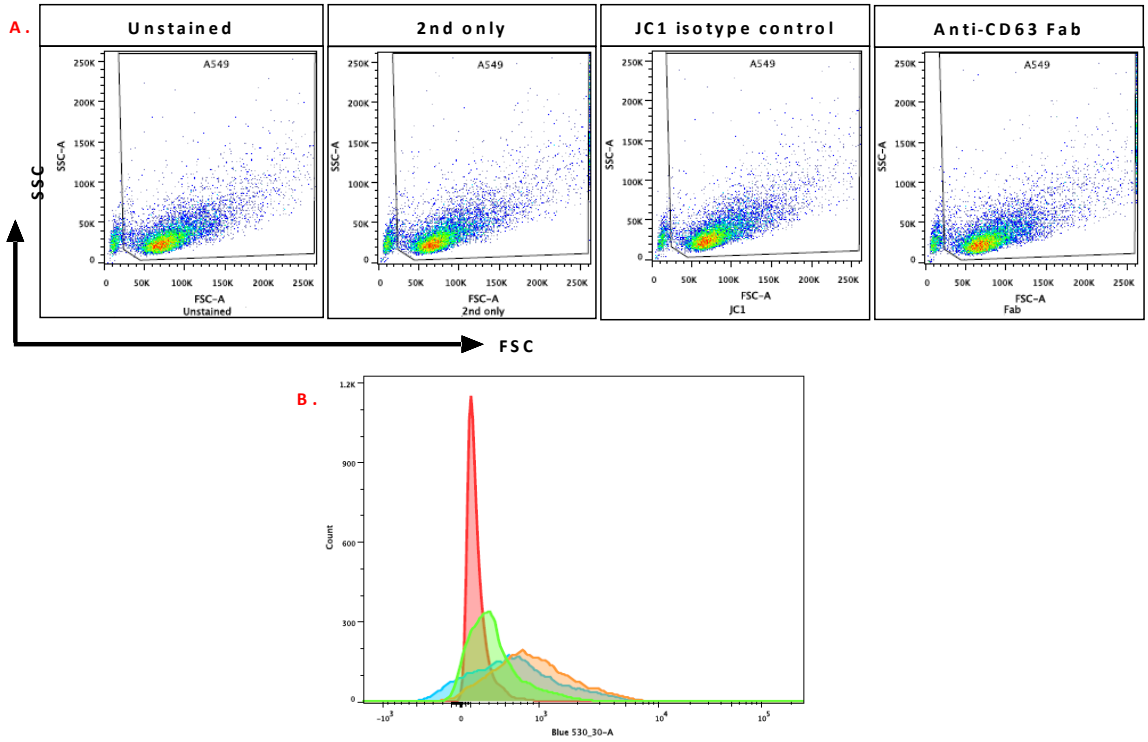


Figure 3.12: Flow cytometry evidence of anti-CD63 Fab fragments inability to bind to target.

A) Cells from the A549 epithelial cell line ($1x10^6$ / condition) were stained with primary antibody directed against JC1 isotype control (10µg/ml) and anti-CD63 Fab fragments old stock (10µg/ml) for 1 hour followed by a 20 minute incubation with 1µl of secondary FITC labeled anti-mouse antibody. A549 cells distribution under different conditions was shown on FSC/SSC dot plots. B) Histogram of A549 labeled cells: red-unstained, blue-secondary antibody only, orange-JC1 isotype control, green-anti-CD63 Fab fragments. Binding to the A549 cells of the JC1 isotype control and anti-CD63 Fab fragments was assessed on a right shift on the Blue 530/30 channel using LSRII flow cytometer. Data obtained from single experiment.

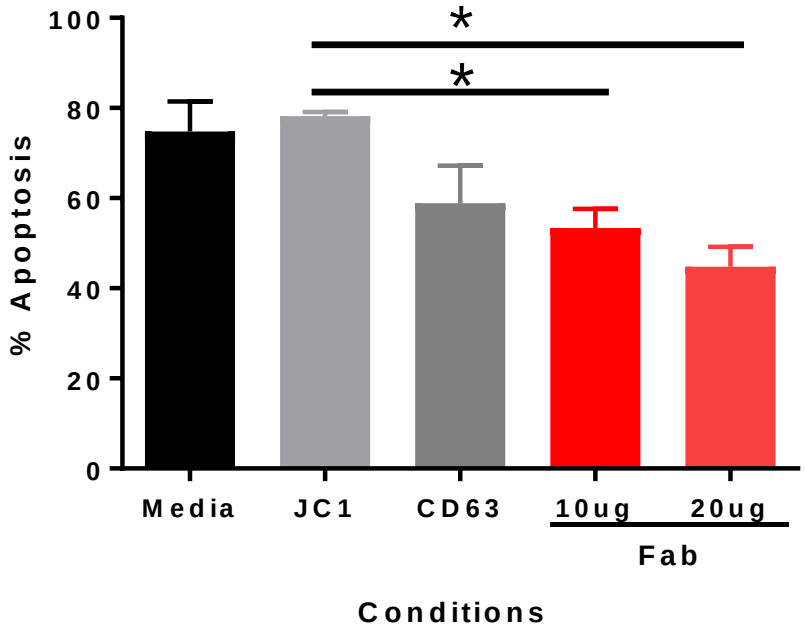


Figure 3.13: Anti-CD63 Fab fragments inhibit neutrophil apoptosis in a concentration dependent manner.

Freshly isolated neutrophils from venous blood of healthy volunteers were incubated for 20 hours with media, JC1 isotype control (20µg/ml), anti-CD63 antibodies (20µg/ml) or different concentrations of anti-CD63 Fab fragments new stock(10 or 20µg/ml). Data from 3 different donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's repeated measures post-test (*$p<0.05$).

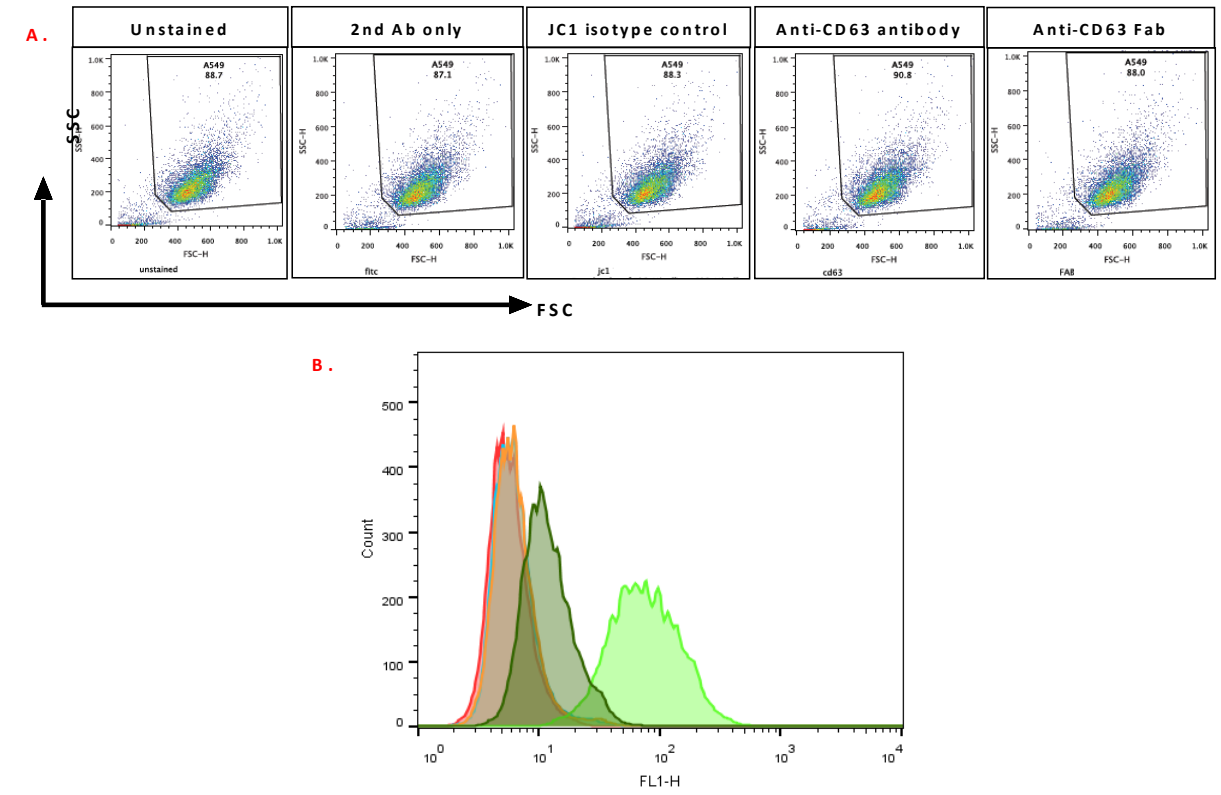


Figure 3.14: Flow cytometry evidence of anti-CD63 Fab fragments ability to bind to target. A) Cells from the A549 epithelial cell line (1x10^6/ conditions) were stained for JC1 isotype control (10µg/ml), anti-CD63 antibodies (10µg/ml) and anti-CD63 Fab fragments new stock (10µg/ml) using the in house protocol of Dr. Lynda Partridge laboratory. Dot plots of A549 cells distribution under different conditions on FSC/SSC scatter. B) Histogram of A549 labeled cells: red-unstained, blue-secondary antibody only, orange-JC1 isotype control, light green- anti-CD63 antibodies, dark green-anti-CD63 Fab fragments. Data obtained from single experiment by Mr. Anesu R. Matamba, Molecular Biology and Biotechnology department, University of Sheffield.

3.2.11. Anti-CD63 antibodies do not regulate neutrophil apoptosis in the presence of accelerating/inhibitory agents of apoptosis

To this point, the effect of anti-CD63 antibodies on constitutive neutrophil apoptosis has been probed. I next investigated whether anti-CD63 antibodies had the potential to regulate neutrophil apoptosis in the presence of agents that either accelerate or inhibit apoptosis. Neutrophils were incubated for 1 hour with anti-CD63 antibodies or JC1 isotype control followed by a 5 hour incubation with pyocyanin (pyo) or a 20 hour incubation with LPS or GM-CSF. Pyocyanin is a *Pseudomonas* toxin, which accelerates neutrophil apoptosis via the production of ROS (Manago et al., 2015; Prince et al., 2008). LPS prolongs neutrophil survival via the NFkB pathway (Remer et al., 2003) while GM-CSF binds to GM-CSF receptors on neutrophils and activates Lyn pathway to induce neutrophil survival (Rapoport et al., 1992; Wei et al., 1996). As expected, constitutive neutrophil apoptosis was significantly inhibited in the presence of anti-CD63 antibodies (Figure 3.15A, B). Apoptosis was significantly increased in the presence of pyocyanin but was not significantly reduced by co-incubation with anti-CD63 antibodies (Figure 3.15A). Although apoptosis was visibly reduced with GM-CSF and even further with LPS, co-incubation of these pro-inflammatory stimuli with anti-CD63 antibodies did not significantly potentiate neutrophil survival (Figure 3.15B). These data suggest that a CD63-mediated survival pathway is most relevant to constitutive, rather than induced, survival.

3.2.12. TIMP-1 does not influence neutrophil survival

TIMP-1 is a tissue inhibitor of metalloproteinases glycoprotein that has been shown, through two-hybrid selection system, to interact with CD63 and to cause cancer cell survival (Chirco et al., 2006; Jung et al., 2006; Toricelli et al., 2013). Therefore, we tested the hypothesis that TIMP-1 is able to cause neutrophil survival. Neutrophils were incubated for 20 hours with 0.05, 0.1, 0.5 or 1µg/ml of recombinant human TIMP-1 protein. Following light microscopy quantification, no modulation of neutrophil apoptosis was seen with TIMP-1 human recombinant protein (Figure 3.16).

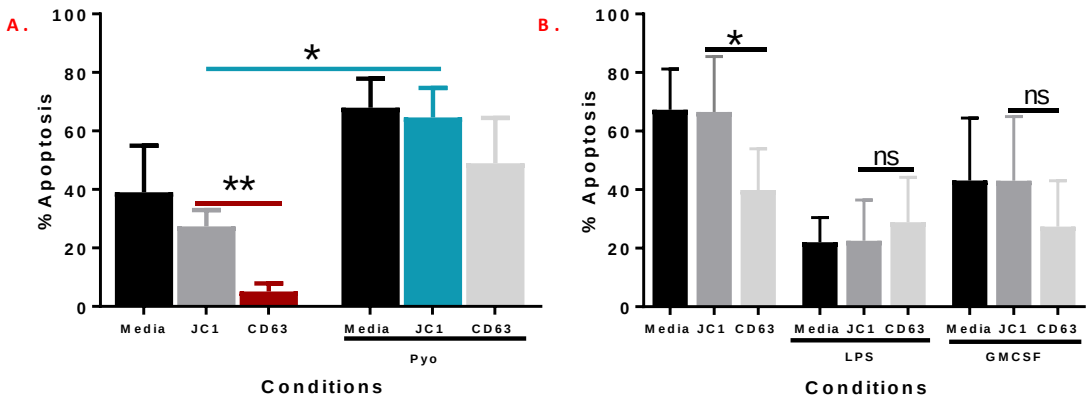


Figure 3.15: Effect of anti-CD63 antibodies on neutrophil apoptosis in the presence of inflammatory and apoptosis inducing/inhibitory stimuli.

Plasma/Percoll-pure neutrophils from healthy volunteers were cultured in the presence of media, anti-CD63 antibodies (20µg/ml) and JC1 isotype control (20µg/ml) for one hour. A further 5 hours incubation with pyo (50µ) (A) or 20 hour incubation with LPS (10ng/ml) or GM-CSF (50 units/ml) was conducted (B). Neutrophil survival was assessed by light microscopy. Data from 4 (A) and 6 (B) different donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's repeated measures post-test with selected columns, comparing anti-CD63 antibody incubated conditions to the equivalent JC1 ones and JC1 to JC1+Pyo (*$p<0.05$, **$p<0.01$).

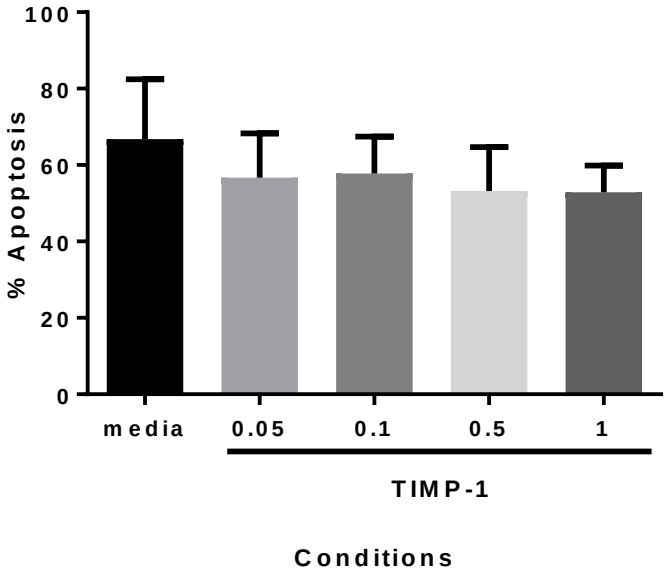


Figure 3.16: Effect of different concentrations of recombinant TIMP-1 protein on neutrophil survival.

Neutrophils isolated from venous blood of healthy volunteers were incubated for 20 hours with 0.05, 0.1, 0.5 or 1µg/ml of recombinant human TIMP-1 protein. Neutrophil survival was assessed by light microscopy. Data from 3 independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's repeated measures post-test.

3.2.13. Cohort analysis indicates different neutrophil survival response to anti-CD63 antibodies

The table in Figure 3.3B showed, albeit using a small sample size, that neutrophil survival rates in the presence of anti-CD63 antibodies varies between donors. This hypothesis was further investigated by performing more experiments and looking at results from a bigger cohort of samples. Results obtained from 44 donors incubated for 20 hours with JC1 isotype control or anti-CD63 antibodies (20μg/ml), show variable reduction in neutrophil apoptosis with anti-CD63 antibodies (Figure 3.17). Some donors have a profound reduction in apoptosis (for the purpose of this study, named 'responsive donors') that can reach up to 90% inhibition. Others show little to no reduction in apoptosis (for the purpose of this study, named 'nonresponsive donors') reaching equal or slightly higher apoptosis rates to the control samples.

As a result, donors were stratified into responders and nonresponders as follows: the donors having a ≥25% decrease in apoptosis in the presence of anti-CD63 antibodies compared to JC1 control where classed as responders while donors having a ≤25% decrease in apoptosis were classed as nonresponders. Out of the cohort, 3 responders and 3 nonresponders were selected. Neutrophils from these donors were incubated for 4 or 20 hours with media, JC1 isotype control (20μg/ml) and anti-CD63 antibodies (20μg/ml) in 3 independent experiments to determine whether variability in the neutrophil isolation techniques rather than donor phenotype was the cause of the responsiveness to anti-CD63 antibodies. Human error was further reduced by maintaining the same phlebotomist and scientist for the neutrophil isolation protocol. Data from each donor was expressed as a mean of the three independent experiments. Statistical analysis showed no significant reduction in neutrophil apoptosis at 4 hours in the presence of anti-CD63 antibodies when compared to the JC1 control for both responsive and nonresponsive donors (Figure 3.18A). However, significant reduction of neutrophil apoptosis at 20 hours with anti-CD63 antibodies when compared to JC1 control is seen in responsive donors (Figure 3.18B). The triplicates from responsive donors at the 20 hour time point were plotted individually (Figure 3.18C.). This data shows minimal variability between the repeats (Figure 3.18C).

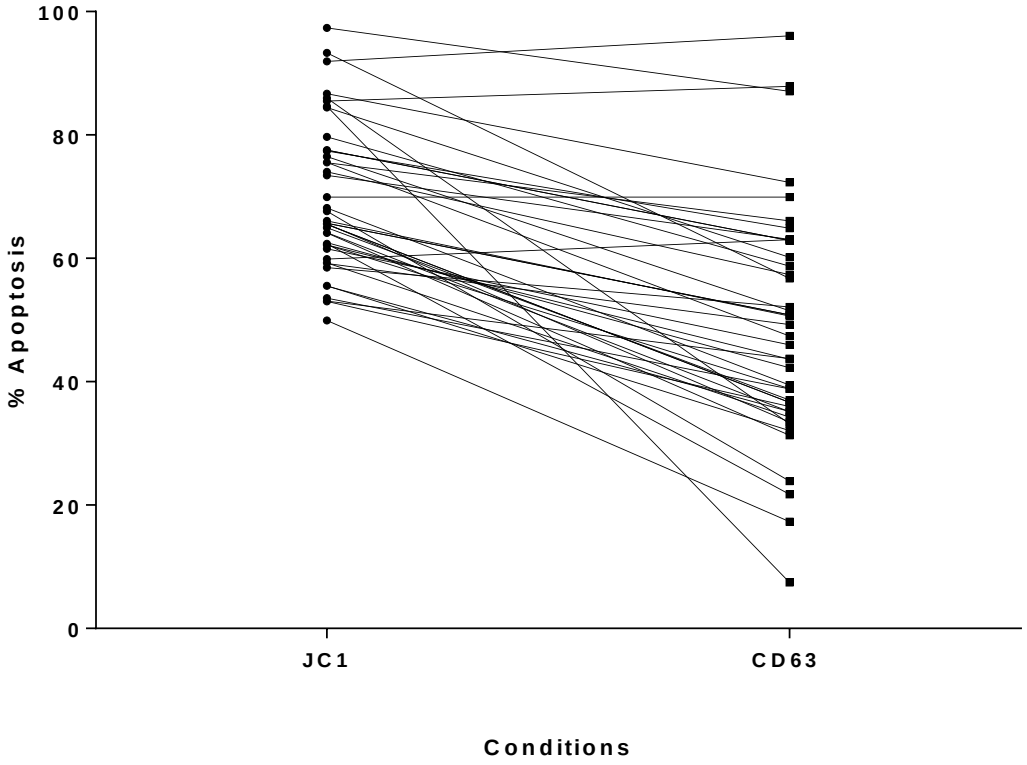


Figure 3.17: Evidence of differential donor neutrophil survival with the anti-CD63 antibodies.

Freshly isolated neutrophils from venous blood of healthy volunteers were cultured for 20 hours with JC1 isotype control and anti-CD63 antibodies at 20µg/ml. Survival was assessed by light microscopy. Data obtained from 44 independent experiments, each line representing an individual donor.

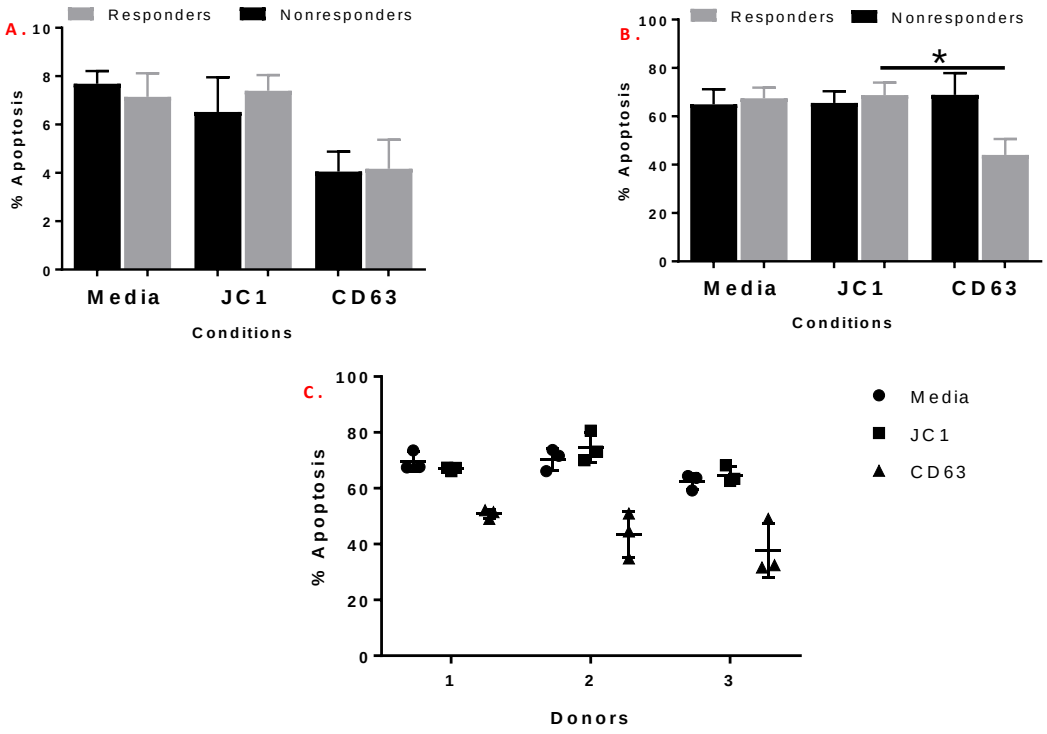


Figure 3.18: Effect of anti-CD63 antibodies on neutrophil survival of responsive and nonresponsive donors.

Percoll-purified neutrophils from responsive and nonresponsive donors incubated for 4 (A) and 20 (B) hours with media, JC1 isotype control and anti-CD63 antibodies at 20µg/ml. Neutrophil survival was assessed by light microscopy. (A-B) Each donor value plotted represents the mean of 3 repeats. Data is expressed as mean±SD. Statistical analysis carried out by One-way ANOVA with non-parametric distribution and Dunn's post-test (*$p<0.05$). (C) Twenty-hour time point individual values from 3 independent, responsive donors. Data is expressed as mean±SD.

3.2.14. No significant variation in CD63 protein expression is seen between responsive and nonresponsive donors and between the time points studied

Considering there is a difference between responsive and nonresponsive donor survival, I hypothesised that responsiveness may correlate with CD63 expression. Hypothesis testing was done by western blotting techniques. Whole cell lysates were prepared at 0, 4 and 20 hours, simultaneously to, and therefore using the same neutrophils as the functional experiments carried out in Section 3.2.13. Lysates from $2X10^6$ neutrophils from each replicate were loaded per well. Membranes were probed for CD63 using the H5C6 antibody clone (Figure 3.19). The blots were imaged at comparable time points: 1289 seconds for the responsive donors and 1073 seconds for the nonresponsive ones. A similar band pattern, 45-60kDa and 30-35kDa, is seen as above in responsive (Figure 3.19 A-C) and nonresponsive (Figure 3.19 D-F) donors. Total unphosphorylated p38 was probed as a loading control and this showed minor variation in the amount of protein loaded for 3 samples, where decreased p38 expression corresponded with lower CD63 protein expression. There is no robust time dependent pattern of CD63 protein expression at 0, 4 and 20 hours. Donor repeats show an inconsistent protein expression, with no obvious differences between responsive and nonresponsive donors. Attempts to quantify the CD63 protein expression from western blots via densitometry, using Fiji software, failed to produce reliable results due to the inconsistent band pattern on the blots. Considering this, it cannot be concluded if there is a difference in CD63 protein expression between responsive and nonresponsive donors.

3.2.15. CD63 expression on neutrophil surface at 4 and 20 hours

A single study has demonstrated increased expression of CD63 on the neutrophil surface as neutrophils age in culture (Beinert et al., 2000). Results from Section 3.2.14. indicated that global analysis of CD63 protein expression did not show any differences between the two different donor groups investigated and between time points, but western blotting is not able to distinguish between intracellular and cell surface expression of CD63. Flow cytometry, the same biochemical method employed by Beinert et al, was used to see if surface expression was regulated during culture. Neutrophils were incubated for 4 and 20

hours, followed by staining with Alexa Fluor 488 pre-stained JC1 isotype control (10µg/ml) or anti-CD63 (10µg/ml) primary antibodies. Neutrophils were gated from contaminating cells/debris based on FSC/SSC profiles (Figure 3.20 A and C). FL-1H histogram showed little to no right shift in $CD63^+$ FL1 with anti-CD63 antibody stained neutrophils when compared to JC1 controls at both 0 (Figure 3.20B) and 20 hours (Figure 3.20D). Furthermore, the FL1-H histogram profile indicates a possible bigger shift in $CD63^+$ fluorescence at 20 hours when compared to the 4 hour one. However, no conclusion can be drawn due to the high JC1 FL1 fluorescence.

3.2.16. Neutrophil apoptosis is significantly reduced at 4 and 20 hours in the presence of anti-CD63 antibodies in age matched HC and COPD patients

Neutrophils are known to drive the pathophysiology of COPD as a consequence of their dysregulated, prolonged survival at the site of inflammation. The literature shows that neutrophils from COPD patients have less CD63 mRNA and cell-surface protein expression than healthy control patients (Zhang et al., 2007). Considering this, the hypothesis investigated was that anti-CD63 antibodies are less effective in inhibiting neutrophil apoptosis in COPD patients. Neutrophils from age matched HC with a mean±SD age of 66±9.42 or from COPD patients with mean±SD age of 63.57±5.29, as indicated in Figure 3.21C table, were incubated for 4 and 20 hours with media, JC1 isotype control and anti-CD63 antibodies at 20µg/ml. Age matched controls are desirable because it accounts for some of the significant variables in the study, where age is considered as one of the most important variable. A great review by Panda et al., 2009 showed that age has been correlated with loss of neutrophil functions such as membrane composition, chemotaxis, priming, activation and most importantly increased neutrophil apoptosis. This therefore shows the importance in considering age as an influential variable in our experiments. Contrary to the hypothesis, apoptosis was significantly reduced at 4 and 20 hours with anti-CD63 antibodies in both age matched HC (Figure 3.21A) and COPD patients (Figure 3.21B).

3.2.17. No significant difference in CD63 protein expression is seen between COPD patients and age matched HC

Global CD63 protein expression from age matched HC and COPD patients was also investigated to determine whether expression correlated with neutrophilic inflammatory disease. Protein extracted from $2X10^6$ neutrophils at 0, 4 and 20 hours from age matched HC and COPD patients was loaded on individual SDS-PAGE gels and probed for CD63. A band pattern at 45-60kDa and 30-35kDa is seen in all blots, indicative of CD63 protein expression (Figure 3.22). However, comparative analysis between age matched HC and COPD patients showed 3 blots with similar or no difference in protein expression between the groups (Figure 3.22 A-C) and two with higher CD63 protein expression in COPD patients compared to the age matched HC (Figure 3.22 D-E).The unphosphorylated p38 control for protein expression showed similar protein load in most samples, with low or no expression of the control correlating with low CD63 expression between 60-45kDa. Attempts to quantify the CD63 protein expression on western blot via densitometry, using Fiji software, failed to produce reliable results due to the inconsistent band pattern on the blots. Looking at COPD and age matched HC, there is no consistent CD63 expression pattern at any of the time points analysed.

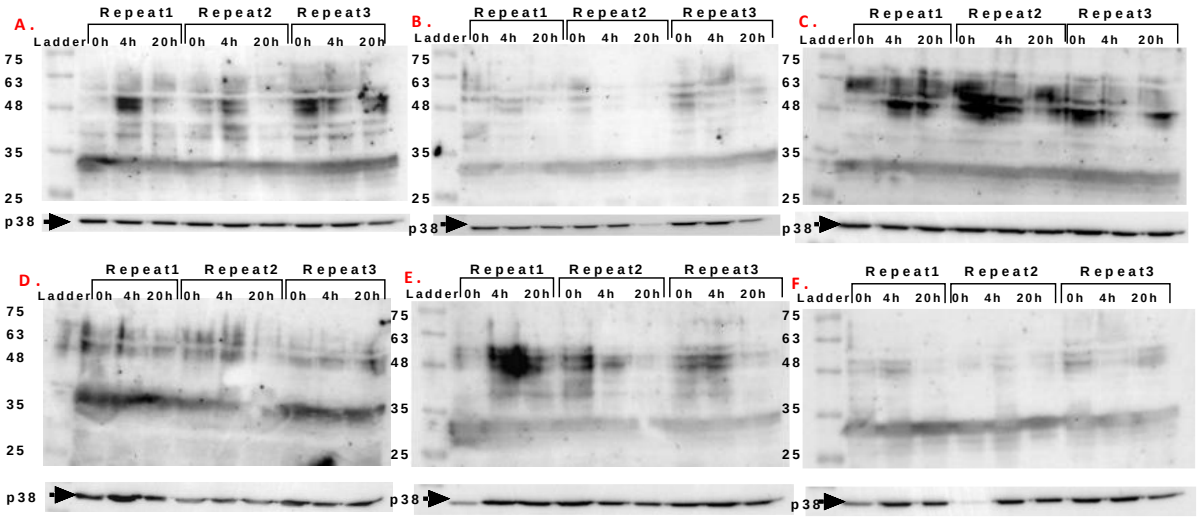


Figure 3.19: Evidence of CD63 expression in responsive and nonresponsive donors.

Whole cell lysates from 3 responsive (A-C) and 3 nonresponsive (D-E) donors were run in triplicates on a 12% SDS-PAGE gel. Protein extracted from $2X10^6$ neutrophils at 0, 4 and 20 hours was loaded in replicates for each donor on individual gels and probed for CD63 (1:600). There are two bands noticeable in both responsive and nonresponsive donors: one between 45-60kDa and one between 30-35kDa. Unphosphorylated p38 (1:4000 dilution) control was used to verify equally loaded protein levels on each panel. Each blot represents an individual donor.

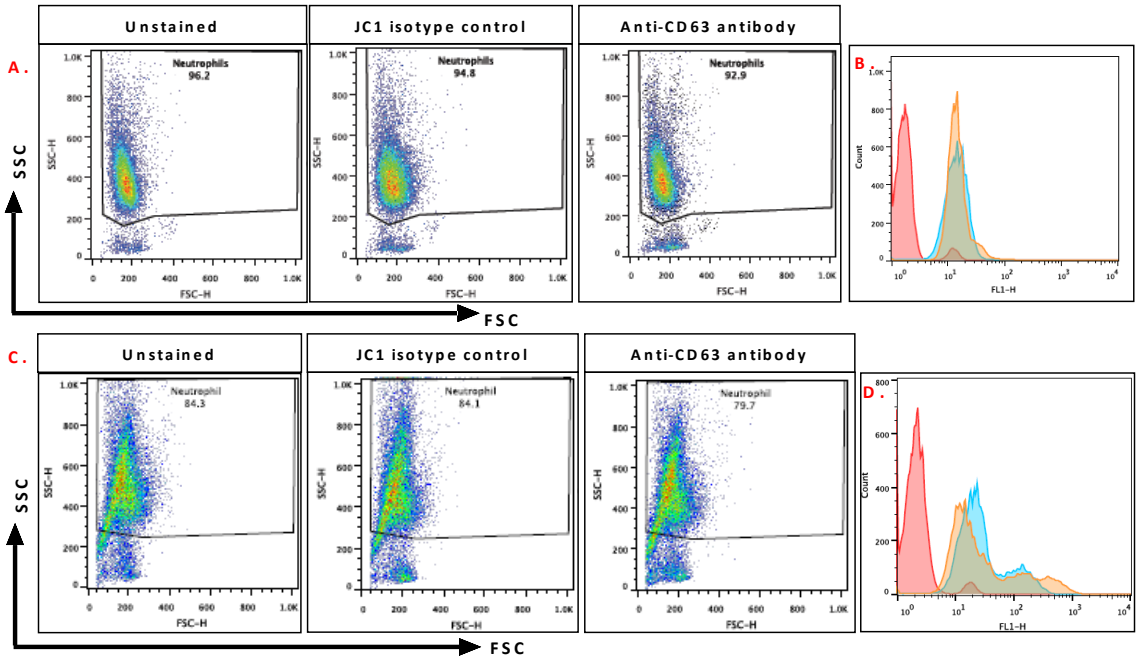
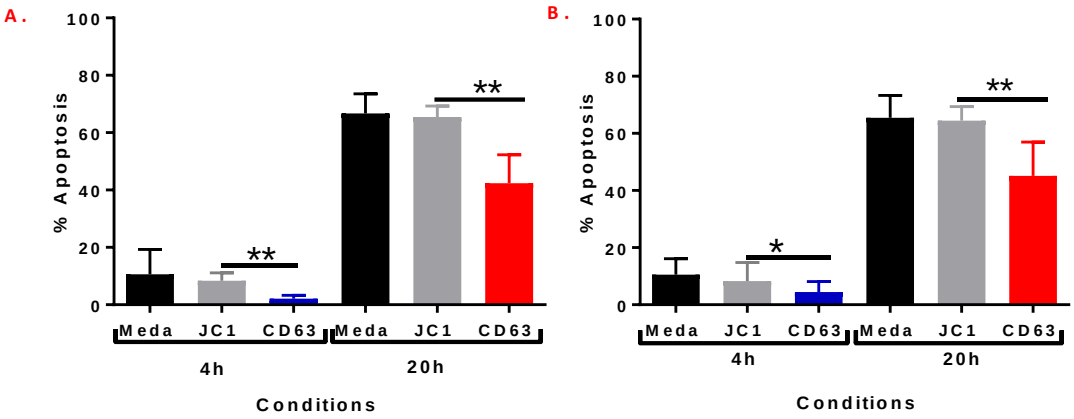


Figure 3.20: CD63 expression at 4 and 20 hours.

Freshly isolated neutrophils from venous blood of healthy volunteers were cultured for 4 (A-B) and 20 hours (C-D). The cells were subsequently incubated for 30 minutes with Alexa Fluor 488 conjugated JC1 isotype control (10µg/ml) or anti-CD63 antibodies (10µg/ml) and analysed using the FACScalibur machine. Distribution of cells under different conditions was visualised on FSC/SSC dot plot (A, C). Histograms of labeled neutrophils at 4 (B) or 20 hours (D): red-unstained, blue-JC1 isotype control, orange-anti-CD63 antibody. Right shift on FL1-H axis is indicative of fluorescence gain. Plots representative of the data obtained from two experiments.



C.

Healthy Controls	Age	COPD patients	Age
SHEFF026	69	SHEFF025	68
SHEFF028	59	SHEFF027	53
SHEFF029	61	SHEFF032	63
SHEFF038	78	SHEFF033	69
SHEFF039	79	SHEFF034	63
SHEFF047	56	SHEFF036	63
SHEFF050	60	SHEFF041	66
Mean±SD	66±9.42	Mean±SD	63.57±5.29

Figure 3.21: Anti-CD63 antibodies inhibit neutrophil apoptosis in age matched HC and COPD patients.

Neutrophils from age matched HC (A) and COPD patients (B) were incubated for 4 and 20 hours with media, JC1 isotype control and anti-CD63 antibodies at 20µg/ml. Neutrophil survival was assessed by light microscopy. Data from 7 HC and COPD patients is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's repeated measures post-test (*$p<0.05$ and **$p<0.01$). (C) Table showing individual age of age matched HC and COPD patient groups used in charts A and B.

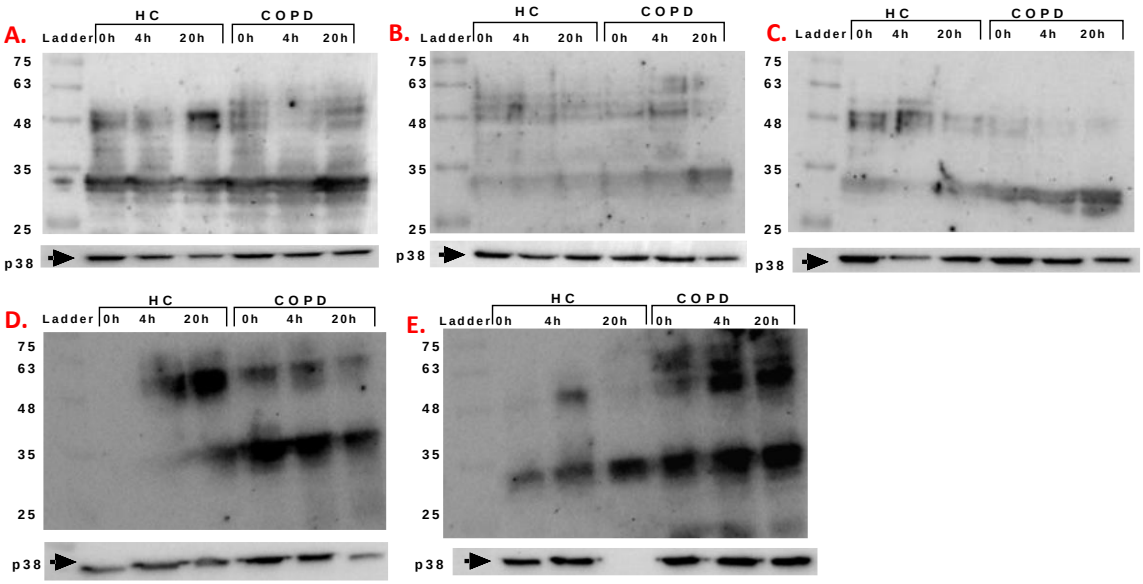


Figure 3.22: Evidence of CD63 expression in COPD patients and age matched HC.

A-E) Whole cell lysates from age matched HC and COPD patients were run on a 12% SDS-PAGE gel. Protein extracted from $2X10^6$ neutrophils at 0, 4 and 20 hours was loaded on individual gels and probed for CD63 expression (1:600). Two sets of bands are seen in both HC and COPD patients: one between 45-60kDa and one between 30-35kDa. Unphosphorylated p38 (1:2000 dilution) control was used to check even protein loading levels on each panel. Each blot represents a COPD patient-age matched healthy control pair.

3.3. Discussion

In this chapter it was shown, using light microscopy and biochemical techniques, that anti-CD63 antibodies significantly inhibit constitutive neutrophil apoptosis. The data was also confirmed by the use of CD63-Fab fragments and additional anti-CD63 antibody clones. Inhibition of CD63-dependent apoptosis was shown to be donor variable, via a mechanism that is yet to be elucidated. The results also indicate similar apoptosis rates between age matched HC and COPD patients, with increased survival in the presence of anti-CD63 antibodies, and no difference in global protein expression. The overall data suggest, therefore, a possible role of CD63 in constitutive neutrophil apoptosis.

The use of antibodies against CD63, but not to the other immune related tetraspanins CD9, CD37 and CD151, reduced neutrophil apoptosis by approximately one third. Alternative anti-CD63 antibody clones modulated neutrophil apoptosis to different degrees, with the most profound seen with the AHN-16.1 clone. The antibodies were derived from different hybridoma cell lines. While the H5C6 antibody comes from a P3X653-Ag8 myeloma cell fused with splenic cells from mice immunised with human splenic and peripheral blood adherent cells, the 6H1 antibody comes from a P3X myeloma cell fused with splenocytes from mice immunised with MTSV 1-7 mammary epithelial cells and the AHN-16.1 antibody is derived from P3X653-Ag8 myeloma cell line fused with splenic cells from mice immunised with eosinophils from a patient with chronic eosinophilia (Azorsa et al., 1991; Berditchevski et al., 1995; Hildreth and August, 1985; Hildreth et al., 1991; Skubitz et al., 1996; Skubitz et al., 1983). Although all of the antibodies come from immunisation with different antigens, all three recognise CD63 protein with the most probable cause for different levels of inhibition in neutrophil survival to be due to the affinity to the epitope. Considering this, the AHN-16.1 antibody clone has the highest affinity, followed by the H5C6 and the 6H1 one, the last clone appearing to have lost its activity due to lack of effect or would have been degraded in storage. In addition, there is evidence in the literature, at least for CD151, that the use of different antibody clones can induce different functions or show different expression results (Geary et al., 2001; Yamada et al., 2008). Such studies were not conducted for anti-CD63 antibodies, probably because there are less available clones. Due to availability reasons, only the H5C6 clone was taken forward in all experiments but, considering the

above information, if more antibody of the potent AHN-16.1 clone could be obtained, it would be interesting to repeat the functional and more importantly the expression experiments to see if the results are altered.

Fab fragments specific to CD63 were used in additional experiments in order to determine whether the anti-CD63 antibodies inhibited neutrophil apoptosis directly or the whole antibody is necessary to produce an indirect effect by cross-linking tetraspanin partner proteins. Results show that Fab fragments are enough to reduce neutrophil apoptosis indicating the mechanism of altering neutrophil apoptosis is via a direct effect on CD63 and not through indirectly affecting antibody cross-linking of CD63 with partner molecules. For instance, CD9 antibody crosslinking with partner molecules can promote degranulation of platelets, eosinophils and basophils while antibody crosslinking of CD9, CD53, CD81 and CD82 mediates activation of T cells (Higginbottom et al., 2000; Kim et al., 1997; Lagaudriere-Gesbert et al., 1997; Naik et al., 1995; Qi et al., 1996). Interestingly, CD63-Fab fragments appear more potent in reducing neutrophil apoptosis, when the opposite is usually the case. This could be due to either the Fab fragments being at higher molar concentration relative to the intact antibody or the antibody was less potent in this experiment. CD63-Fab fragments can also disrupt CD63-partner protein interaction, although at lower magnitude to the antibodies, which can lead to changes in signalling or protein distribution in the cell membrane. Were we to find that targeting CD63 may be therapeutically useful in patients with inflammation, Fab fragments are more desirable in this context since they limit the risk of antibody cross-reactivity through the Fc domain or antibody failure to reach a target due to their size. However, the most advantageous therapeutics are peptide fragments due to their characteristics of being small, highly permeable as well as being fast and cheaper to produce compared to whole or fragments of antibodies. What is more, recent published data, showed the high efficacy of peptides (~16 amino acids) representing the EC2 region of CD9 in preventing bacterial adherence, indicating potential therapeutic benefits for other tetraspanins (Ventress et al., 2016). It would therefore be interesting to test in the future the effects of synthetic peptides based on CD63 on neutrophil survival.

Antibodies are comprised of a $F(ab)_2$ region and an Fc region(Murphy, 2012). While the $F(ab)_2$ region is involved in antigen recognition, the Fc region can interact with the Fc

receptor on neutrophil surface and activate the neutrophil (Murphy, 2012; Porges et al., 1994; Swanson, 2008). Consequently, anti-CD63 antibodies could inhibit neutrophil apoptosis either by direct target binding of the antibody or through the antibody Fc region that could potentially interact with the Fc receptors on adjacent cells. The latter mechanism could potentially involve pro-survival cytokine activation, such as IL-8, upon Fc region- Fc receptor interaction, resulting in decreased apoptosis of neighboring cells (Vogelpoel et al., 2015). However, the use of anti-CD63 Fab fragments, demonstrated that the Fab antibody region is sufficient to reduce significantly neutrophil apoptosis. This suggests that the mechanism of reducing neutrophil apoptosis at 20 hours with the anti-CD63 antibodies does not depend on the antibody Fc region interacting with the Fc receptors on adjacent cells.

My results show that recombinant TIMP-1 protein does not inhibit neutrophil apoptosis at any of the concentrations used. In accordance with our data, a recent *in vivo* study demonstrated that TIMP-1 does not regulate neutrophil apoptosis but regulates neutrophil induction of granulopoiesis (Kobuch et al., 2015). However, recombinant TIMP-1 protein binding to CD63 must be checked in our experimental model to determine if a lack of interaction could have caused the results. TIMP-1-CD63 interaction can be probed using fluorescent microscopy to determine their co-localisation.

Our results show that the GST-CD63 protein constructs are contaminated with LPS. Reducing the LPS concentration in the protein construct preparation would allow us to see if the effect of the antibodies can be replicated by them as it was done in previous studies (Green et al., 2011; O'Driscoll et al., 2013). The GST-CD63 constructs were obtained by Dr. John Palmer in the Partridge laboratory via affinity purification, with purities for GST-CD63 above 0.5ng/µg protein and for GST at 0.16ng/µg protein. This indicates, at least for GST-CD63 construct, a high contamination with LPS that could alter neutrophil functions. Therefore, to use the peptides in future experiments, without altering their survival, reduction of LPS levels below the detectable limit of 0.020ng/mL is of key importance (Wakita, 2014). While a number of methods can be used currently to reduce LPS levels within a sample, none are able to completely remove it (Wakelin et al., 2006). Still, a technique was recently published where LPS concentrations could be reduced below

detectable limit by using porous supports bearing polymeric lipid membranes (Wakita, 2014). Alternatively, the endotoxin can be neutralised by adding polymyxin B, a lipid A binding agent, however this agent can stimulate cytokine production (Damais et al., 1987).

In our experiments, neutrophils were incubated only with antibodies and antibody fragments directed against a single tetraspanin. However, the literature shows us that tetraspanins are more likely to form TEMs where multiple tetraspanins have complementary and often redundant role in these complexes (Huang et al., 2005; Sugiura and Berditchevski, 1999). There is proof in the literature that CD63 can interact and form complexes with other tetraspanins such as CD9, CD151, CD81 and CD82 (Hammond et al., 1998; Radford et al., 1996; Sincock et al., 1997). It could therefore be hypothesised that a more profound neutrophil apoptosis inhibition can be seen by using anti-CD63 antibodies/fragments in combination with antibodies/fragments against CD63 interacting tetraspanins. The sole use of anti-CD9 and anti-CD151 antibodies resulted in no direct effect on neutrophil survival. This suggests perhaps that either these tetraspanins are not involved directly in mediating neutrophil survival or a function compensation by other tetraspanins could take place. However, involvement of other tetraspanins, their collective role as well as their redundancy in neutrophil survival pathways, would need to be investigated in the future by using a mixture of anti-tetraspanin antibodies/fragments. The advantage of this experiment would be that it could allow us to understand the role of tetraspanins in neutrophil survival pathways but also hint towards a more universal mechanism of tetraspanin rearrangement in TEMs to induce functional changes in cells.

My data shows that individual donors respond differently when incubated with the anti-CD63 antibodies with some having a profound inhibition of cell apoptosis while others showing little to no effect. When a small cohort of responsive and nonresponsive donors were investigated, results reached statistical significance at 20 hours for the responsive donors in the presence of anti-CD63 antibodies when compared to JC1 control. However, according to current literature, donor variability is not an uncommon phenomenon in neutrophils. For instance, *in vivo* studies show that interferon γ (IFNγ) can induce different neutrophilic post-translational modifications which trigger different donor HLA-DR expression in neutrophils (Reinisch et al., 2003). This supports the possibility of donor

variation in our study. Still, donor variability can be induced by other factors. It is known that complement activation during blood sampling procedure causes an increase in CD11b/CD18 expression on the neutrophil cell surface (Wehlin et al., 1998). CD63 is also known to associate at high levels only with externalised CD11b/CD18 (Skubitz et al., 2000). This suggests that if the cells become activated during preparation (and therefore upregulate CD11b/CD18) then there is an increased likelihood of CD63 being expressed on cell surface. A good way to investigate complement activation and CD11b/CD18 expression on neutrophil surface is by conducting the experiments in whole blood. The 20 hour triplicates of the 3 responsive donors demonstrate that there is little variability in the results between the 3 repeats, due to the use of the same phlebotomist and the same scientist to conduct plasma/Percoll neutrophil purification. Furthermore, flow cytometry experiments looking at CD63 expression in responsive and nonresponsive donors could be conducted in the future in parallel with survival experiments to look if there is different expression of CD63 on the neutrophil cell surface of each group. However, other factors such as age, gender, diet or environmental factors could potentially alter the survival results, a hypothesis that would be interesting to test in the future (Spitzer and Zhang, 1996). The ethics for this study prohibited the use of age and sex to correlate with neutrophil apoptosis but these factors would need to be investigated in the future to understand the variable donor response to anti-CD63 antibodies. To understand the variability between donors I explored CD63 expression between responsive and nonresponsive donors. The western blot data (Figure 3.19) did not show any visible difference between responders and nonresponders or, within the same donor, between the 3 time points studied. Considering the fact that neutrophils contain many proteases, it is possible that some of the protein was cleaved, which could have resulted in inaccurate results. The lysis buffer used in protein extraction contained protease inhibitors, however, a loading control such as the use of the PLB-985 or A549 cells was not run alongside the experiment to check for protease cleavage (El Azreq et al., 2010). Still, other aspects of protein modification could be investigated to see if there is a clear segregation between the two group responses. For instance, literature shows that the degree of glycosylation of CD63 or other tetraspanins can impact on their function (Metzelaar et al., 1991; Schroder et al., 2009; van Gisbergen et al., 2005; Yoshida

et al., 2009). Considering that CD63 is one of the most heavily glycosylated tetraspanins with 4 glycosylation sites, it is possible that there will be a variability of glycosylation at protein level between responders and nonresponders. While glycosylation results in a difference in molecular weight and theoretically can be visualised by western blotting, mass spectrometry would be a more quantitative technique. For example, the smear that is observed by western blot at the higher molecular weights impairs an accurate interpretation of the results. Furthermore, western blotting of whole cell lysates allows only an overall view of protein expression and not trafficking, which is a key regulator of function. Time-lapse fluorescent microscopy would give a better understanding of the localisation of CD63 on the plasma membrane or azurophilic granule of neutrophils in the presence of anti-CD63 antibodies. A possible internalisation of CD63 upon the use of antibodies could explain the increased neutrophil survival due to internalisation of the interacting partners, consequences that will be explained in the following paragraphs.

The light microscopy data indicated that neutrophil apoptosis at 4 and 20 hours in both COPD and age matched HC patients is inhibited by anti-CD63 antibodies. Western blot analysis of whole cell lysates from age matched HC and COPD patients at three time points showed that there is no obvious difference between the two groups in terms of protein expression. This outcome was expected considering the similar apoptosis rates between the two groups. In addition, no difference in CD63 protein expression between time points within the same donor is seen. The literature shows that CD63 mRNA and plasma membrane protein level is reduced in COPD patients compared to HC (Zhang et al., 2007). Considering the study used flow cytometry to look at cell surface CD63 protein expression, it suggests that although the protein level can be similar between HC and COPD patients, there could be variations between plasma membrane and intracellular expression of CD63. It is however interesting that neutrophils from COPD patients and HC respond similarly to the anti-CD63 antibodies.

Anti-CD63 antibodies were shown to be more effective in modulating constitutive neutrophil apoptosis since incubation with survival/apoptosis modulating factors such as pyocyanin, LPS and GM-CSF with anti-CD63 antibodies did not affect neutrophil survival further. This may be because the pro-apoptotic/pro-survival stimulus engaged pathways

that overrode the function of CD63 in regulating survival. While pyocyanin accelerates apoptosis via ROS induction, cathepsin D, cytochrome C release and mitochondria permeabilisation; LPS prevents apoptosis by activating the MYD88 pathway and GM-CSF activates Lyn pathway to induce neutrophil survival (Manago et al., 2015; Prince et al., 2008; Rapoport et al., 1992; Remer et al., 2003; Wei et al., 1996). The lack of anti-CD63 antibodies effect to modulate further neutrophil apoptosis when 3 different, important signaling neutrophil pathways were targeted suggests that perhaps this tetraspanin is modulating constitutive neutrophil apoptosis. Probably other tetraspanins that have not been investigated yet could be studied in the context of induced neutrophil apoptosis. For instance, in monocytes LPS is able to induce the co-clustering of TLR4, Fcγ-RIIa, CD81 and CD14 while in macrophages CD9 can modulate CD14, a TLR4 cofactor, expression on the cell membrane in the presence of LPS (Pfeiffer et al., 2001; Takeda et al., 2015). Considering the involvement of these 2 tetraspanins in TLR signaling, future experiments could explore if they are involved in regulating LPS induced neutrophil survival.

An important question concerns the mechanism of CD63-mediated neutrophil survival. The constitutive neutrophil apoptosis pathway occurs through the intrinsic/mitochondrial pathway and is the result of increased pro-apoptotic Bcl-2 factors compared to anti-apoptotic members which go on to activate apoptosome formation and caspase activation (Adams, 2003; Adams and Cory, 1998; Murphy et al., 2003; Zou et al., 1997). Bcl-2 is an anti-apoptotic factor that was shown in tumor cells to be regulated by AP-2 α-Rb (adaptor protein-2 α-retinoblastoma) activation complex (Morales et al., 2011). Despite the fact that CD63 can bind to AP-2 it is also know that neutrophils do not express the Bcl-2 anti-apoptotic factor, therefore leading to a dead end for this mechanism (Hirst et al., 1999; Moulding et al., 1998). The pro-apoptotic protein Bax transcription was shown in colonic epithelial cells to require the transcription factor AP-1 to initiate apoptosis (Mandal et al., 2001). According to the literature, there is no evidence of CD63 interacting with AP-1 protein. Although a mechanism for CD63 involvement in constitutive neutrophil apoptosis cannot be proposed yet, it could be speculated that via CD63-AP-2 interaction in other cell types the tetraspanin could be involved in modulating cell survival by promoting their trafficking. However, it may as well be the case that the tetraspanin may regulate

expression of one or more Bcl-2 family members by activating different signalling pathways, as seen in acute myelogenous leukemia cells (Nishioka et al., 2015).

CD63 can be transported to the cell surface from the cytoplasm and recycled back (Pols and Klumperman, 2009). However, there is evidence that indicates that antibody binding induces rapid internalisation of CD63 via its internalisation/lysosomal C-terminal targeting motif (Mantegazza et al., 2004; Rous et al., 2002; Smith et al., 1995). Considering the literature, it is plausible that CD63-protein interactions and the formation of TEMs to be abrogated by the recycling of the CD63 to the lysosomal compartment. This may result in loss of function by abrogating formation of signalling platforms that may be involved in promoting neutrophil apoptosis.

A possible mechanism by which CD63 inhibits constitutive neutrophil apoptosis is by affecting the function or transport of protein kinase C (PKC) δ signalling molecule to the cell surface. PKCδ is an important pro-apoptotic neutrophil signaling molecule that once translocated to the plasma membrane, aids PS externalisation (Nusbaum et al., 2005; Pongracz et al., 1999; Webb et al., 2000). Interestingly, Plaszczyca et al., 2014 showed in fibrous histiocytoma, that PKCδ catalytic genes can fuse with the CD63 gene resulting in constitutive activation of this kinase. It is possible that either anti-CD63 antibodies prevent recruitment or translocation of the PKCδ to the cell surface or the antibody-CD63-PKCδ complex prevents PS externalisation by preventing kinase activation. A possible way to investigate if this interaction takes place in neutrophils is by using fluorescent microscopy and fluorescently tagged antibodies in co-localisation studies. Co-immunoprecipitation could be another way to investigate CD63-PKC interactions, although this technique causes problems in neutrophils due to the proteases that can degrade proteins. In addition, the place of interaction, if present, between the tetraspanin and the kinase can be determined by using markers for the different endosomal compartments involved in protein trafficking such as Ras-related protein 5 (Rab-5) for early endosomes, lysososmal-associated membrane protein 1 (LAMP-1) for lysosomes and Rab7 for late endosomes (Chavrier et al., 1990; Meresse et al., 1995; Peters et al., 1991).

In a different neutrophil study, CD63 was also shown to interact with αMβ2 integrin and Src and Lyn protein kinases (Skubitz et al., 1996). This resulted in kinase phosphorylation and activation leading to neutrophil migration and adhesion to HUVEC epithelial cells. This adhesion was increased in the presence of anti-CD63 antibodies. Recent data also shows that ligation of SMIP-0166 drug with CD37 protein situated on chronic lymphoid leukemia cells led to phosphorylation of the N-terminus immunoreceptor tyrosine-based activation -like motif of CD37 by Lyn kinase, leading to Src homology region 2 domain-containing phosphatase-1 -dependent BIM upregulation and cell death (Lapalombella et al., 2012). This suggests a possible mechanism of action for anti-CD63 antibodies in preventing cell death. However, one of the antibodies used by Skubitz et al., 1996, was used in a single experiment (Figure 3.5) to show a profound inhibition of neutrophil apoptosis. The association of the kinases with CD63, in a fairly big complex, was also demonstrated in the presence of Brij 58 detergent (Skubitz et al., 2000). It is possible that actually the Lyn and Syk kinases to associate with other proteins but because they are part of a big complex, to appear as it interacts with CD63. The cytokine GM-CSF was also shown to activate Lyn kinase resulting to inhibition of neutrophil apoptosis (Wei et al., 1996). Results in this chapter showed no cumulative role for GM-CSF and anti-CD63 antibodies (Figure 3.15B) in inhibiting further neutrophil apoptosis when compared to separate incubation of neutrophils with the two reagents. As a result, in this context, it is improbable a role for CD63 role in Lyn kinase activation.

Another possible mechanism of action CD63 is involved in to modulate neutrophil apoptosis, is via PI4-K type II. CD63 is known to interact almost entirely in the intracellular compartment with the PI4-K type II kinase (Yauch and Hemler, 2000). PI4-K type II participates in phosphoinositide synthesis at the plasma membrane and these lipids are involved, amongst many functions, in modulating cell survival (Tolias and Cantley, 1999). The binding of anti-CD63 antibodies, as previously described, could promote rapid internalisation of CD63 from the plasma membrane which can result in PI4-K type II internalisation. This would result in the inhibition of phosphoinositide synthesis or reduced production of phosphoinositides that could affect cell membrane properties and as a result increase cell survival.

Still, CD63 involvement in neutrophil survival may be a more complicated process than expected since CD63 acts as a transporter for many proteins, some of which are involved in neutrophil survival, degranulation and cell homeostasis. Using a zebrafish model, it was shown that CD63 is involved in embryo hatching by altering the function of the secretory machinery either by disrupting CD63-integrin function or by failing to deliver the hatching enzymes to the granules (Trikic et al., 2011). A similar morphology as the hatching model was seen in a neutrophil *in vitro* model, where the authors showed that CD63 knockout, by siRNA in COS cells, resulted in failure to transport the precursor of neutrophil elastase to the lysozyme granules (Kallquist et al., 2008). Neutrophil elastase is a key enzyme in neutrophil functions since mutations in the neutrophil elastase ELANE gene can result in severe congenital neutropenia and cyclic neutropenia, exhibited by fewer than 1.5×10^9neutrophils/L and lasting for more than 3 months (Dale et al., 2000; Rosenberg et al., 2006). The mutations in the ELANE gene results in protein unfolding, mislocalisation accumulation in the cytoplasm and cell death (Grenda et al., 2007; Horwitz et al., 2007; Kollner et al., 2006). However, the *in vitro* experiments conducted did not show any severe neutrophil survival pathology with the anti-CD63 antibodies, nor did the phenotyping of *in vivo* mouse model showed any neutrophil abnormalities (Schroder et al., 2009). These results suggest that perhaps modulation of neutrophil apoptosis by CD63 is through a different mechanism since tampering with neutrophil elastase would most probably result in cell death, considering the known literature, rather than survival.

Another mechanism CD63 can be involved in regulating to reduce neutrophil apoptosis is the transport of the H,K-ATPase. Current literature shows, using COS7 cells, that CD63 sequential association with the AP-2 and AP-3 adaptor complexes, allows CD63 interaction with the gastric H,K-ATPase β-subunit to move it from the cell surface to early and late endosomes to lysosomal compartment (Duffield et al., 2003). Western blotting techniques also showed that human neutrophils have similar H,K-ATPase β-subunit to the gastric one (Ritter et al., 1998). In addition to this, neutrophils have vacuolar H-ATPase that is involved in preserving neutrophil homeostatis by pumping protons in the extracellular space and in the organelles (Lafourcade et al., 2008). Furthermore, in the presence of G-CSF, β-subunit synthesis is upregulated in the cytosolic compartment and increased translocation of the

vacuolar H-ATPase to the neutrophil plasma membrane can be seen (Niessen et al., 1997). There is also evidence that in the presence of G-CSF, there is an upregulation of the vacuolar H-ATPase (Gottlieb et al., 1995). Considering the above literature, it is possible that rapid internalisation of CD63 from the cell surface to lysosomal compartment, due to the use of anti-CD63 antibodies, results in the possible fail to internalise the H,K-ATPase and vacuolar H-ATPase to the intracellular compartment. The overall effect seen would be more protons being pumped out preventing intracellular acidification, an early step in the apoptosis program. It is possible that co-incubation of GM-CSF and anti-CD63 antibodies targeted the same process and as a result a more prominent reduction in neutrophil apoptosis, however not significant, was seen with both in comparison to the GM-CSF alone.

My results show that although there is good CD63 protein expression, as indicated by western blotting, the flow cytometry experiments failed to show a CD63 protein surface expression on unstimulated neutrophil. There are a number of studies that show, however, that neutrophils have low CD63 protein expression on the plasma membrane, with the majority of the protein being localized in the primary granules (Beinert et al., 2000; El Azreq et al., 2010; Tohami et al., 2004; Zhang et al., 2007). Although flow cytometry analysis failed to show CD63 protein expression on unstimulated neutrophil cell surface, stimulation of neutrophils with *S. aureus* resulted in the detection of CD63 when compared to JC1 control (Figure 3.9). Our results are in accordance with the literature since, in the presence of other degranulation stimuli, the same effect is seen. For instance, HL-60 cell incubation with zymosan results in CD63 recruitment to the phagocytic cup while fMLP incubation increases CD63 expression to the surface (Nordenfelt et al., 2009). This suggest that the anti-CD63 antibody clone used in the experiments can detect CD63. Though low CD63 surface expression of the unstimulated neutrophils correlated with possibly a false positive signal resulting from JC1 isotype antibody binding to the high amounts of Fc receptors that neutrophils express on their surface, resulting in inadequate detection of the tetraspanin on neutrophil surface in this thesis. Although studies show CD63 expression on unstimulated neutrophil surface, additional experiments would need to be done to show this in our model system. To reduce the false positive signal future experiments could use human serum to block the Fc receptors.

Similarly, the flow cytometry CD63 expression data obtained showed that a conclusion could not be reached about CD63 upregulation on cell surface with age. This could be also due to the high amounts of Fc receptors that neutrophils express on their surface that would allow JC1 isotype antibody binding to these receptors and give a false positive signal. *In vitro* study conducted by Beinert et al., 2000 showed that CD63 expression increases on cell surface with age. However, in the study the authors used gentamicin to inhibit neutrophil apoptosis for 48 hours. Gentamicin can affected the internal gene transcription as well as translation and expression of protein potentially resulting in skewed results (Healy et al., 2002).

An in depth understanding of CD63 role in neutrophil survival could be achieved by deleting the CD63 gene in neutrophils. However, neutrophils have a very short life and are genetically intractable. To get around this problem, PLB-985, a myeloid-leukemia cell line, can be differentiated into cells that are functionally similar to neutrophils and express $CD63^{+}$ granules (Pivot-Pajot et al., 2010). As a consequence, studying CD63 function in neutrophil survival can be done by knocking down the tetraspanin using small interfering RNA (siRNA). Furthermore, PLB-985 can be used to fine tune experiments that would otherwise require large neutrophil numbers and are limited by blood availability. For instance, to understand what survival pathway CD63 feeds into, CD63 interacting partners could be studied. Pull-down or immunoprecipitation assays, which require more protein and are difficult to optimise with primary neutrophils due to high number of degrading proteases, is more feasible with differentiated PLB-985 due to readily availability in high numbers and lower levels of proteases. Alternatively, neutrophils fromCD63 knockout mice could be obtained, albeit at the time of the study this was not possible due to mice breeding problems.

Alternatively CD63 role in neutrophil survival can be investigated *in vitro* by overexpressing the tetraspanin. CD63 is highly expressed in neutrophil granules but overexpressing CD63 could have a different effect on neutrophil survival (Cham et al., 1994). Despite the fact that neutrophils cannot be manipulated in this way, previous studies conducted by our group showed that neutrophils can be stably transfected with lentivirus encoding a dominant negative protein to overexpress and study the protein of interest (Dick et al., 2009). This

technique can be used to induce neutrophils CD63 overexpression to investigate its function.

The effects of CD63 on neutrophil survival has only been studied in this thesis in an *in vitro* model or by using ex vivo patient samples. However, *in vitro* models present limitations due to inability of seeing a more integrated global effect as seen with an *in vivo* model. To put the data in a more clinical context and to understand better the role of CD63 in neutrophil survival, *in vivo* studies using mice or zebrafish models must be done. Considering that mouse and human DNA is 95% similar, the mouse is a suitable model for studying neutrophils. CD63 knock-out mice that could be used in our study were previously obtained by knocking out the two CD63 gene copies on C57BL/6J background mice (Schroder et al., 2009). A similar strain is also commercially available (Taconic, n.d.). Moreover, an elastase/LPS induced mouse model of COPD could also be used in conjunction with anti-CD63 antibodies to study the role of CD63 in an in vivo model, giving a more clinical context of the tetraspanin role (Ganesan et al., 2012). When considering the zebrafish model, the human and zebrafish CD63 proteins are 62.2% similar with comparable key features: 3 glycosylation identical sites in the EC2 domain and the C-terminus internalisation motif (Trikic et al., 2011). However, complete knock-down using morpholinos of CD63 in zebrafish was shown to result in impaired hatching of the embryos (Trikic et al., 2011). At present, Sanger institute is working on a stable line with a nonsense mutation to be available in the near future, that will probably overcome the hatching problem noticed by Trikic et al., 2011 (Welcome trust Sanger institute, 2017) Furthermore, when selecting an appropriate *in vivo* model to confirm this study, it should be considered the CD63 knockout mouse model did not demonstrate any severe physiological changes as seen in the zebrafish model. This indicates perhaps that mammals have more redundancy in the tetraspanin network where if a tetraspanin is absent or malfunctioning, other tetraspanins could compensate for its function.

The findings presented in this chapter show for the first time that CD63 plays a role in constitutive neutrophil apoptosis, independent of small levels of PBMCs contamination. In addition, neutrophils from COPD patients showed similar apoptosis results to age matched HC controls when incubated with anti-CD63 antibodies. Protein analysis data did not show

a difference in protein expression, failing to hint toward a potential mechanism. All things considered, the results allow for many questions to be opened about the mechanism of CD63-dependent regulation of neutrophil apoptosis.

Chapter 4: A role for CD151 in neutrophil interactions with *S. aureus* and other respiratory pathogens

4.1. Introduction

4.1.1. Neutrophil defence against pathogens

Phagocytosis is one of the most important processes by which neutrophils protect the host from invading pathogens. It involves pathogen engulfment and activation of the downstream processes involved in pathogen killing. To do this, neutrophils recognise conserved pathogen structures via pathogen recognition receptors described in detail in Chapter 1 (Schymeinsky et al., 2007; Takeuchi and Akira, 2010). The neutrophils ingest the pathogen through a zipper model, forming a phagocytic cup which seals to form the phagosome. Fusion of granules to the phagosome forms the phagolysosome, which is progressively acidified and exposed to antimicrobial components and ROS, ultimately resulting in bacterial death (Borregaard and Cowland, 1997b; Groemping and Rittinger, 2005; Swanson, 2008). *S. aureus*, as well as other pathogens have evolved mechanisms to evade recognition by neutrophils and escape from the phagolysosome (Bera et al., 2005; Bestebroer et al., 2007; Bestebroer et al., 2009; Haas et al., 2005; Haas et al., 2008; Laarman et al., 2012; Malachowa et al., 2011; Mishra et al., 2011; Prat et al., 2006; Prat et al., 2009). It is therefore important to study the mechanisms underpinning neutrophil phagocytosis, which may ultimately reveal novel therapeutic strategies for patients with life threatening and/or chronic infections.

4.1.2 The involvement of tetraspanins in phagocytosis

Previous studies have shown that tetraspanins play a role in bacterial phagocytosis (Artavanis-Tsakonas et al., 2011; Artavanis-Tsakonas et al., 2006; Chang and Finnemann, 2007; Noda et al., 2013). For instance, CD81, via the αvβ5 integrin, has a role in retinal pigment epithelial phagocytosis of photoreceptor segment fragments (Chang and Finnemann, 2007). In dendritic cells, CD82 is recruited to phagosomes containing fungi and bacteria, prior to their fusion with the lysosomes, but its role in this process is yet to be elucidated (Artavanis-Tsakonas et al., 2011). The same group has previously shown CD63

dependent recruitment of the *Cryptococcus neoformans* to the acidified phagolysosome (Artavanis-Tsakonas et al., 2006). Furthermore, CD9 was shown to be part of the macrophage efferocytosis pathway (Noda et al., 2013). The role of tetraspanins in neutrophil phagocytosis has not been investigated.

4.1.2. Evidence of tetraspanins role in *S. aureus* adhesion

S. aureus interacts with host cells by mechanisms that may involve tetraspanins. Assays showed that the strong CD9 and CD151 interaction with β1 integrins can mediate *S. aureus*-fibronectin adhesion while CD9 interaction with heat shock protein 70 (Hsp70) and αVβ3 integrin is able to mediate *S. aureus* intestinal cell adhesion via Hsp70 (Dziewanowska et al., 2000; Guerrero and Moreno, 2012; Peddibhotla et al., 2013; Rubinstein et al., 1994; Yauch et al., 2000). Additionally, CD9 binds to fibronectin in intestinal cells, a *S. aureus* adhesion component, but it also promotes shedding fibrinogen receptor on platelet cells to prevent *S. aureus* adhesion via it (Guerrero and Moreno, 2012; Hato et al., 1988; Piroth et al., 2008). Previous data from Dr. Lynda Partridge and Prof. Peter Monk groups also showed that CD9, CD63 and CD151 are involved in *S. aureus* adhesion to epithelial cells through an unknown mechanism (Green et al., 2011). A recent paper by Virreira Winter et al., showed that ADAM10 bound and internalised less *S. aureus* pore-forming toxin α hemolysin when cells were deficient in TSPAN14 (Virreira Winter et al., 2016).

The studies, although CD9 oriented, suggest a role for tetraspanins in *S. aureus* interaction with host cells and potential subversion of host defences. However, none of these studies explore the role of tetraspanins in neutrophil interaction with *S. aureus*. As a result the **hypothesis** investigated in this chapter is that tetraspanins play a role in the interaction with *S. aureus* and possibly other respiratory pathogens. The first aim of the project was to identify the tetraspanin(s) involved in *S. aureus* interactions using different anti-tetraspanin antibodies. The second aim was to see if this tetraspanin(s) is involved in modulating neutrophil interactions with different *S. aureus* strains or other Gram positive/negative respiratory pathogens. Lastly, I proceeded to investigate the mechanism by which this tetraspanin(s) modulates neutrophil interaction with *S. aureus* or other respiratory pathogens.

4.2. Results

4.2.1 Neutrophil interaction with HK *S. aureus* increases with MOI

Neutrophils are professional phagocytes that readily phagocytose *S. aureus* (Odell and Segal, 1991). To validate the interaction range of *S. aureus*, neutrophils were incubated for 2 hours with heat-killed (HK) *S. aureus* at MOI of 2.5, 5, 10 and 20. The phagocytic index (PI) was calculated to account for the percentage of neutrophils that phagocytose bacteria, but also the number of bacteria each cell has phagocytosed. Light microscopy analysis showed that interaction with HK *S. aureus* increased with MOI, reaching a mean±SD of 529.5±128.1 at MOI20 (Figure 4.1). Light microscopy does not discriminate between phagocytosed and surface bound bacteria therefore in all experiments where the technique is used the results will be referred to as showing a neutrophil-bacterial interaction.

4.2.2. Anti-CD151 antibodies inhibit neutrophil interaction with HK *S. aureus*

Results from the groups of Dr. Lynda Partridge and Prof. Peter Monk, as well as others show that tetraspanins are involved in *S. aureus* adhesion and interaction with epithelial cells (Dziewanowska et al., 2000; Green et al., 2011; Ventress et al., 2016; Virreira Winter et al., 2016). With the exception of Tohami et al., 2004 who showed that neutrophils from patients with pneumonia and urinary tract infections express reduced levels of CD81 and CD82, the role of tetraspanins in neutrophil bacterial infections has not been investigated. To explore this, neutrophils were incubated for 1 hour with anti-tetraspanin antibodies directed against CD9, CD37, CD63 and CD151 at 10µg/ml. These tetraspanins are expressed at higher levels, but are not restricted to myeloid cells and therefore good candidates for study in this context. Media and JC1 IgG1 isotype (10µg/ml) were used as controls. HK *S. aureus* MOI20 was then added for a further 2 hours. Photomicrograph of JC1 control (Figure 4.2A) incubated neutrophils showed the presence of *S. aureus* within phagosomes. Phagocytic index quantification showed a significant difference between JC1 and anti-CD151 antibodies (517.71±135.6 Mean±SD in JC1 control and 402.66±132.7 Mean±SD in anti-CD151 antibody incubated neutrophils (Figure 4.2B)). Antibodies to CD9, CD37 and CD63 were without effect. Expression of individual phagocytic index in a table (Figure 4.2C) shows

variation of PI between donors. Overall, the data indicates a potential role for CD151 in neutrophil interaction with HK *S. aureus* and this was explored in further experiments.

4.2.3. Anti-CD151 antibodies inhibit neutrophil interaction with HK *S. aureus* at 10 µg/ml

Since anti-CD151 antibodies inhibited neutrophil interactions with HK *S. aureus*, their working concentration was further refined to determine the optimal use. To do this, neutrophils were incubated for 1 hour with JC1 isotype control (10µg/ml) and low (0.5-1µg/ml) (Figure 4.3A) or high (5-20µg/ml) (Figure 4.3B) concentrations of anti-CD151 antibodies. HK *S. aureus* MOI10 was added for a further 2 hours. While the low concentrations of anti-CD151 antibodies had no effect on reducing neutrophil interaction with HK *S. aureus*, the higher concentration of 10µg/ml significantly reduced neutrophil interaction with HK *S. aureus*. Although, 20µg/ml of anti-CD151 antibodies led to a visible reduction in phagocytic index, this was not significantly different, probably due to variations in the results obtained with that concentration. For this reason, 10µg/ml of anti-CD151 antibodies was used in all further experiments.

4.2.4. Different anti-CD151 antibody clones inhibit neutrophil interaction with HK *S. aureus*

To confirm further that CD151 plays a role in neutrophil interactions with HK *S. aureus*, a commercially available anti-CD151 was compared to the in-house antibody used above. Neutrophils were incubated for 1 hour with anti-CD151 antibodies (10µg/ml) or their equivalent isotype controls (10µg/ml), followed by a 2 hour incubation with HK *S. aureus* at MOI10. Phagocytic index counts indicate a significant reduction in interaction of neutrophils with HK *S. aureus* when both anti-CD151 antibody clones were used, by comparison to their equivalent isotype controls (Figure 4.4). These results confirm further the involvement of CD151 in neutrophil phagocytosis of HK *S. aureus*.

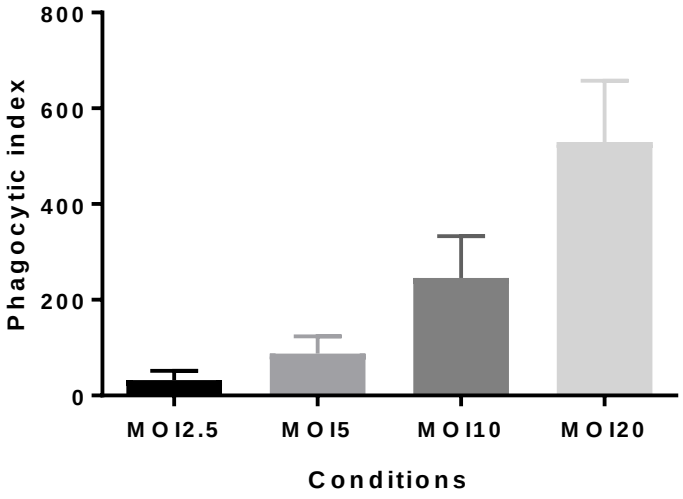
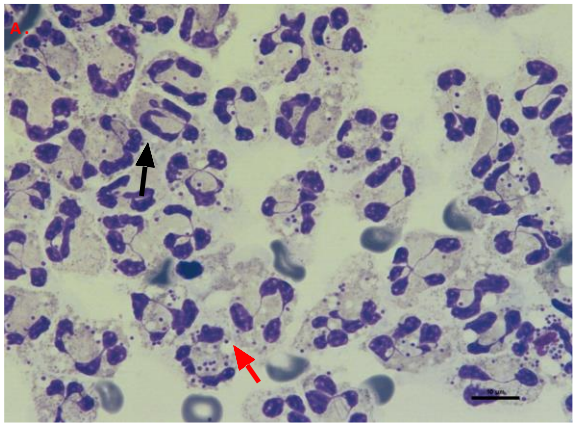
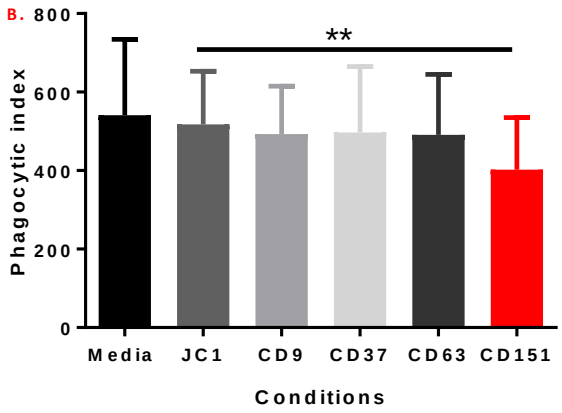


Figure 4.1: Evidence of increased neutrophil interaction with HK *S. aureus* with MOI.

Plasma/Percoll-pure neutrophils were incubated for 2 hours with HK *S. aureus* at MOI 2.5, 5, 10 and 20. Phagocytosis was assessed by light microscopy. Data from 4 different experiments is expressed as mean±SD.





C.

Donor	Media	JC1	CD9	CD37	CD63	CD151
D1	439.82	391.28	403.78	322.82	298.54	278.26
D2	721.67	533.95	461.89	442.23	542.05	437.44
D3	319.68	444.66	433.01	497.52	458.12	320.8
D4	682.51	700.98	672.9	724.42	664.96	574.14
Mean±SD	540.92±193.1	517.71±135.6	492.89±122.3	496.75±168.4	490.92±153.8	402.66±132.7

Figure 4.2: The effect of different anti-tetraspanin antibodies on neutrophil interaction with HK *S. aureus*.

Freshly isolated neutrophils from venous blood of healthy volunteers by plasma/Percoll centrifugation were cultured for 1 hour with media or 10µg/ml of anti-tetraspanin antibodies against CD9, CD37, CD63 and CD151 or JC1 isotype control, followed by a 2 hour incubation with HK *S. aureus* at MOI20. Neutrophil interaction with *S. aureus* was assessed by light microscopy. A) Photomicrograph of neutrophil interaction with HK *S. aureus* in the presence of JC1 isotype control. Neutrophils-black arrow, HK *S. aureus* in the phagolysososme- red arrow. Scale bar 10µm. B) Phagocytic index of the data from 4 independent repeats is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test (*$p<0.05$). C) Table showing different phagocytic index rates obtained from the donors in the presence of different anti-tetraspanin antibodies.

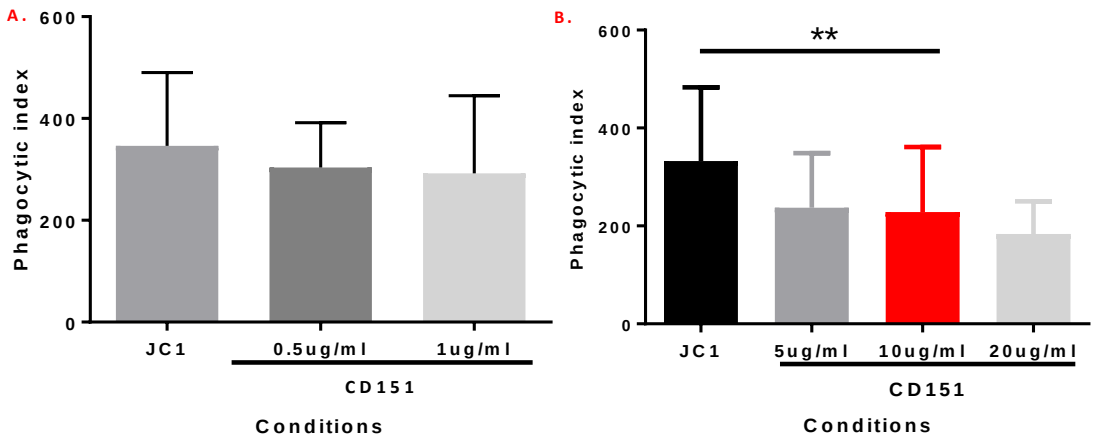


Figure 4.3: Anti-CD151 antibodies inhibit neutrophil interaction with HK *S. aureus* at 10µg/ml.

Freshly isolated neutrophils from venous blood of health volunteers by plasma/Percoll centrifugation were incubated for 1 hour with JC1 isotype control (10µg/ml) and low (0.5-1µg/ml) (A) or high (5-20µg/ml) (B) concentrations of anti-CD151 antibodies followed by a 2 hour incubation with HK *S. aureus* at MOI10. Neutrophil interaction with HK *S. aureus* was assessed by light microscopy. Data from 4 (A) and 5 (B) independent experiments is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures (**p<0.01).

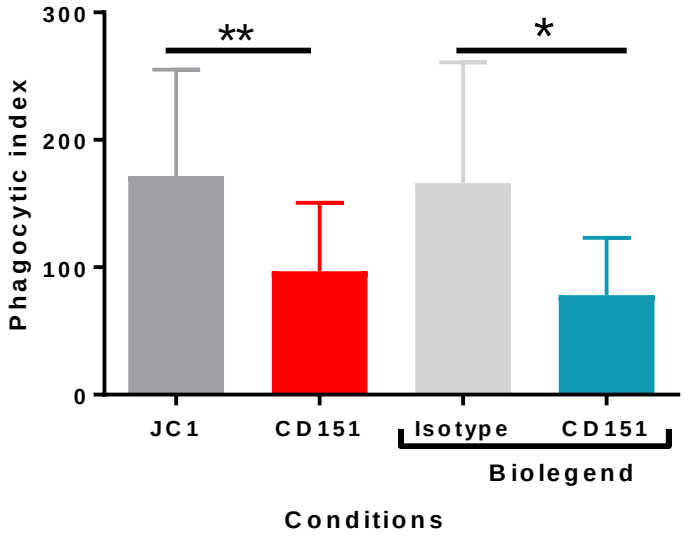


Figure 4.4: Commercially available and in house obtained anti-CD151 antibodies inhibit neutrophil interaction with HK *S. aureus*.

Freshly isolated neutrophils from venous blood of health volunteers via plasma/Percoll centrifugation were incubated for 1 hour with anti-CD151 antibodies or isotype controls (10µg/ml), either obtained in house by Dr. Lynda J. Partridge or commercially available from Biolegend, followed by a 2 hour incubation with HK *S. aureus* at MOI10. Neutrophil interaction with *S. aureus* was assessed by light microscopy. Data from 6 independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures (*p<0.5, **p<0.01).

4.2.5. Anti-CD151 antibodies inhibit neutrophil interaction with HK *S. aureus* at MOI5 and 10 and at 60 and 120 minutes post incubation

I next wanted to determine the potential of anti-CD151 antibodies in inhibiting neutrophil interaction with HK *S. aureus* across a range of MOIs and time points. Neutrophils were incubated for 1 hour with JC1 isotype control or anti-CD151 antibody at 10µg/ml followed by a further 2 hour incubation with HK *S. aureus* at MOI1, 2.5, 5 or 10 (Figure 4.5A) or by a 30, 60 or 120 minute incubation with HK *S. aureus* at MOI10 (Figure 4.5B). Neutrophil interactions with HK *S. aureus* was significantly inhibited at all MOIs and time points, reaching statistical significance at MOI5 and 10 (Figure 4.5A) and after 60 and 120 minutes (Figure 4.5B).

4.2.6. Anti-CD151 antibodies inhibit neutrophil interaction with HK *S. aureus* WT and JE2 strains

There are over a thousand strains of *S. aureus* with some being more pathogenic than others (van Belkum and Melles, 2009). To show the role for CD151 was not restricted to a single strain (SH1000-WT), the USA300 background (JE2) was also tested. Considering that *S. aureus* SH1000-WT is predominantly a laboratory strain, the most common community-associated MRSA clinical isolate USA300, has increased pathogenicity and potentially altered mechanism of cell entry (Kennedy et al., 2008; Strobel et al., 2016). Neutrophils were incubated for 1 hour with JC1 isotype control or anti-CD151 antibodies at 10µg/ml, followed by a 2 hour incubation with JE2 and WT strains of HK *S. aureus* at MOI10. Interaction of both strains of *S. aureus* with neutrophils was significantly inhibited in the presence of anti-CD151 antibodies (Figure 4.6). This indicates that CD151 is not restricted to a single strain of *S. aureus*.

4.2.7. Anti-CD151 antibodies inhibit interaction of live *S. aureus* with neutrophils

So far, only heat inactivated strains of *S. aureus* were used. However, heat inactivation of bacteria can change the structural proteins and lipoproteins on the surface of the cell and therefore may alter rates of phagocytosis. In addition, the bacteria is no longer able to secrete factors that may ordinarily prevent engulfment (Ingmer and Brondsted, 2009;

Kumar, 2015). As a result, to elucidate further the role of CD151 in *S. aureus* interaction with neutrophils, the cells were incubated for 1 hour with anti-CD151 antibodies and JC1 isotype control (10µg/ml) followed by a 45 minute incubation with live *S. aureus* at MOI5. Interaction of neutrophils with live *S. aureus* was significantly inhibited with anti-CD151 antibodies (Figure 4.7) when compared to JC1 control.

4.2.8. Anti-CD151 antibodies do not inhibit interaction of HK *S. pneumoniae* with neutrophils at any MOI tested or any incubation time points used

Neutrophils are equipped to protect a wide variety of respiratory pathogens that are often associated with pneumoniae and cause serious and often fatal exacerbations in people suffering from chronic inflammatory disease (Craig et al., 2009; Eldika and Sethi, 2006). Consequently, CD151 involvement in neutrophil interaction with another Gram positive respiratory pathogens, *S. pneumoniae,* was investigated. Neutrophils were incubated for 1 hour with JC1 isotype control or anti-CD151 antibody at 10µg/ml followed by a 2 hour incubation with opsonised HK *S. pneumoniae* at MOI 5, 10 or 20 (Figure 4.8A-C) or by a 30, 60, 120 or 180 minute incubation with opsonised HK *S. pneumoniae* at MOI10 (Figure 4.8D). Light microscopy shows a similar *S. pneumoniae* load in JC1 isotype treated neutrophils (Figure 4.8A) and anti-CD151 antibody treated cells (Figure 4.8B). A zoomed in photomicrograph shows *S. pneumoniae* as purple rods arranged in a chain structure inside clear phagolysosomes. Neutrophil interaction with HK *S. pneumoniae* was not significantly different in the presence of anti-CD151 antibodies compared to JC1 control at any of the MOI used (Figure 4.8C) or after any of the incubation time point employed (Figure 4.8D).

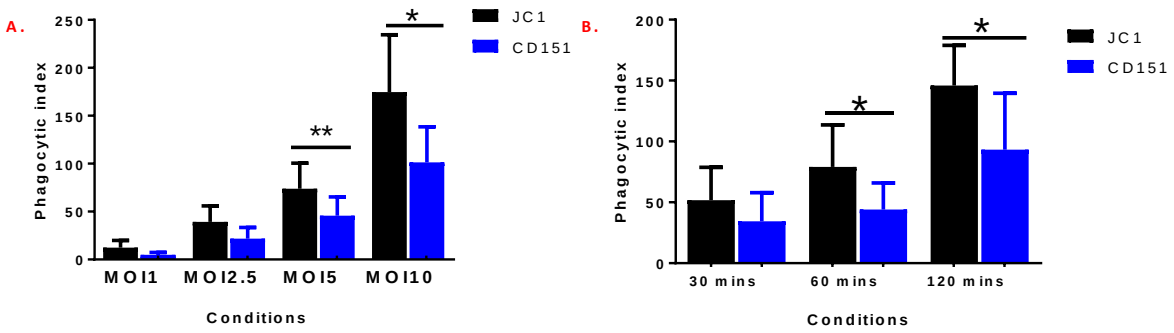


Figure 4.5: Anti-CD151 antibodies significantly inhibit neutrophil interaction with HK *S. aureus* at MOI5 and 10 and at 60 and 120 minutes post incubation.

Freshly isolated neutrophils from venous blood of health volunteers via plasma/Percoll centrifugation were incubated for 1 hour with JC1 isotype control or anti-CD151 antibodies at 10µg/ml (Figure 4.5 A, B) followed by a 2 hour incubation with HK *S. aureus* at MOI 1, 2.5, 5 or 10 (Figure 4.5 A) or by a 30, 60 or 120 minutes (mins) incubation with HK *S. aureus* at MOI 10 (Figure 4.5 B). Neutrophil interaction with *S. aureus* was assessed by light microscopy. Data from 5 (A) or 4 (B) independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures (*p<0.5, **p<0.01).

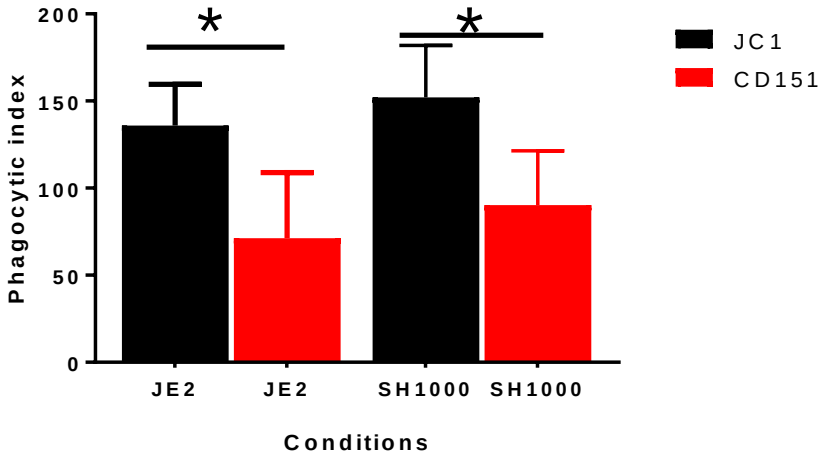


Figure 4.6: Anti-CD151 antibodies inhibit neutrophil interaction with JE2 and SH1000 (WT) strains of HK *S. aureus*.

Plasma/Percoll-purified neutrophils were incubated for 1 hour with JC1 isotype control or anti-CD151 antibodies at 10µg/ml, followed by a 2 hour incubation with JE2 or SH1000 (WT) strains of HK *S. aureus* at MOI10. Neutrophil interaction with both *S. aureus* strains was assessed using light microscopy. Data from 4 different donors is expressed as mean±SD (one-way ANOVA with Bonferroni's repeated measures post-test (*$p<0.05$)).

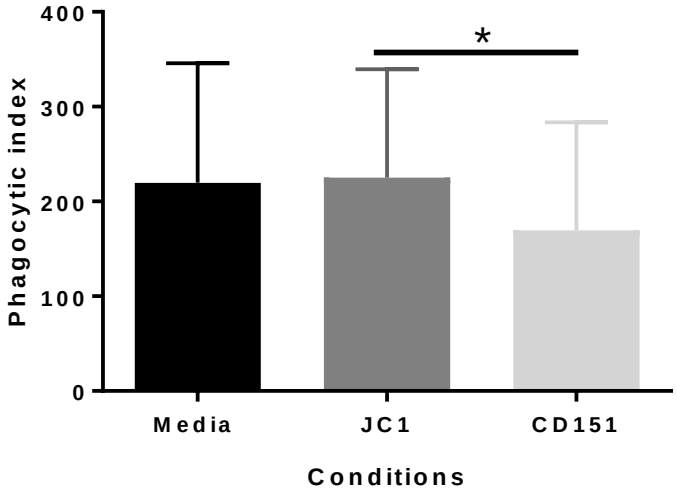


Figure 4.7: Anti-CD151 antibodies inhibit neutrophil interaction with live *S. aureus*.

Freshly isolated neutrophils from venous blood of health volunteers via plasma/Percoll-centrifugation were incubated for 1 hour with media, anti-CD151 antibodies and JC1 isotype control (10µg/ml) followed by a 45 minute incubation with live *S. aureus* at MOI 5. Neutrophil interaction with *S. aureus* was assessed by light microscopy. Data from 11 independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures (*$p<0.5$).

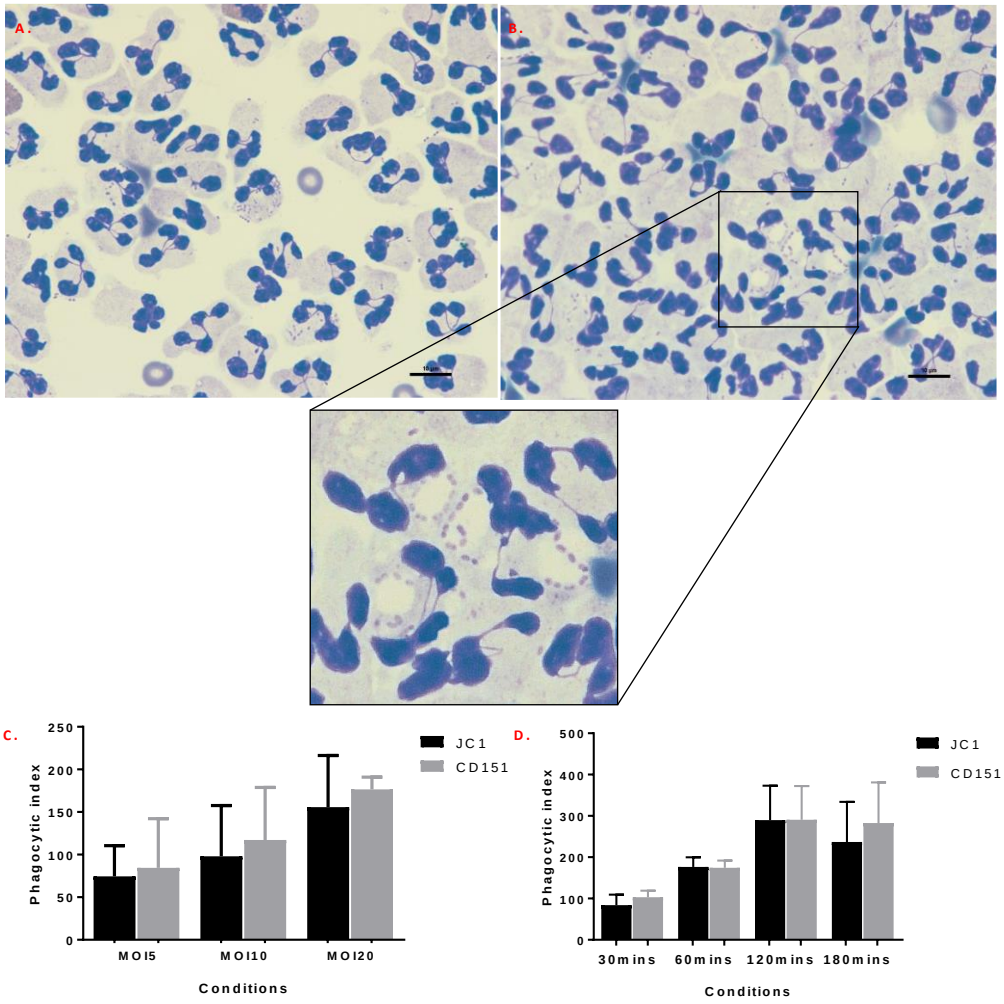


Figure 4.8: The effect of anti-CD151 antibodies on neutrophil interaction with different MOI of HK *S. pneumoniae* and after different incubation times.

Freshly isolated neutrophils from venous blood of health volunteers via plasma/Percoll centrifugation were incubated for 1 hour with JC1 isotype control or anti-CD151 antibodies at 10µg/ml (A-D) followed by a 2 hour incubation with opsonised HK *S. pneumoniae* at MOI5, 10 or 20 (A-C) or by a 30, 60, 120 or 180 mins incubation with opsonised HK *S. pneumoniae* at MOI 10 (D). Neutrophil interaction with *S. pneumoniae* was assessed by light microscopy. Photomicrographs of neutrophil interaction with HK *S. pneumoniae* at MOI10 in the presence of JC1 isotype control (A) and anti-CD151 antibodies (B). Enlarged area depicting HK *S. pneumoniae* in the clear phagolysosomes (B). C, D) Data from 3 independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures.

4.2.9. Anti-CD151 antibodies do not inhibit neutrophil interaction with live *S. pneumoniae*

As stated above, bacterial heat inactivation can damage cellular structures, resulting in altered neutrophil phagocytosis. To elucidate further the role of CD151 in neutrophil interaction with *S. pneumoniae,* live bacteria was used. Neutrophils were incubated for 1 hour with media, anti-CD151 antibodies (10µg/ml) and JC1 isotype control (10µg/ml). Opsonised live *S. pneumoniae* MOI10 was added for a further 1 hour. Neutrophil interaction with live *S. pneumoniae* was not significantly inhibited with anti-CD151 antibodies (Figure 4.9), compared to the control, confirming further that CD151 does not play a role in neutrophil interaction with *S. pneumoniae*.

4.2.10. Anti-CD151 antibodies have no impact on *S. aureus*-induced cell death

S. aureus can result in rapid neutrophil lysis following phagocytosis via the production of cell lysis factors such as staphylococcal α-phenol soluble modulins, γ-hemolysin and panton valentine leukocidin (PVL) (Cribier et al., 1992; Kobayashi et al., 2010; Konig et al., 1997; Surewaard et al., 2013; Voyich et al., 2005). Considering that anti-CD151 antibodies inhibit *S. aureus* phagocytosis by neutrophils, I hypothesise that the antibodies will also reduce the *S.-aureus*-induced cell death. ToPro-3 is a vital dye that intercalates between double stranded DNA when cells become permeable, making it a good tool to investigate neutrophil lysis (Babiychuk et al., 2008; Cantinieaux et al., 2004; Solito et al., 1998; Vanhooijdonk et al., 1994). Neutrophils were incubated for 1 hour with media, JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml) followed by a 2.5 hour incubation with live *S. aureus* at MOI5. Neutrophils were then stained with ToPro-3 and analysed by flow cytometry. FSC/SSC dot plots allowed gating of cells from debris (Figure 4.10A). RL1-A histogram showed a total right shift in ToPro-3 positivity. Lysed cells represent the positive (+ve) control while unstained and stained media incubated neutrophils represent the negative controls. Co-incubation of *S. aureus* with media, JC1 isotype and anti-CD151 antibodies indicated a moderate increase of ToPro-3^{+}cells (Figure 4.10B). % ToPro-3^{+} cell calculation indicated no difference in cell lysis between the media, JC1 and anti-CD151 antibodies incubated neutrophils with *S. aureus* (Figure 4.10C). The results indicate similar neutrophil cell lysis in both anti-CD151 antibody incubated cells and controls.

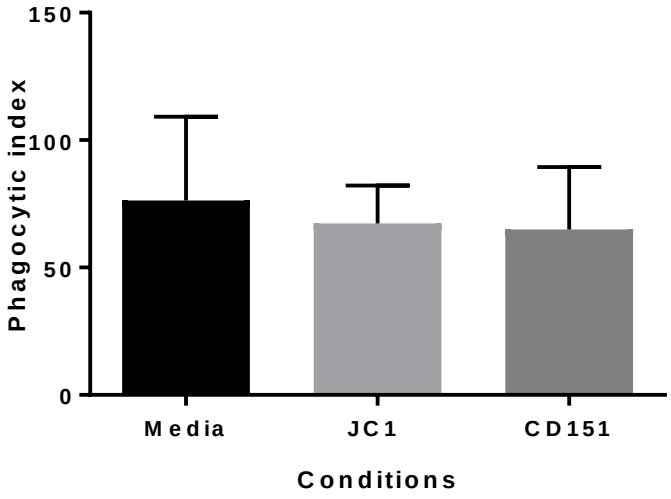


Figure 4.9: Effect of anti-CD151 antibodies on neutrophil interaction with live *S. pneumoniae*.

Freshly isolated neutrophils from venous blood of health volunteers via plasma/Percoll centrifugation were incubated for 1 hour with media, JC1 isotype control or anti-CD151 antibodies (10µg/ml) followed by a 1 hour incubation with opsonised live *S. pneumoniae* MOI 10. Neutrophil interaction with *S. pneumoniae* was assessed by light microscopy. Data from 3 independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures.

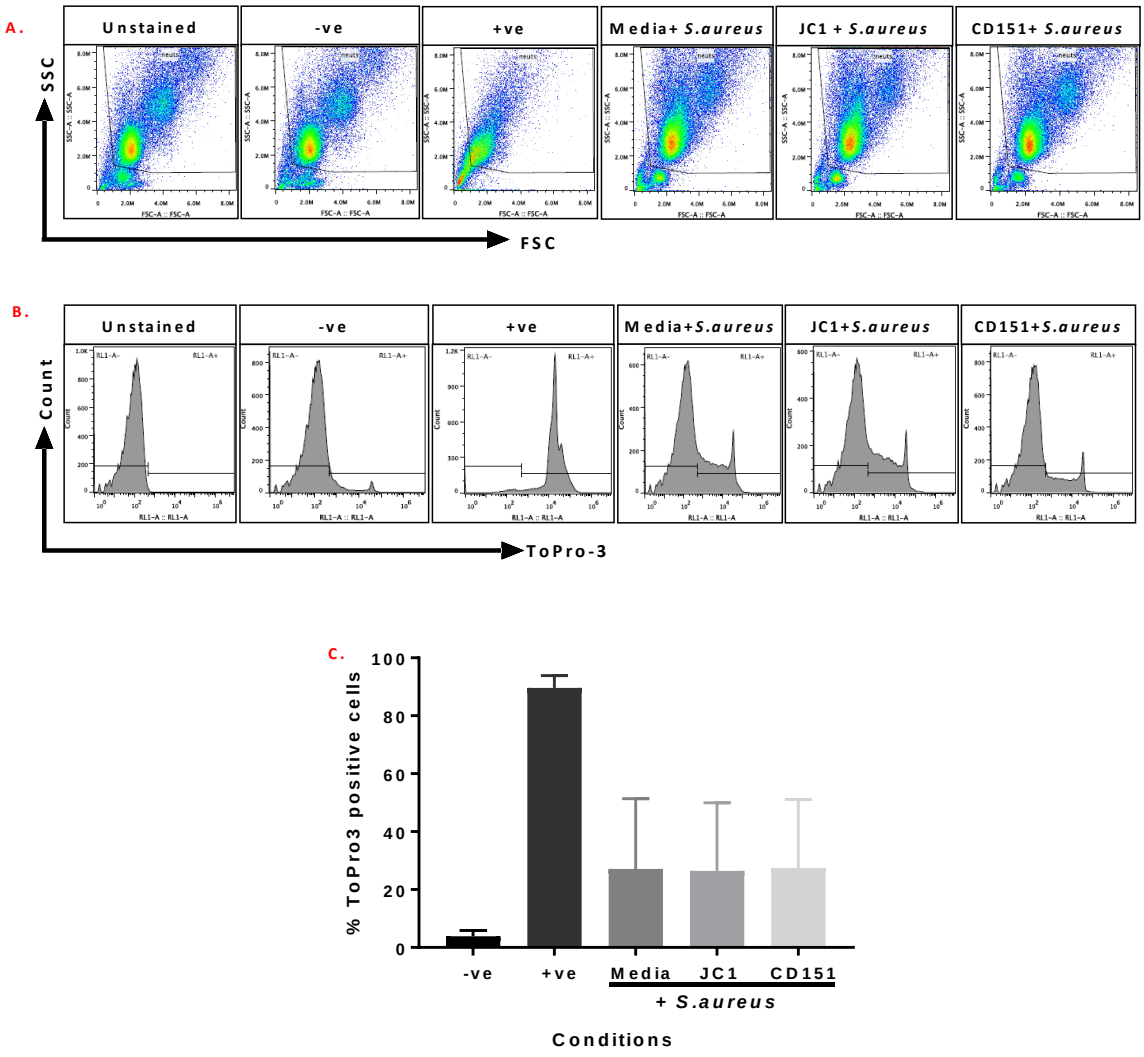


Figure 4.10: Effect of anti-CD151 antibodies on *S. aureus*-induced cell death.
Plasma/Percoll isolated neutrophils from venous blood of healthy volunteers were incubated for 1 hour with media, JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml) followed by a 2.5 hours incubation with live *S. aureus* at MOI5. Permeability was assessed by ToPro-3 staining. Unstained, media stained and lysed cells are representative of negative and positive (+ve) controls. A.) FSC/SSC dot plot of neutrophil separation from debris. B) RL1-A histogram representative of ToPro-3^{+} fluorescence, obtained using Attune flow cytometer. C) % ToPro-3 positive cells quantification of neutrophils obtained under different conditions. Data expressed from 4 independent donors as mean±SD.

4.2.11. Trypan Blue quenches *S. aureus* (WT chr-GFP) GFP fluorescence with increased MOI

There is no knowledge if anti-CD151 antibodies inhibit *S. aureus* adhesion or internalisation by neutrophils. To test this, a biochemical method can be used to test between the two categories. Such a method uses trypan blue (TB) to quench FITC or GFP fluorescence of extracellular bacteria (Nuutila and Lilius, 2005). To probe the suitability of this method to distinguish between intracellular and extracellular bacteria in our experiments, neutrophils were incubated with live *S. aureus* (WT chr-GFP) at MOI 5, 10 or 20 for 45 minutes. To determine the right amount of TB required and TB incubation time, cells were incubated with TB at either 1:4 (0.1% TB) or 1:2 (0.2% TB) dilutions for 1 minute or 15 minutes. FSC/SSC dot plots show cell separation from debris (Figure 4.11 A-C). FL1-H histograms right shift show gain of *S. aureus* (WT.chr-GFP) fluorescence (Figure 4.11 D-F). TB quenching of GFP fluorescence is seen as a decrease in the fluorescence intensity peak and a left shift on the FL1-H axis. Little FL1-H left fluorescence shift with an MOI5 of *S. aureus* (WT.chr-GFP) was seen when using the two TB dilutions and incubation times (Figure 4.11D). By contrast, when an MOI10 of *S. aureus* (WT.chr-GFP) (Figure 4.11E) or MOI20 (Figure 4.11F) was used, a noticeable left shift fluorescence change is seen. Neutrophil incubation with *S. aureus* (WT.chr-GFP) MOI20 and MOI10 showed the most profound fluorescence quenching when TB was used at 1:2 dilution for 15 minutes. For this reason, in future experiments TB was used at 1:2 dilution for 15 minutes.

4.2.12. TB fluorescence quenching technique cannot be used to accurately differentiate between internalised and surface attached *S. aureus* (WT.chr-GFP) when neutrophils are incubated with anti-CD151 antibodies

As previously described, *S. aureus* neutrophil incubation can result in spontaneous cell lysis following phagocytosis. If this were to happen when neutrophils were incubated *S. aureus* (WT.chr-GFP), TB would gain entry in the cells and quench internal bacteria fluorescence leading to skewed results. To account for this leakage, ToPro-3 was used as a marker for cell lysis (Vanhooijdonk et al., 1994). Media control neutrophils were incubated for 1 hour at 37°C/ 5%CO_2 or 4°C while JC1 isotype control (10µg/ml) or anti-CD151 antibody (10µg/ml)

neutrophil incubation was done for 1 hour at 37°C/ 5%CO_2. Live *S. aureus* (WT chr-GFP) MOI10 was then added for a further 45 minutes to the cells. TB at 1:2 dilutions for 15 minutes and ToPro-3 (1:10,000) were added to neutrophils prior to the analysis. FSC/SSC dot plots show cell separation from debris (Figure 4.12A-E). FL1-H histogram indicates a left shift upon GFP fluorescence quench by TB (Figure 4.12F) while FL4-H histogram indicates a right shift upon ToPro-3^+ staining of lysed cells (Figure 4.12G). A 4°C neutrophil negative control was used, showing a peak in the FL1-H and FL4-H unstained region of the axis ($<10^1$). At 4°C neutrophils metabolic functions are reduced resulting in impaired phagocytosis (Magnusson and Holst, 1998). The FL1-H histogram shows a low, similar mean fluorescence intensity (MFI) reduction between equivalent anti-CD151 antibody and control conditions when TB is used (Figure 4.12F). Conversely, ToPro-3 positivity indicates a right FL4-H shift in all 37°C *S. aureus* (WT chr-GFP) incubated neutrophil conditions (Figure 4.12G). The results obtained therefore indicate that *S. aureus* (WT chr-GFP) induces cell lysis in all of the conditions used, making the use of TB fluorescent quenching technique unsuitable to distinguish between neutrophils internalised and surface-bound bacteria in our experimental model.

4.2.13. Optimisation of HK *S. aureus* staining with Alexa Fluor 647 and pHrodo dyes

Light microscopy is considered a gold standard technique to count bacteria that interacts with neutrophils, however phagocytosed and adherent bacteria cannot be distinguished due to sample viewing from one focal point. *In vivo* work conducted using a zebrafish model showed that by co-conjugating *S. aureus* with pHrodo and Alexa Fluor 647, phagocytosed and neutrophil surface adherent *S. aureus* can distinguish and quantified, making it a suitable technique to understand better the role of CD151 in *S. aureus* interaction with neutrophils (Ellett et al., 2015). PHrodo is a pH sensitive dye that only fluoresces in an acidic, phagolysosomal environment (3-6 pH) while Alexa Fluor 647 is a highly stable dye, visible an acid- alkaline environment (Aziz et al., 2013; Ellett et al., 2015). Additionally, pHrodo (Ex -560nm, Em -585nm) and Alexa Fluor 647 (Ex -650nm, Em -665nm) dyes have well separated emission spectra, allowing their simultaneous usage.

To test this, neutrophils were initially incubated with HK *S. aureus* MOI10 pre-stained for either pHrodo or Alexa Fluor 647, following the published protocol of Ellett et al., 2015. The neutrophil cell membrane was then stained using PKH67 cell tracker. Cells were then cytocentrifuged and fixed on glass slides. Analysis of fluorescent images show that there appears to be a good staining of the HK *S. aureus* with both dyes (Figure 4.13). However bacterial numbers are low with no more than 10 pHrodo or Alexa Fluor 647 stained S. *aureus* per image. This indicates the possibility of bacterial loss during the staining process.

Thus, the bacteria loss was investigated to determine if it could be reduced by using 1% BSA or 1% FBS during the *S. aureus* dye washing steps by supplementing the washing buffers with either of the compounds (Bulard et al., 2012; Ellett et al., 2015). As seen from Figure 4.14, there is more Alexa Fluor 647 and pHrodo stained HK *S. aureus* when 1% BSA supplemented washing buffers were used when compared to the condition where 1% FBS supplemented washing buffers were used. This indicates that by using 1% BSA in the washing buffers bacteria yield is increased. As a result, all future *S. aureus* staining with pHrodo were conducted using 1% BSA in the washing buffers.

4.2.14. Alexa Fluor 647 stained HK *S. aureus* poor visualisation via fluorescent microscopy

Following the bacterial staining optimisation process, the effect of anti-CD151 antibody usage on neutrophil HK *S. aureus* internalisation and adhesion was investigated using *S. aureus* co-stained for Alexa Fluor 647 and pHrodo. Neutrophils were pre-incubated for 1h with JC1 control or anti-CD151 antibodies at 10µg/ml, followed by a 45 minute incubation with HK *S. aureus* MOI10 stained for both pHrodo (red) and Alexa Fluor 647 (green). The well separated emission spectra of the Alexa Fluor 647 and pHrodo dyes makes them suitable to be used in conjunction. Alexa Fluor 647 fluorescence will be seen on all bacteria regardless of them being phagocytosed or not while pHrodo will fluoresce only in an acidic, phagolysosomal environment. Co-staining with the two dies (yellow) would show phagocytosed bacteria while adherent/internalised only bacteria will be visualised via the Alexa Fluor 647 (green) specific channel. Fluorescent microscopy merge images showed that overall there are fewer HK *S. aureus* present in the presence of anti-CD151 antibodies when compared to JC1 control (Figure 4.15). However, there are visibly less Alexa Fluor 647

stained HK *S. aureus* when compared to the pHrodo stained bacteria (Figure 4.15). This indicates that probably the Alexa Fluor 647 staining of HK *S. aureus* is not uniformly distributed on all bacteria.

To test for this, unstained (Figure 4.16A) or stained bacteria for both pHrodo and Alexa Fluor 647 dyes (Figure 4.16B) were run through the LSRII flow machine. The Blue 610/20 histogram shows a small right shift in pHrodo^{+} fluorescence while the Red 660/20 histogram shows a prominent right shift in Alexa Fluor 647^{+} fluorescence, indicative of a uniform staining of the HK *S. aureus* cells with the Alexa Fluor 647 dye. This indicates the possibility of other factors being involved which do not allow visualisation of Alexa Fluor 647 stained bacterial cells using fluorescent microscopy. Due to this consideration, further fluorescent microscopy experiments were conducted using only the pHrodo stain.

4.2.15. Significant inhibition of neutrophil phagocytosis of live *S. aureus* but not of HK *S. aureus* in the presence of anti-CD151 antibodies, as shown by fluorescent microscopy

PHrodo stained *S. aureus* was used to probe if there is less phagocytosed *S. aureus* in the presence of anti-CD151 antibody when compared to JC1 control. Freshly isolated neutrophils were incubated for 1 hour with media, JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml), followed by a 30 minutes incubation with pHrodo pre-stained live *S. aureus* MOI5 (Figure 4.17 and Figure 4.19A) or by by a 45 minute incubation with pHrodo pre-stained HK *S. aureus* MOI10 (Figure 4.18 and Figure 4.19B). The neutrophil cell membrane was then stained with PKH67 cell tracker dye. Fluorescent microscopy images showed that in the presence of anti-CD151 antibodies there appears to be less pHrodo stained live *S. aureus* in neutrophils compared to the controls (Figure 4.17) but similar pHrodo stained HK *S. aureus* levels in neutrophils from the controls or incubated with anti-CD151 antibodies (Figure 4.18). Quantification of the images, using Fiji program with an algorithm designed by Dr. Darren Robinson, showed similar results with significant less pHrodo stained live *S. aureus* with anti-CD151 antibodies compared to the controls (Figure 4.19A) but with visible but not significant difference in HK *S. aureus* levels between anti-CD151 antibody incubated neutrophils and JC1 control (Figure 4.19B).

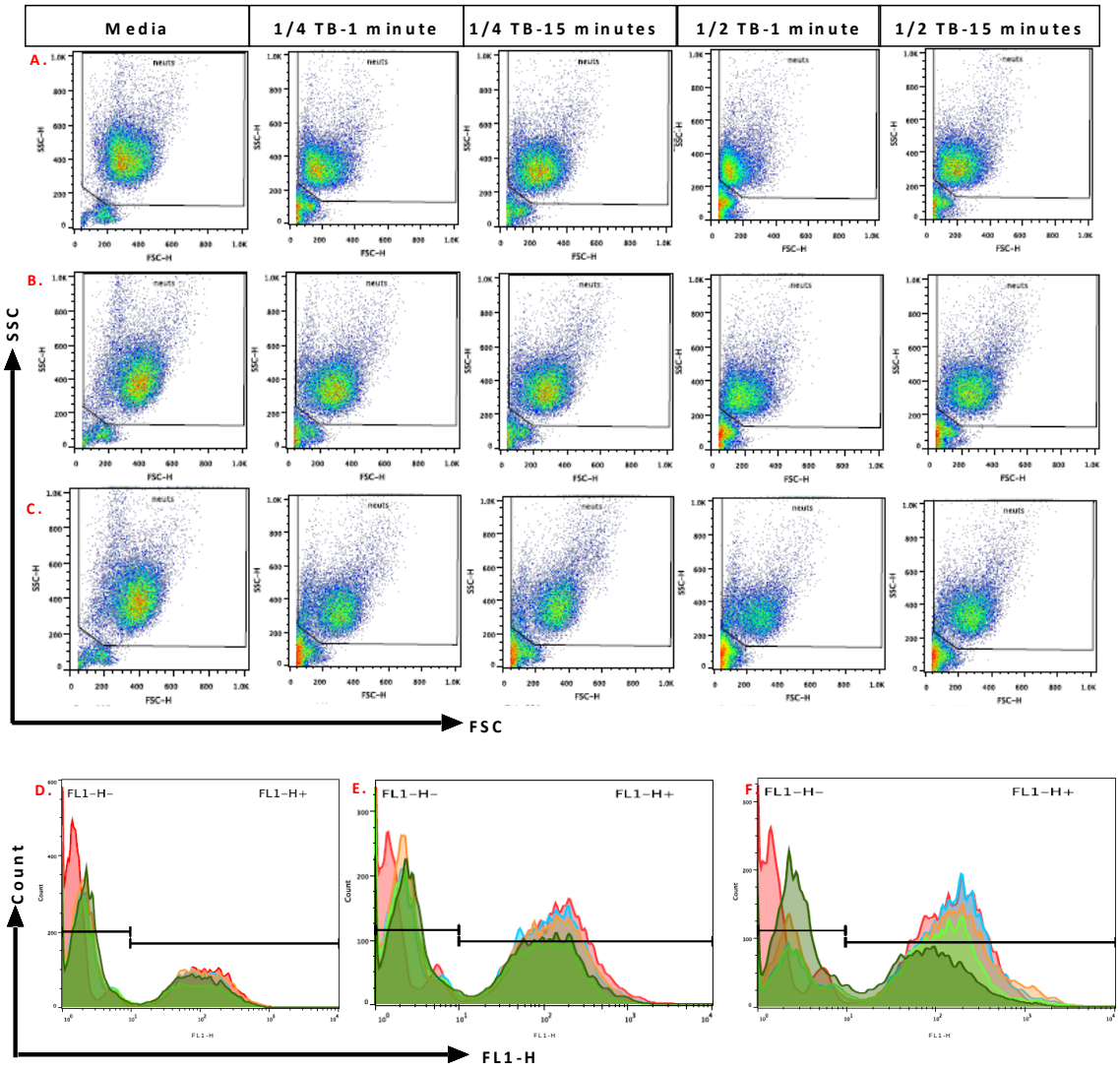


Figure 4.11: Effect of TB fluorescence quenching on different MOI of live *S. aureus* (WT chr-GFP).

Plasma/Percoll isolated neutrophils from venous blood of healthy volunteers were incubated with live *S. aureus* (WT chr-GFP) at MOI 5, 10 or 20 for 45 minutes. The cells were then incubated with TB at either 1/4 or 1/2 dilutions for 1 minute or 15 minutes. A-C) FSC/SSC dot plot of neutrophils incubated with MOI5 (A), MOI10 (B) or MOI20 (C) of live *S. aureus* (WT chr-GFP), separated from debris. D-F) FL1-H histograms of neutrophils incubated with MOI5 (D), MOI10 (E) or MOI20 (F) of *S. aureus* (WT chr-GFP), representing GFP^+ FL1-H right shift in fluorescence: red- neutrophil+ *S. aureus* (WT chr-GFP), blue- neutrophil+ *S. aureus* (WT chr-GFP)+ 1/4TB for 1 minute, orange- neutrophil+ *S. aureus* (WT

chr-GFP)+1/4 TB for 15 minutes, light green- neutrophil+ *S. aureus* (WT chr-GFP)+1/2 TB for 1 minute, dark green- neutrophil+ *S. aureus* (WT chr-GFP)+1/2 TB for 15 minutes. Black segmented line delimitation between unstained and stained peaks. Data obtained from single experiment.

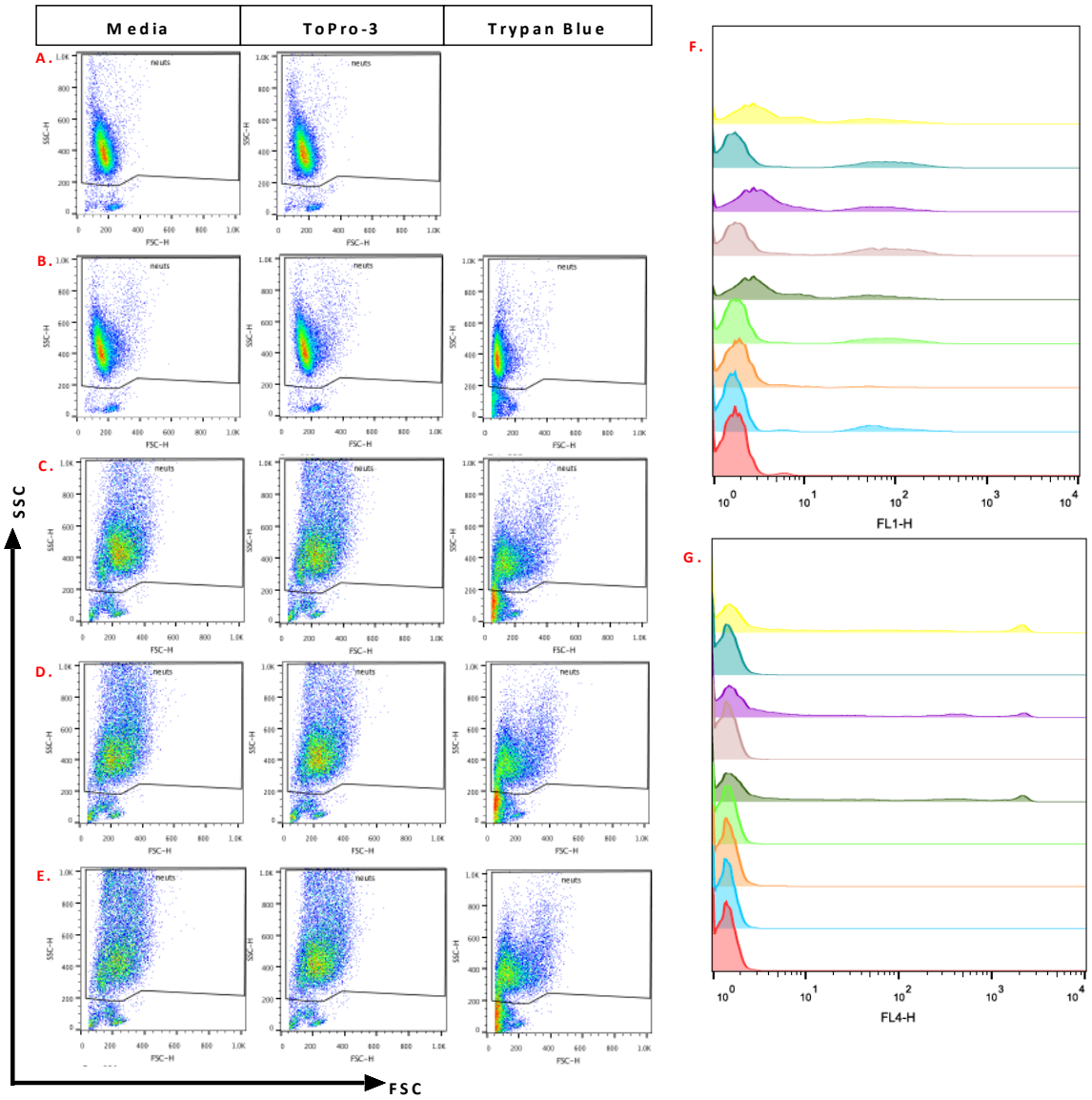


Figure 4.12: Anti-CD151 antibodies effect on *S. aureus* (WT chr-GFP) fluorescence quenching by TB.

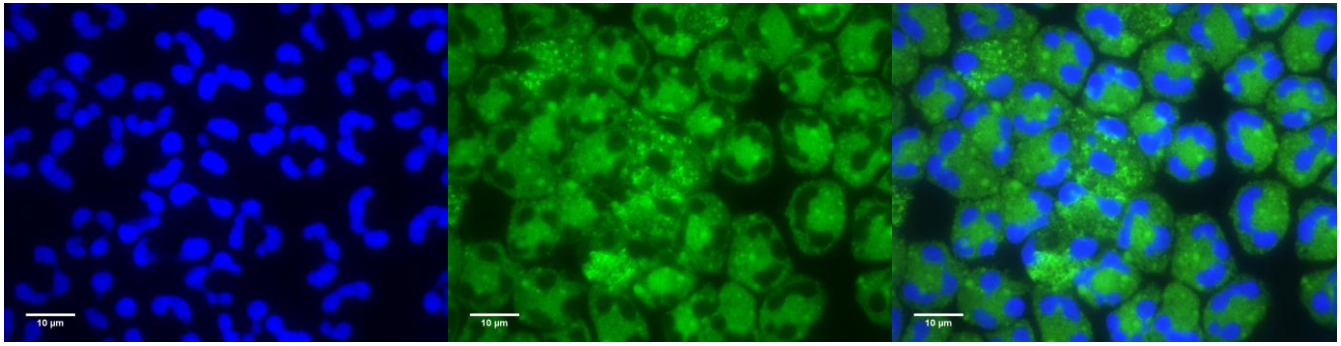
Plasma/Percoll isolated neutrophils from venous blood of healthy volunteers were incubated for 1 hour with media at 37°C/ 5%CO_2 or 4°C or with JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml) at 37°C/ 5%CO_2. Live *S. aureus* (WT chr-GFP) at MOI10 was then added to the cells for a further 45 minutes. Cells were stained with TB at 1/2 dilutions for 15 minutes and ToPro-3 just before analysis. A-E) FSC/SSC dot plots of: neutrophils only (A), neutrophil+ *S. aureus* (WT chr-GFP) 4°C (B) neutrophil+ *S. aureus* (WT chr-GFP) 37°C/ 5%CO_2 (C), neutrophil+ *S. aureus* (WT chr-GFP) + JC1 37°C/ 5%CO_2 (D),

neutrophil+ *S. aureus* (WT chr-GFP)+anti-CD151 antibodies 37°C/ 5%CO_2 (E). F-G) FL1-H and FL4-H histogram representative of right shift on GFP^+ FL1-H fluorescence (F) or ToPro-3^+ FL4-H fluorescence (G) of neutrophils incubated with *S. aureus* (WT chr-GFP):

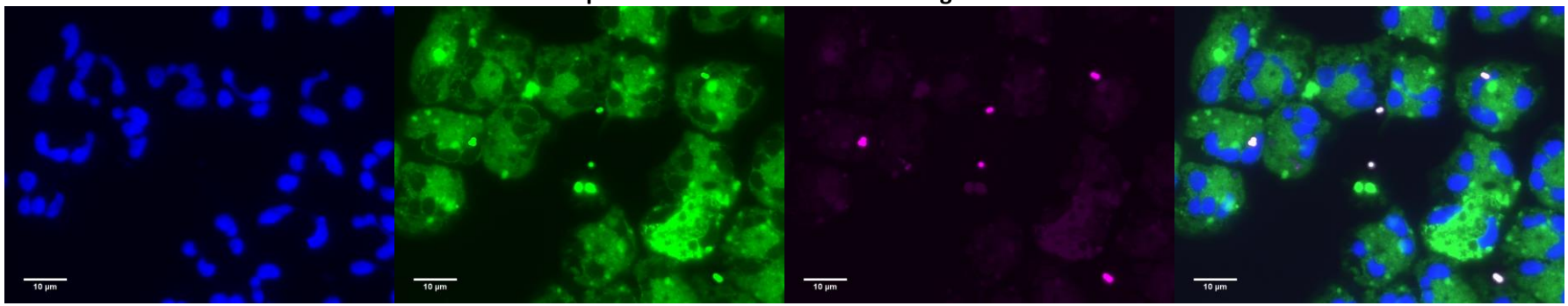
F) red- neutrophils , light blue- neutrophil+*S. aureus* (WT chr-GFP) 4°C, orange- neutrophil+ *S. aureus* (WT chr-GFP) 4°C + TB, light green- neutrophil+ *S. aureus* (WT chr-GFP) 37°C/ 5%CO_2, dark green- neutrophil+ *S. aureus* (WT chr-GFP) 37°C/ 5%CO_2+ TB, light purple- neutrophil+ *S. aureus* (WT chr-GFP) + JC1 37°C/ 5%CO_2, dark purple- neutrophil+ *S. aureus* (WT chr-GFP) + JC1 37°C/ 5%CO_2+ TB, dark blue- neutrophil+ *S. aureus* (WT chr-GFP) +anti-CD151 antibodies 37°C/ 5%CO_2, yellow- neutrophil+ *S. aureus* (WT chr-GFP) +anti-CD151 antibodies 37°C/ 5%CO_2+ TB.

G)red- neutrophils + ToPro-3, light blue- neutrophil+*S. aureus* (WT chr-GFP) 4°C, orange- neutrophil+ *S. aureus* (WT chr-GFP) 4°C + ToPro-3, light green- neutrophil+ *S. aureus* (WT chr-GFP) 37°C/ 5%CO_2, dark green- neutrophil+ *S. aureus* (WT chr-GFP) 37°C/ 5%CO_2+ ToPro-3, light purple- neutrophil+ *S. aureus* (WT chr-GFP) + JC1 37°C/ 5%CO_2, dark purple- neutrophil+ *S. aureus* (WT chr-GFP) + JC1 37°C/ 5%CO_2+ ToPro-3, dark blue- neutrophil+ *S. aureus* (WT chr-GFP) +anti-CD151 antibodies 37°C/ 5%CO_2, yellow- neutrophil+ *S. aureus* (WT chr-GFP) +anti-CD151 antibodies 37°C/ 5%CO_2+ ToPro-3.

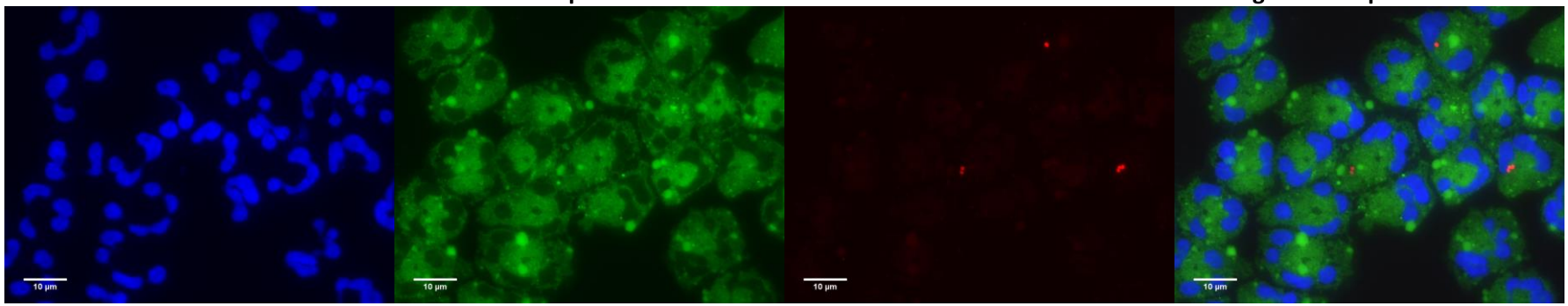
Data obtained from single experiment.



DAPI-nucleus FITC-neutrophil membrane Merge-Media



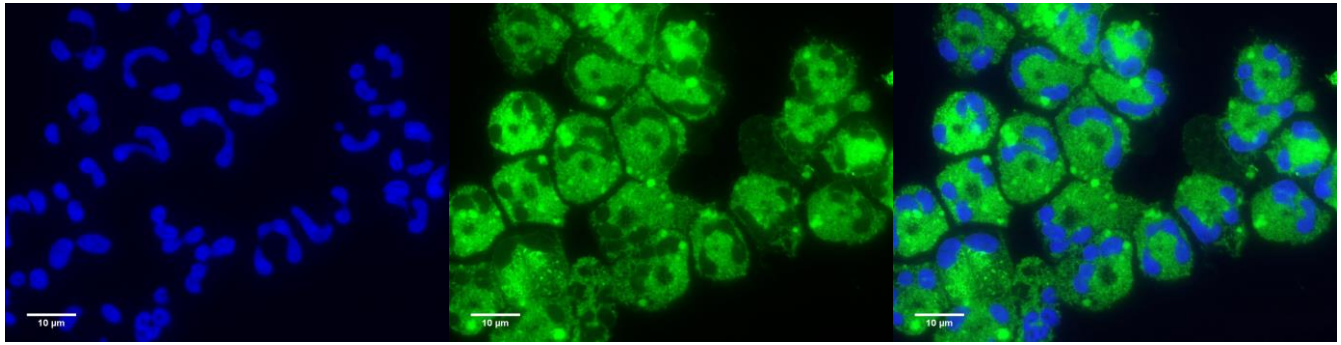
DAPI-nucleus FITC-neutrophil membrane Alexa Fluor 647-HK *S. aureus* Merge- Neutrophil+ HK *S.aureus*



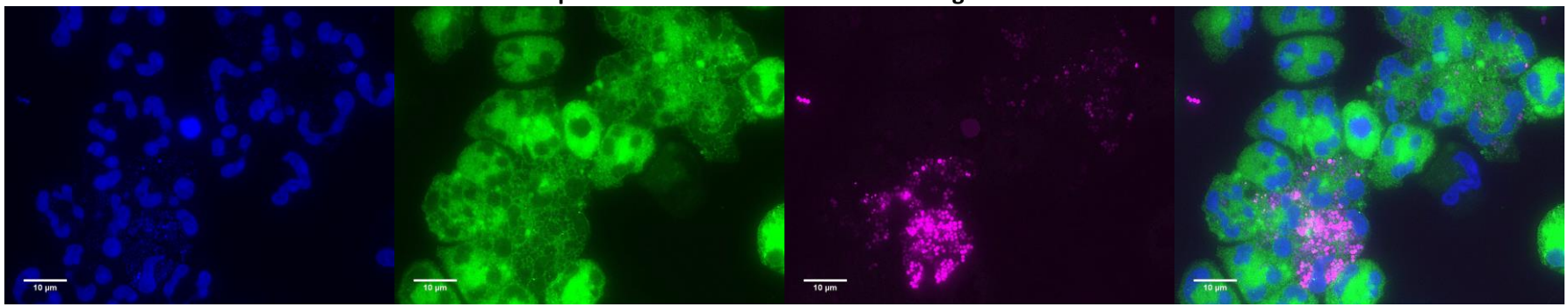
DAPI-nucleus FITC-neutrophil membrane pHrodo-HK *S. aureus* Merge- Neutrophil+ HK *S. aureus*

Figure 4.13: Visualisation of neutrophils incubated with HK *S. aureus* stained with Alexa Fluor 647 and pHrodo

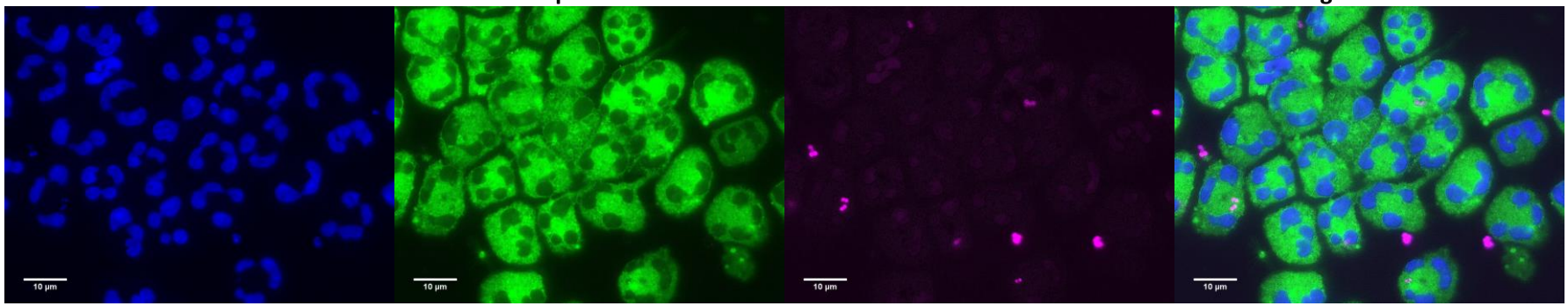
Freshly isolated neutrophils were incubated for 45 minutes with Alexa Fluor 647 or pHrodo stained HK *S. aureus* MOI10 followed by neutrophil membrane staining with PKH67 cell tracker. The cells were then fixed on glass slides using 4% paraformaldehyde and nucleus stained for DAPI. The slides were then imaged using Olympus upright epifluorescence system with 100x oil immersion. Photomicrographs of infected and non-infected cells: Nucleus- blue; neutrophil membrane- green; Alexa Fluor 647 HK *S. aureus* - magenta; red pHrodo HK *S. aureus* -red. Data obtained from single experiment. Images were adjusted to reduce blurriness and enhance contrast using Fiji program.



DAPI-nucleus FITC-neutrophil membrane Merge- Media



DAPI-nucleus FITC-neutrophil membrane Alexa Fluor 647-HK *S. aureus* Merge- 1%BSA



DAPI-nucleus FITC-neutrophil membrane Alexa Fluor 647-HK *S. aureus* Merge- 1% FBS

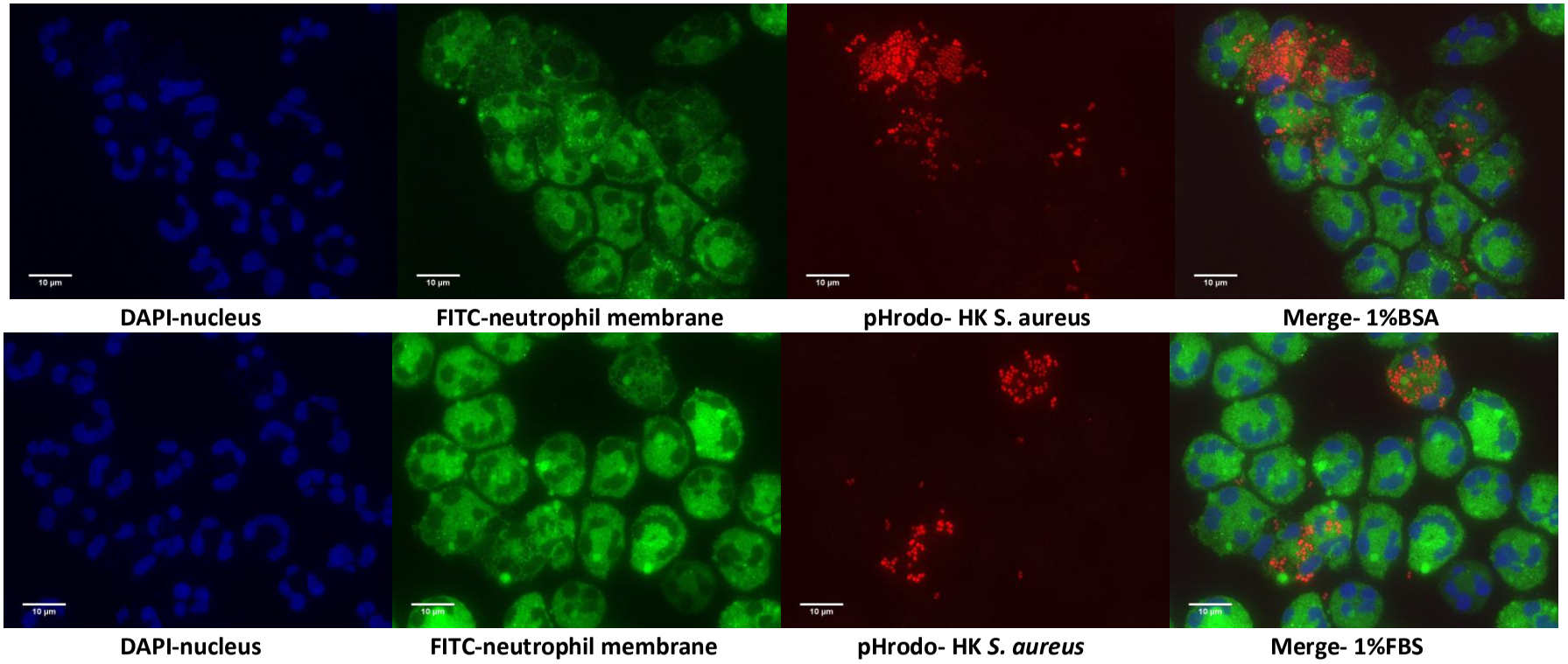


Figure 4.14: Loss of HK *S. aureus* during Alexa Fluor 64 and pHrodo staining when using 1%BSA or FBS during the washing process.

HK *S. aureus* staining with either pHrodo or Alexa Fluor 647 was done using neat washing buffers or containing either 1%FBS or 1%BSA. Freshly isolated neutrophils were incubated for 45 minutes with Alexa Fluor 647 or pHrodo pre-stained HK *S. aureus* MOI10. Neutrophil cell membrane was stained for PKH67 followed by cell fixing on glass slides using 4% paraformaldehyde and nucleus staining for DAPI. The slides were then imaged using Olympus upright epifluorescence system with 100x oil immersion. Photomicrographs of infected and non-infected cells: nucleus- blue; neutrophil membrane- green; Alexa Fluor 647 HK *S. aureus* - magenta; pHrodo HK *S. aureus* -red. Data obtained from single experiment. Images were adjusted to reduce blurriness and enhance contrast using Fiji program.

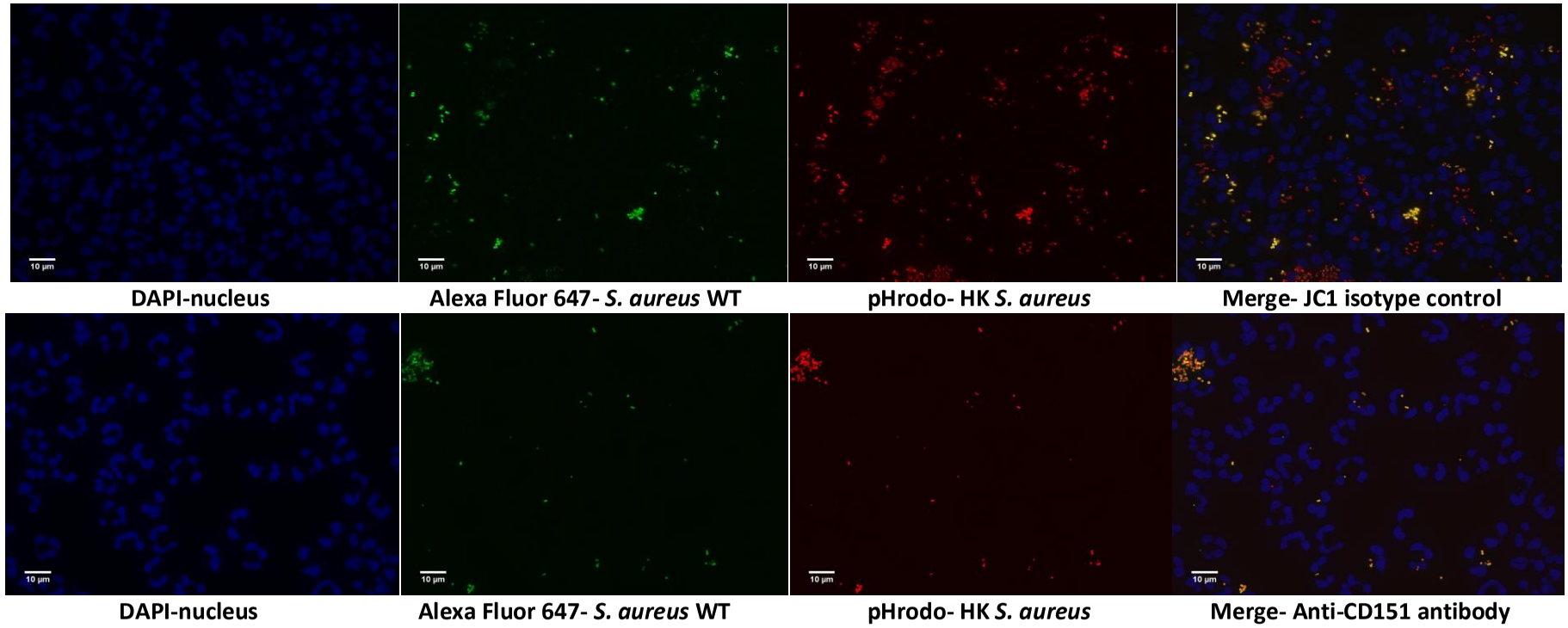


Figure 4.15: Co-localization of pHrodo and Alexa Fluor 647 stained HK *S. aureus*.

HK *S. aureus* was co-stained with pHrodo and Alexa Fluor 647 using washing buffers containing 1%BSA. Freshly isolated neutrophils were pre-incubated for 1h with JC1 control or anti-CD151 antibodies at 10µg/ml followed by a 45 minute incubation with Alexa Fluor 647 and pHrodo stained HK *S. aureus* MOI10. The cells were then fixed on glass slides using 4% paraformaldehyde and nucleus staining for DAPI. The slides were imaged using Olympus upright epifluorescence system with 60x oil immersion. Photomicrographs of infected cells: nucleus- blue; Alexa Fluor 647 HK *S. aureus* WT- green; pHrodo HK *S. aureus* -red. Co-localization of the two dyes is seen as a yellow dot. Data obtained from single experiment. Images were adjusted to reduce blurriness and enhance contrast.

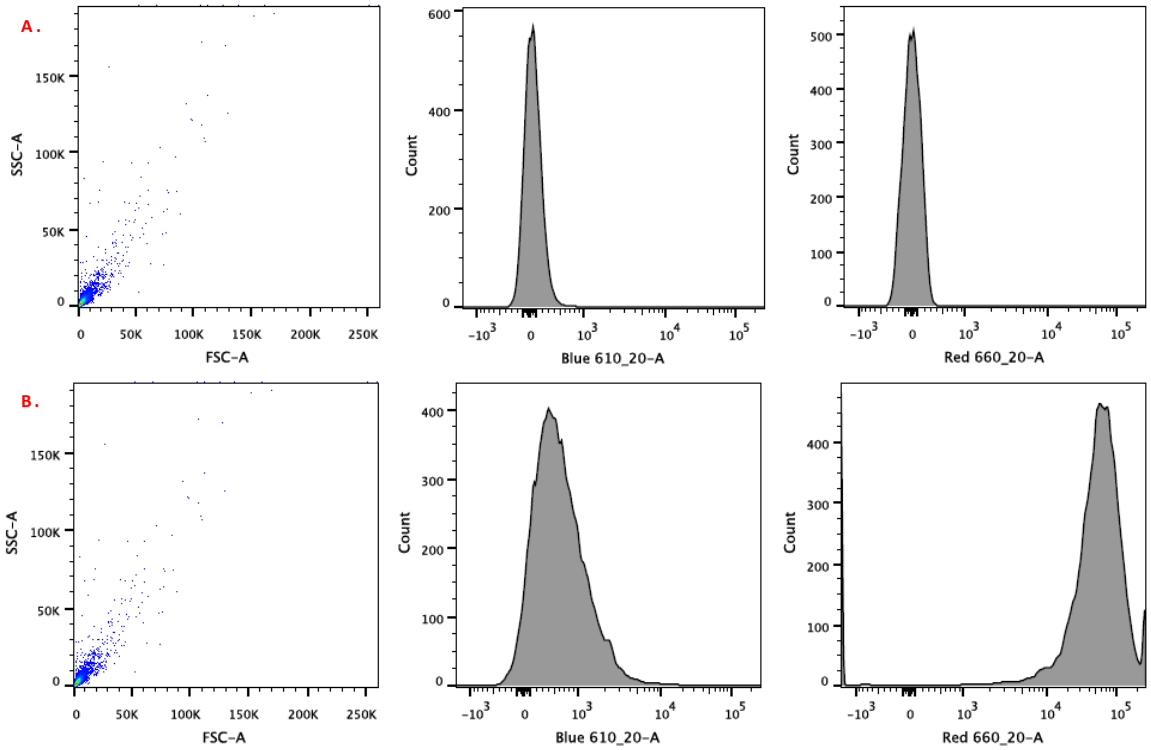
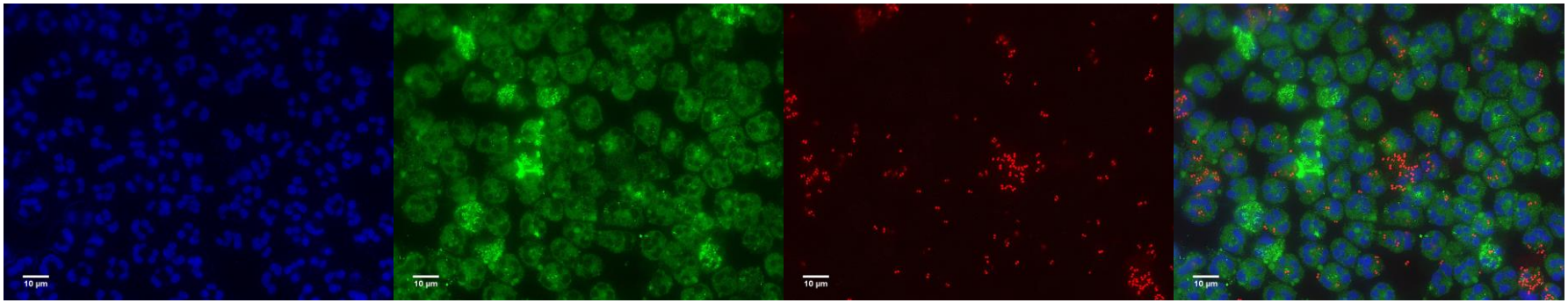
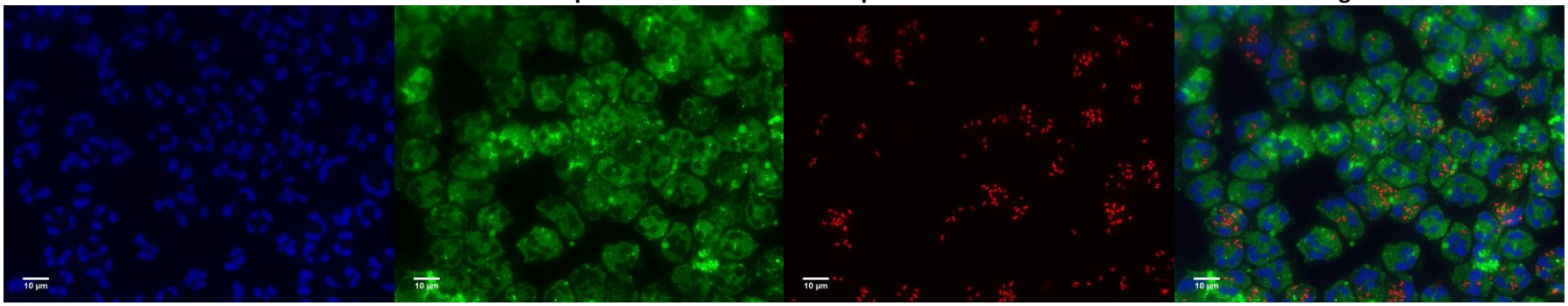


Figure 4.16: HK *S. aureus* staining with Alexa Fluor 647 and pHrodo.

Unstained (A) or co-stained HK *S. aureus* for Alexa Fluor647 and red pHrodo (B) were analysed using the LSRII flow cytometer. FSC/SSC dot plot of HK *S. aureus* log scale. Blue 610/20-A histogram is indicative of a right shift in pHrodo^{+} cells while the Red660/20-A histogram indicates a right shift in Alexa Fluor 647^{+} cells. Data obtained from single experiment.


DAPI-nucleus
FITC- neutrophil membrane
pHrodo- live S. aureus
Merge- Media


DAPI-nucleus
FITC- neutrophil membrane
pHrodo-live S. aureus
Merge- JC1 isotype control

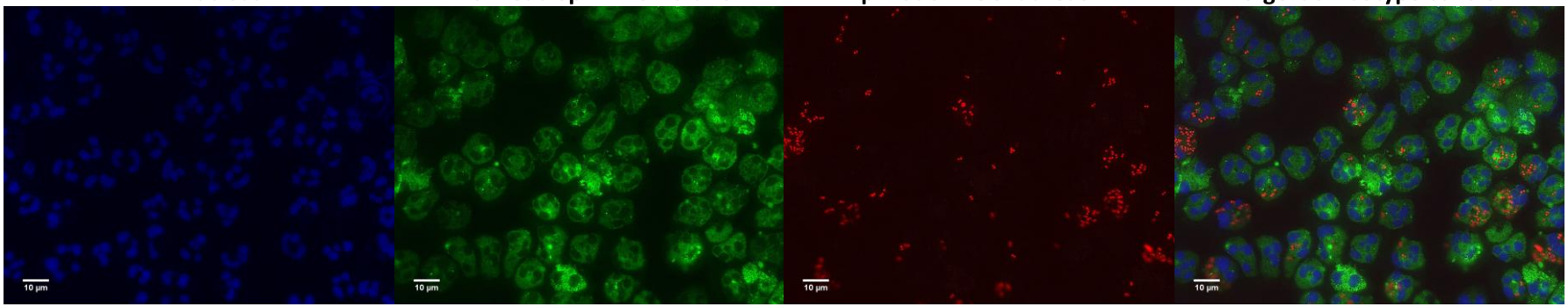
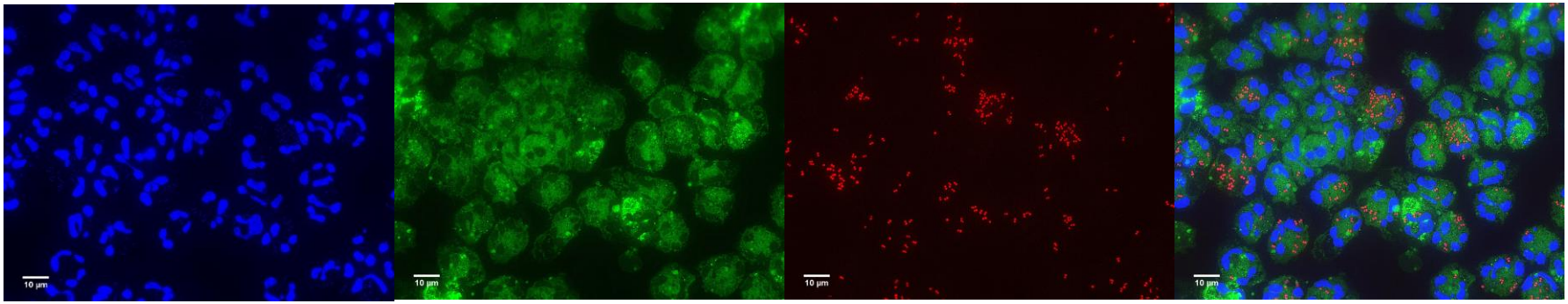
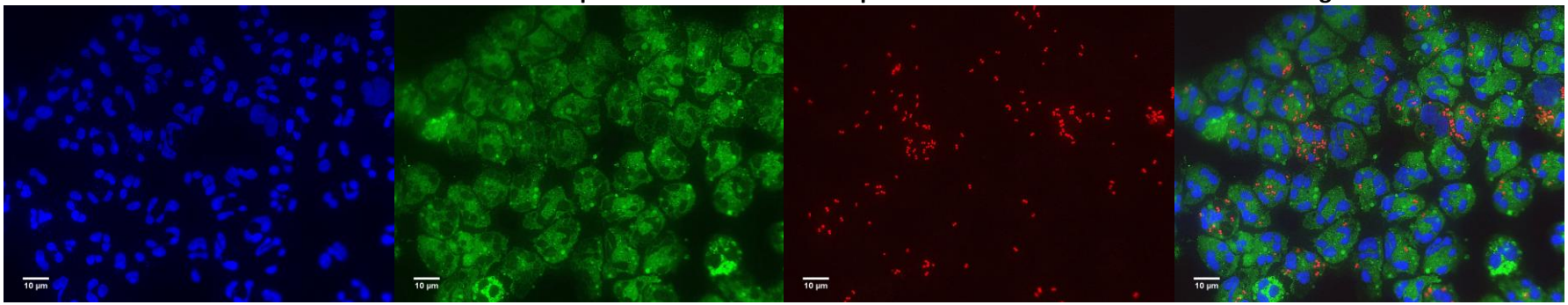

DAPI-nucleus
FITC- neutrophil membrane
pHrodo-live S. aureus
Merge- anti-CD151 antibody

Figure 4.17: The use of anti-CD151 antibodies results in less pHrodo stained live *S. aureus* in neutrophils.

Live *S. aureus* was stained with pHrodo using washing buffers containing 1% BSA. Freshly isolated neutrophils were incubated for 1 hour with media, JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml), followed by a 30 minute-incubation with pHrodo stained live *S. aureus* MOI5. Neutrophil membrane was stained for PKH67 cell tracker followed by centrifugation, neutrophil fixing on glass slides using 4% paraformaldehyde and nucleus staining for DAPI. The cells were then imaged using the Olympus upright epifluorescence system with 60x oil immersion. Photomicrographs of infected and non-infected cells: Nucleus- blue; neutrophil membrane- green; pHrodo live *S. aureus* -red. Data representative of 4 independent experiments. Images were adjusted to reduce blurriness and enhance contrast using Fiji program.


DAPI-nucleus
FITC- neutrophil membrane
pHrodo- HK S. aureus
Merge- Media


DAPI-nucleus
FITC- neutrophil membrane
pHrodo- HK S. aureus
Merge- JC1 isotype control

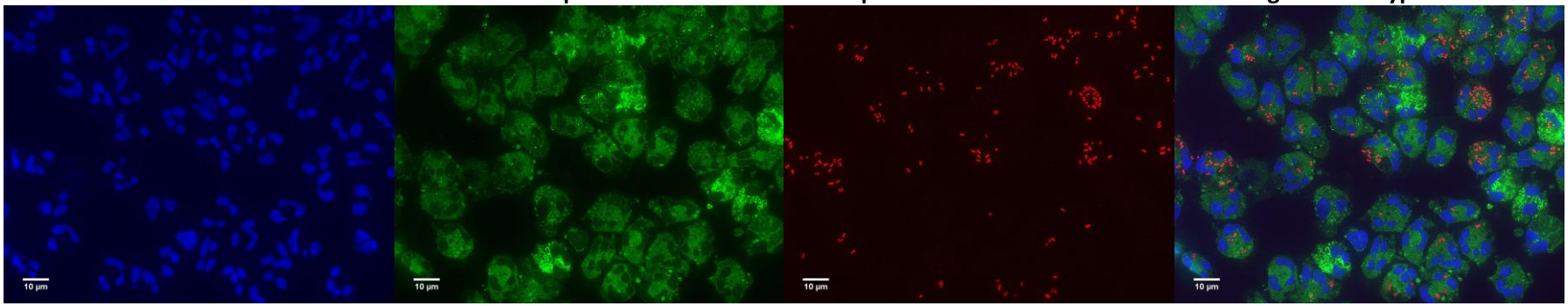

DAPI-nucleus
FITC- neutrophil membrane
pHrodo- HK S. aureus
Merge- anti-CD151 antibody

Figure 4.18: The use of anti-CD151 antibodies does not show less pHrodo stained HK *S. aureus* in neutrophils.

HK *S. aureus* was stained with pHrodo using washing buffers containing 1%BSA. Freshly isolated neutrophils were incubated for 1 hour with media, JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml), followed by a 30 minute incubation with pHrodo stained HK *S. aureus* MOI10. Neutrophil membrane was stained for PKH67 cell tracker followed by centrifugation, neutrophil fixing on glass slides using 4% paraformaldehyde and nucleus staining for DAPI. The cells were then imaged using the Olympus upright epifluorescence system with 60x oil immersion: nucleus- blue; neutrophil membrane-green; pHrodo live *S. aureus* -red. Data representative of 4 independent experiments. Images were adjusted to reduce blurriness and enhance contrast using Fiji program.

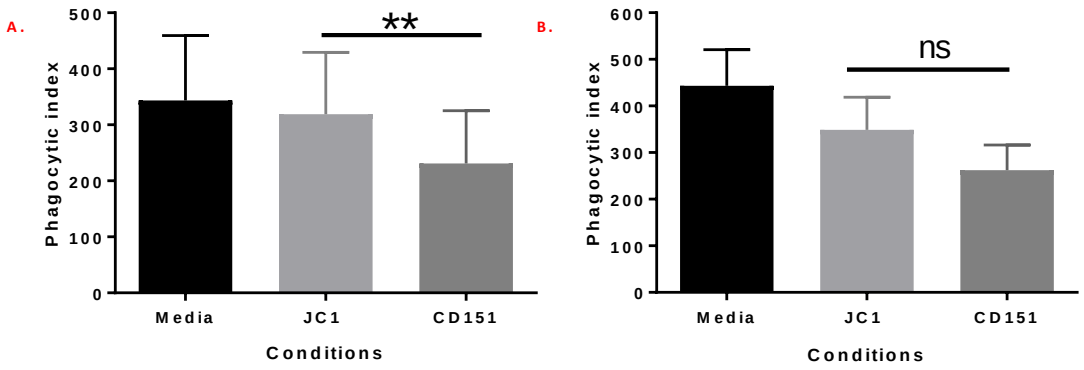


Figure 4.19: Anti-CD151 antibodies significantly inhibit neutrophil interaction with pHrodo stained live *S. aureus* but not with HK *S. aureus*.

Plasma/Percoll purified neutrophils from venous blood of healthy volunteers were incubated for 1 hour with media, JC1 isotype control or anti-CD151 antibodies at 10µg/ml, followed by a 30 minute incubation with live pHrodo pre-stained *S. aureus* at MOI5 (A) or by a 45 minute incubation with pHrodo pre-stained HK *S. aureus* at MOI10. Neutrophil interaction with pHrodo stained *S. aureus* was visualised using upright epifluorescence microscope. Data was assessed using Fiji program with the algorithm designed by Dr. Darren Robinson to count the bacteria. Data from 4 independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures (**p<0.01, ns-nonsignificant).

4.2.16. Anti-CD151 antibodies promote significantly less internalisation of live *S. aureus* by neutrophils

Another technique that can differentiate between adherent and internalised bacteria is the gentamicin protection assay and this was used to understand better *S. aureus*- neutrophil interaction in the presence of anti-CD151 antibodies (Vaudaux and Waldvogel, 1979). Neutrophils were incubated for 1 hour with JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml) followed by a 45 minute incubation with live *S. aureus* at MOI5. The extracellular and cell surface bacteria were then killed using gentamicin followed by cell lysis for estimation of the intracellular CFU. Results show that *S. aureus* internalisation by neutrophils was significantly inhibited with anti-CD151 antibodies when compared to JC1 control (Figure 4.20), supporting further the hypothesis that CD151 plays a role in neutrophil interaction with *S. aureus*.

4.2.17. The use of anti-CD151 antibodies results in inability of neutrophils to internalise and kill live *S. aureus*

S. aureus internalisation and killing by neutrophils is often a fast process (Lu et al., 2014; Nielsen et al., 1995). To understand this process further when using anti-CD151 antibodies, a later time point was included in the gentamicin protection assay. Neutrophils were incubated for 1 hour with JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml) followed by a 45 minute incubation with live *S. aureus* at MOI5. Extracellular and cell adherent bacteria were killed with gentamicin for 30 minutes and then cultures were either lysed or incubated for a further 75 minutes in media containing gentamicin, before neutrophil lysis for estimation of the intracellular CFU. Cytospins were also made 45 minutes post *S. aureus* addition and after the extracellular and cell adherent bacteria were killed. There is significantly less *S. aureus* with JC1 control condition at second time point compared to the first one indicating intracellular killing of *S. aureus* over time (Figure 4.21A). By contrast, the CFU/ml in the anti-CD151 antibody condition showed an inconsistent, nonsignificant trend between the two time points, indicating a possible donor dependent killing of *S. aureus* over time in the presence of anti-CD151 antibody (Figure 4.21B). In addition, while significantly less *S. aureus* was seen at 75 minutes with anti-CD151

antibodies when compared to JC1 control, after 120 minutes there were similar viable *S. aureus* rates in the JC1 control and anti-CD151 antibody incubated neutrophils (Figure 4.21C). Phagocytic index count showed significantly less *S. aureus* in anti-CD151 incubated cells when compared to JC1 control after 45 minutes and with similar JC1 and anti-CD151 antibody PI 75 minutes post incubation (Figure 4.21D).

4.2.18. Anti-CD151 antibodies do not inhibit live NTHi internalisation by neutrophils

Only Gram positive bacteria have been used to this point to understand CD151 involvement in respiratory pathogenic bacteria-neutrophil interactions. As a result, the respiratory Gram negative pathogen NTHi, which often promotes exacerbation and COPD progression, was further used to study CD151 function in neutrophil-bacteria interactions (Eldika and Sethi, 2006; Patel et al., 2002). Neutrophils were incubated for 1 hour with media, JC1 isotype control (10µg/ml) or anti-CD151 antibody (10µg/ml) followed by 1 hour incubation with live NTHi MOI10. Extracellular and cell adherent bacteria were killed using gentamicin before neutrophils were lysed for intracellular CFU estimation. Light microscopy could not be conducted to determine the phagocytic index, because of the small size of NTHi. No significant NTHi internalisation differences were seen between JC1 and anti-CD151 antibody incubated neutrophils (Figure 4.22).

4.2.19. Anti-CD151 antibodies effect on MDMs internalisation and killing of *S. aureus*

Macrophages are professional phagocytes that can engulf *S. aureus*, however their ability to completely kill the bacteria is low and poorly understood (Jubrail et al., 2016). Considering that CD151 appears to play a role in *S. aureus* phagocytosis by neutrophils, I hypothesised that the tetraspanin also plays a role in macrophage phagocytosis of *S. aureus*. To test this, MDMs differentiated for 14 days were incubated for 1 hour with JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml). The cells were then incubated with live *S. aureus* for 1 hour on ice followed by a further 1 hour at 37°C/ 5%CO_2. The extracellular and cell adherent bacteria were killed with gentamicin for 30 minutes followed by cell lysis (2h time point on the graph) or maintained in low gentamicin dose for a further 1.5 hours (4h time point on the graph). MDMs lysis was then conducted for intracellular CFU

estimation. There is no significant change at 2 hours between anti-CD151 antibody incubated MDMs and media or JC1 controls (Figure 4.23A). In the JC1 control, there is significantly less *S. aureus* at 4 hours compared to the 2 hour-time point (Figure 4.23B). By contrast, no significant changes were seen between the two time points when anti-CD151 antibody was used (Figure 4.23C). Furthermore, the variation in MDMs donor response with anti-CD151 antibody indicates a possible donor specific response when the antibody is used. The data is, however, insufficient to conclude if CD151 has a role in MDMs internalisation and killing of *S. aureus*.

4.2.20. CD151 is expressed at protein level in neutrophils

Considering that there is some debate in the literature about CD151 expression by neutrophils with one study saying that neutrophils do not express CD151 while another showing low expression of the tetraspanin in neutrophils (Sincock et al., 1997; Tohami et al., 2004), CD151 expression at the neutrophil protein level was explored. Neutrophil whole cell lysates from 3 independent donors were subjected to SDS-PAGE and immunostained with the anti-CD151 antibody. The staining confirmed CD151 expression in all 3 donors at the expected band of 30-32kDa (Figure 4.24.), indicating CD151 presence in neutrophils.

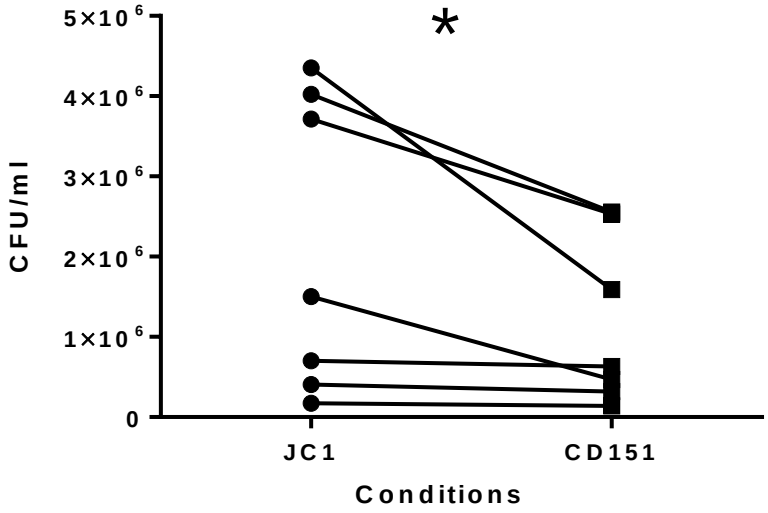


Figure 4.20: Anti-CD151 antibodies inhibit neutrophil internalisation of live *S. aureus*.
Freshly isolated neutrophils from venous blood of healthy volunteers via plasma/Percoll centrifugation were incubated for 1 hour with JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml) followed by a 45 minute incubation with live *S. aureus* at MOI5. Following gentamicin killing of extracellular and cell adherent bacteria, neutrophils were lysed for intracellular CFU estimation. Data obtained from 7 independent donors. Statistical analysis carried out by paired t-test (*$p<0.05$).

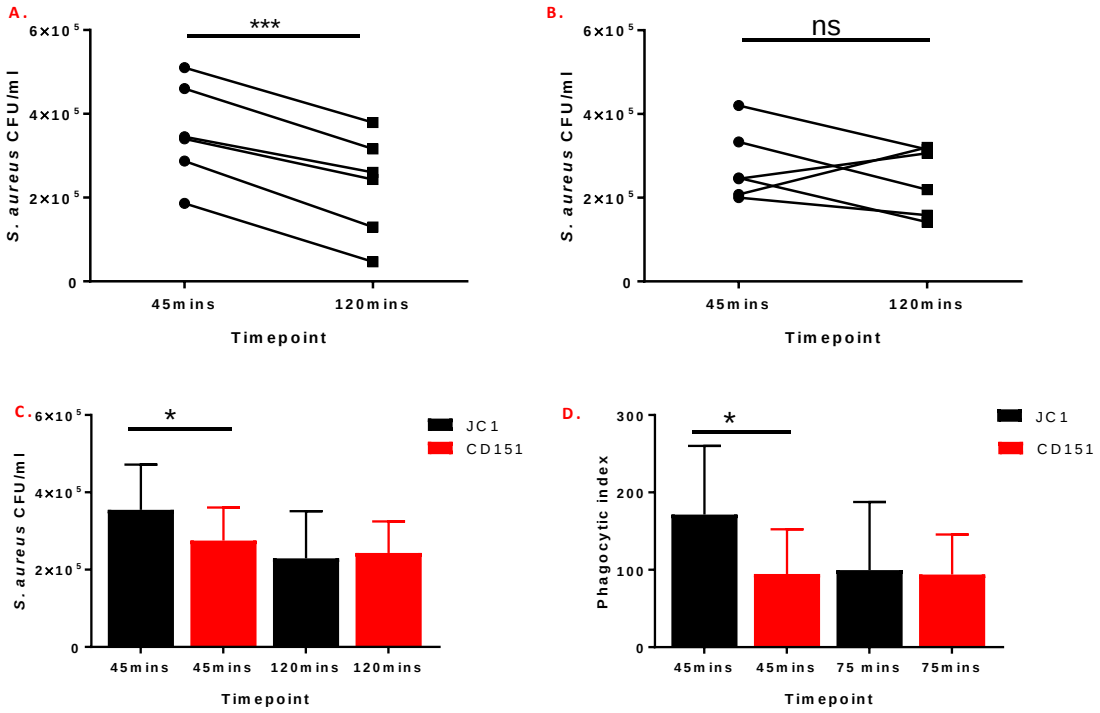


Figure 4.21: Anti-CD151 antibodies effect on neutrophil internalisation and killing of *S. aureus*.

Freshly isolated neutrophils from healthy volunteers via plasma/Percoll centrifugation were incubated for 1 hour with JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml) followed by 45 minute incubation with live *S. aureus* at MOI5. (A-C) Extracellular and cell adherent bacteria were killed with gentamicin for 30 minutes (75 minutes on the graph)and then cultures were either lysed or incubated for a further 75 minutes (120 minutes on the graph) in media containing gentamicin, before neutrophil lysis for estimation of the intracellular CFU. D) Light microscopy cytospins count 45 minutes post *S. aureus* addition and after the extracellular bacteria was killed (75 minutes on the graph). A-D) Data from 6 independent donors is expressed as Mean±SD. A-B) Statistical analysis carried out by paired t-test on JC1 incubated neutrophils (A) and anti-CD151 incubated neutrophils (B).C-D) Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures (*p<0.5, ***p<0.001).

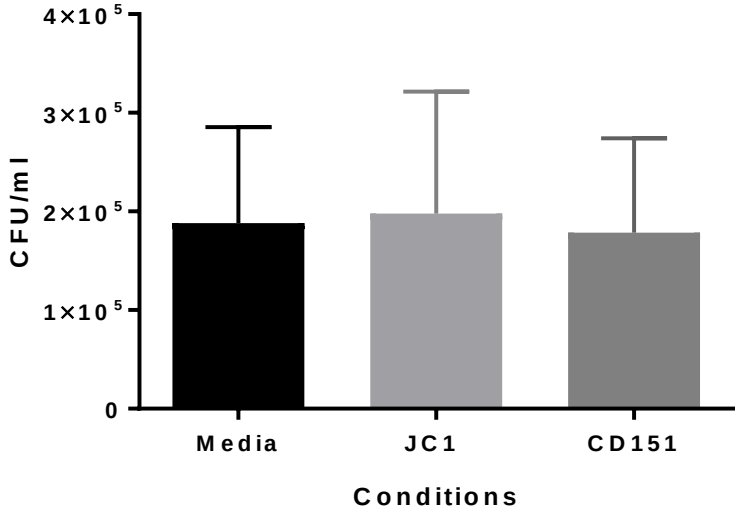


Figure 4.22: The effect of anti-CD151 antibodies on neutrophil internalisation of live NTHi. Freshly isolated neutrophils from healthy volunteers via plasma/Percoll centrifugation were incubated for 1 hour with media, JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml) followed by 1 hour incubation with live NTHi MOI10. Extracellular and cell adherent bacteria were killed with gentamicin followed by cell lysis for intracellular CFU estimation. Data from 5 independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures.

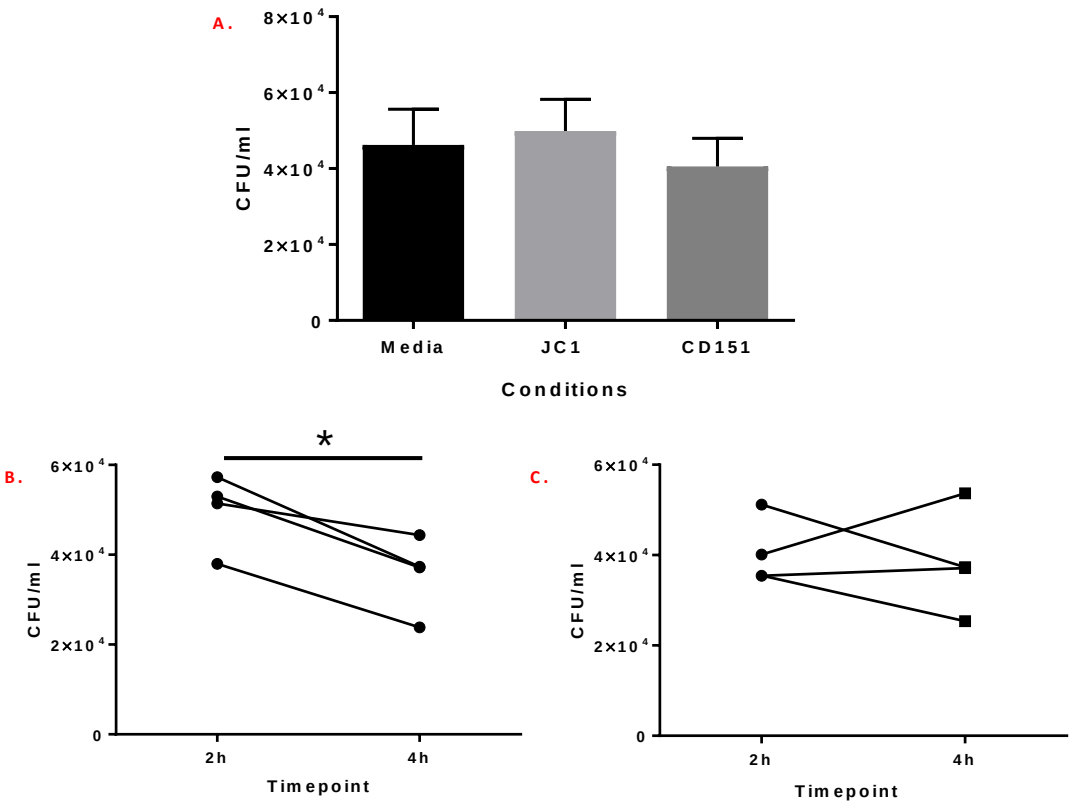


Figure 4.23: Anti-CD151 antibodies effect on MDMs internalisation of live *S. aureus*.

A-C) Freshly isolated PBMCs from healthy volunteers via plasma/Percoll centrifugation were differentiated into MDMs for 2 weeks. On the 14th day the cells were incubated for 1 hour with media, JC1 isotype control (10µg/ml) or anti-CD151 antibodies (10µg/ml). The cell were subsequently incubated with live *S. aureus* MOI5 for 1 hour on ice followed by a further one hour incubation at 37°C/ 5%CO_2. The extracellular and cell adherent bacteria were then killed using gentamicin for 30 minutes followed by cell lysis (2h time point on the B-C graphs) or by a further 1.5 MDM maintenance in low gentamicin dose (4h time point on the B-C graphs). MDMs lysis was then conducted for intracellular CFU estimation. A-C) Data from 4 independent donors is expressed as mean±SD. A) Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures. B-C) Statistical analysis carried out by paired t-test on JC1 incubated MDMs (B) and anti-CD151 antibody incubated MDMs (C) (*$p<0.5$).

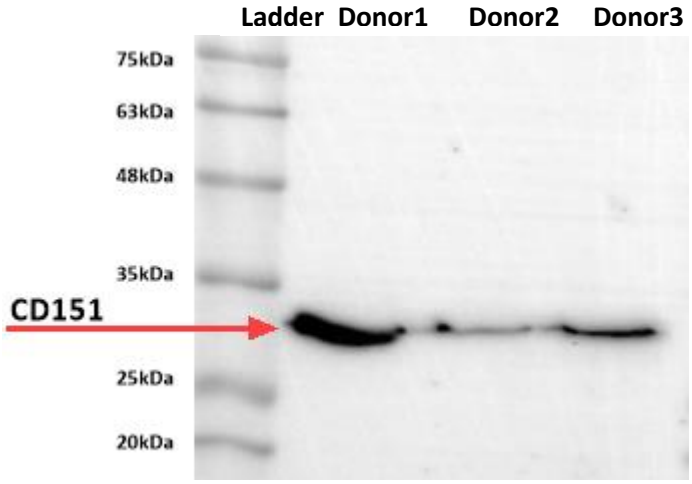


Figure 4.24: CD151 protein expression by neutrophils.

Whole cell lysates from freshly isolated neutrophils from three donors were run on 12% SDS-PAGE gel. Protein extracted from $2X10^6$ neutrophils of each donor was loaded on each lane and probed for CD151 (1:600). CD151 expression is seen at 30-32kDa. Red line represents the expected molecular weight of CD151.

4.2.21. Quantification of CD151 expression on neutrophil surface cannot be accurately done

CD151 is mainly expressed on neutrophil surface as well as cytoplasmic compartments (Baldwin et al., 2008; Berditchevski et al., 2001). Considering the changes in neutrophil interaction with *S. aureus* in the presence of anti-CD151 antibodies, I speculated that these changes are due to CD151 recruitment to cell surface upon infection. To investigate this, neutrophils were incubated for 15 minutes with media or HK *S. aureus* at MOI10. The Fc cell surface receptors were then blocked for 15 minutes with 10% goat serum. The cells were then stained with primary JC1 isotype control or anti-CD151 antibodies at 10µg/ml for 20 minutes followed by a further 20 minute incubation with a similar amount of FITC labelled secondary anti-mouse IgG antibody. Unstained and secondary only antibody negative and positive controls were used. FSC/SSC dot plots showed a right and upwards shift of neutrophils in the presence of HK *S. aureus*, indicative of neutrophil activation and interaction with the bacteria (Figure 4.25A). FL1-H histogram indicates right fluorescence shift upon FITC labelling. The right shift in the $FITC^+$ FL1-H florescence for infected and noninfected cells is decreased in anti-CD151 antibody incubated neutrophils when compared to their equivalent JC1 controls (Figure 4.25B). A secondary antibody only right shift in $FITC^+$ FL1-H florescence is also seen, indicative of secondary antibody nonspecific binding (Figure 4.25B).

To eliminate the background noise due to binding of the secondary antibody, the above experiment was repeated using JC1 isotype control and anti-CD151 antibodies (10µg/ml) that were conjugated with Alexa Fluor 488. FSC/SSC dot plots show a right and upwards shift of neutrophils in the presence of HK *S. aureus*, indicative of neutrophil activation and interaction with the bacteria (Figure 4.26A). FL1-H histogram showed a similar right shift in the Alexa $Fluor488^+$ FL1-H florescence in infected or noninfected neutrophils when incubated with anti-CD151 antibody or JC1 control (Figure 4.26B). The small fluorescence peak in all conditions indicates the possibility that the antibodies have failed to properly stain.

As a consequence, investigation of conjugation potency between CD151 antibody and Alexa Fluor 488 dye was done using HEC-1-B epithelial cell line. This line stably expresses high levels of CD151 on the cell surface(Green et al., 2011). HEC-1-B were blocked for 15 minutes with 10% goat serum. The cells were then incubated on ice with Alexa Fluor 488 JC1 isotype control, non-conjugated anti-CD151 antibodies, Alexa Fluor 488 anti-CD151 antibodies at 10µg/ml or 5µl of commercially available APC isotype and anti-CD151 antibodies for 30 minutes. The cells incubated with non-conjugated anti-CD151 antibodies were incubated further for 30 minute on ice with the same amount, to the primary antibody, of FITC labelled secondary anti-mouse IgG antibody. The commercially available APC antibodies were used as positive controls. FSC/SSC dot plots shows HEC-1-B separation from debris (Figure 4.27A). FL1-H histogram right shift indicates Alexa Fluor 488 and FITC gain of fluorescence (Figure 4.27B). Only a slight, right shift in Alexa Fluor 488^{+} FL1-H fluorescence can be seen with the Alexa Fluor 488 pre-stained anti-CD151 antibody when compared to the more prominent right shift in $FITC^{+}$ FL-1 fluorescence of cell incubated with primary anti-CD151 followed by FITC labelled secondary antibodies (Figure 4.27B). FITC labelled secondary antibodies produced no nonspecific binding in HEC-1-B cells. The results therefore indicate poor labelling of the anti-CD151 antibody with Alexa Fluor 488. FL4-H histogram shows a slight right shift in APC^{+} fluorescence with anti-CD151 antibody when compared to the isotype control (Figure 4.27C). A high APC fluorescence right shift is however seen with the isotype.

Considering that the APC labelled commercially available antibodies give a better binding signal and there is no need for secondary antibody that could result in nonspecific binding, CD151 externalisation in infected and noninfected neutrophils was investigated further using these antibodies. Neutrophils were incubated for 15 minutes with media or HK *S. aureus* at MOI10 followed by a 30 minute incubation with commercially available, conjugated APC isotype control or anti-CD151 antibodies at 10µg/ml. FSC/SSC dot plots show a right and upwards shift of neutrophils in the presence of HK *S. aureus*, indicative of neutrophil activation and interaction with *S. aureus*. (Figure 4.28A). FL4-H histogram shows a bigger, right shift in APC^{+} fluorescence with the isotype control conditions when compared to the equivalent anti-CD151 incubated cells (Figure 4.28B). This section failed to accurately quantify CD151 expression on neutrophil surface.

4.2.22. Investigation of PI4-K type II protein expression in neutrophils incubated with different *S. aureus* MOI and time points

CD151 was shown to have a strong interaction with the 55kDa PI4-K type II protein (Yauch and Hemler, 2000). PI4-K type II forms a stable complex with α3β1 and CD151 to promote cell migration in epithelial cell lines (Yauch et al., 1998). PI4-K type II is involved in PtdIns(4)P synthesis, a precursor of the phagosome shaping lipids PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 and Ins(3,4,5)P_3, but it also has an important role in endosome and vesicle trafficking due to its AP-3 sorting motif (Craige et al., 2008; Wang et al., 2007). These data suggest that CD151 may promote a PI4-K type II dependent neutrophil interaction with *S. aureus*, that could result in uptake of the bacteria via phagolysosome cup formation or via its trafficking inside the cell via the PI4-K type II AP-3 sorting motif. To investigate this, neutrophils were incubated with HK *S. aureus* at MOI 20 for 15, 30, 60, 120, 180 or 240 minutes (Figure 4.29A). Neutrophils were also incubated with HK *S. aureus* at MOI 2.5, 5, 10, 20 and opsonised HK *S. pneumoniae* at MOI 10, 20, 40 for 2 hours (Figure 4.29B). *S. aureus* and PLB-985 were used as controls. Neutrophil, *S. aureus* and PLB-985 whole cell lysates were subjected to SDS-PAGE and immunostained with the anti-PI4-K type II antibody. PLB-985 cells present a band at 55kDa, indicative of PI4-K type II protein expression (Figure 4.29A). The various incubation time points and MOI of neutrophils with HK *S. aureus* present consistent, multiple bands ranging from 45-75kDa (Figure 4.29A, B). Clear bands can be seen when an MOI2.5 of HK *S. aureus* WT is used, showing binding of the antibody at about 38, 52 and 60kDa but not at 55kDa (Figure 4. 29B). Neutrophil HK *S. pneumoniae* incubation, on the other hand, showed no bands on the blot when probed for PI4-K type II (Figure 4.29B). Phagocytic index numbers under each lane show, however, variation in *S. aureus* numbers. A single band at 35kDa is seen in the neutrophil only lane. Unphosphorylated p38 control shows similar protein loading amounts for neutrophils incubates with *S. aureus* and more in neutrophil only lane and neutrophils with HK *S. pneumoniae*. Antibodies used for interrogating PI4-K type II expression in the two blots were purchased from different companies, both showing high amounts of non-specific binding.

4.2.23. PI4-K type II expression cannot be confirmed in neutrophils when using different strains of HK *S. aureus*

Looking at the section above, most of the non-specific PI4-K type II binding pattern appears to be due to HK *S. aureus* proteins. As a consequence, a HK *S. aureus* protein A (SpA) mutant was used to try to reduce some of the non-specific binding. This mutant was chosen since protein A is one of the most abundant proteins *S. aureus* produces (Forsgren and Nordstro.K, 1974). Neutrophils were incubated with HK *S. aureus* WT or SpA at MOI 10 or 20 for 2 hours (Figure 4.30A). The effect of anti-CD151 antibodies on PI4-K type II expression in neutrophils incubated with Spa was also probed by pre-incubating for 1 hour the neutrophils with JC1 isotype control or anti-CD151 antibodies at 10µg/ml followed by a 2 hour incubation with HK *S. aureus* SpaA or HK *S. pneumoniae* or beads at MOI10 (Figure 4.30B). Neutrophil and *S. aureus* whole cell lysates were subjected to SDS-PAGE and immunostained with the anti-PI4K type II antibody. Figure 4.30A shows non-specific binding with HK *S. aureus* WT from 23-80kDa while with SpA mutant the non-specific binding is seen from 45-80kDa. A similar band pattern is seen with the anti-CD151 antibodies when neutrophils are incubated with *S. aureus* but no bands are seen in the presence of *S. pneumoniae* or beads (Figure 4.30B). Phagocytic index numbers indicate good neutrophil *S. aureus* interaction rates but due to lack of unphosphorylated p38 control, even protein load between conditions could not be confirmed (Figure 4.30A). In Figure 4.30B there appears to be overall less protein expressed in JC1 control incubated neutrophils with SpA compared to anti-CD151 incubated cells and media control. However unphosphorylated p38 protein loading control confirms less protein loading in that lane compared to the others. PI4-K type II probing indicates that although less bands are seen with neutrophils incubated with SpA mutant, the non-specific binding in the 55kDa region is not eliminated making it hard to distinguish the protein expression changes between conditions.

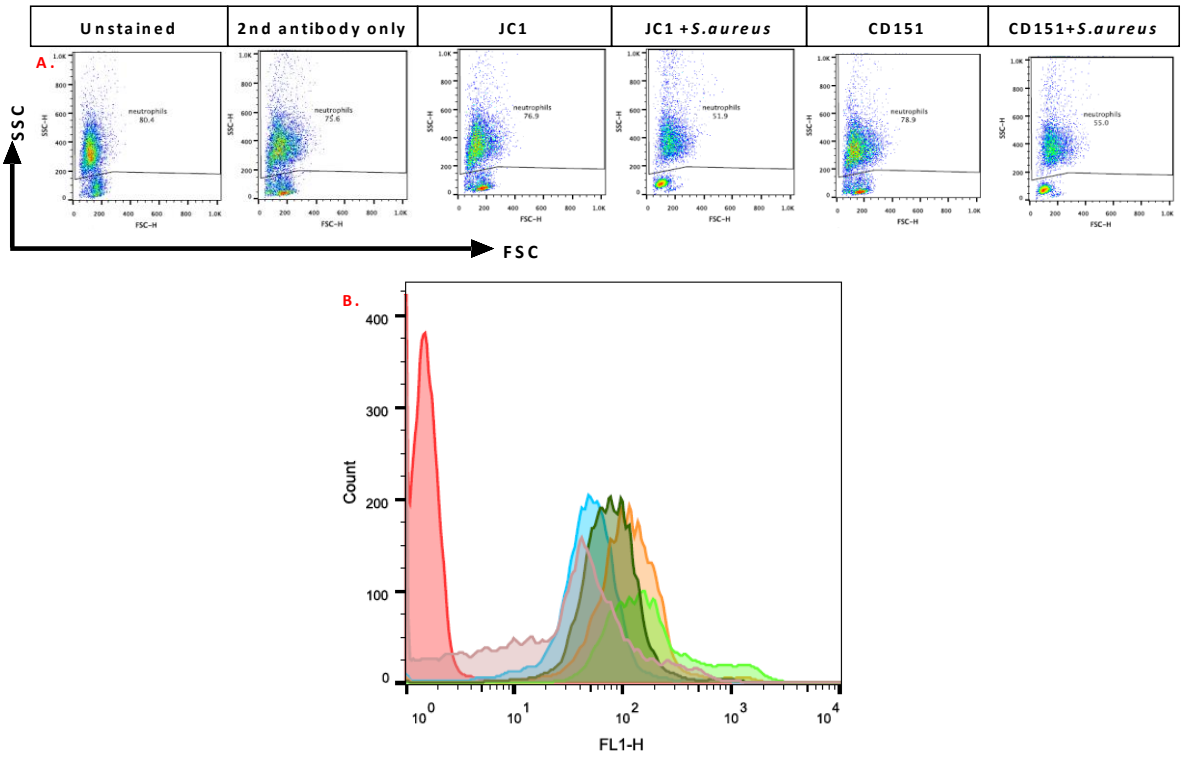


Figure 4.25: Flow cytometry analysis of CD151 expression on neutrophil surface.

Neutrophils were incubated for 15 minutes with media or HK *S. aureus* at MOI10. The cells were blocked for 15 minutes with 10% goat serum. Neutrophils were then stained with primary JC1 isotype control or anti-CD151 antibodies at 10µg/ml for 20 minutes followed by a further 20 minute incubation with an equivalent amount of FITC labelled secondary anti-mouse IgG antibodies. Unstained and secondary antibody only were used as negative controls. A) FSC/SSC dot plot of neutrophils under different conditions with the delimitations from contaminating cells and debris. B) FL1-H histogram indicative of a right shift in $FITC^{+}$ FL1-H fluorescence of neutrophils under different conditions: red-unstained neutrophils, blue-neutrophils+ secondary antibodies only, orange- neutrophils+ JC1 isotype control, light green-neutrophils+ HK *S. aureus*+ JC1 isotype control, dark green- neutrophils+ anti-CD151 antibodies, purple- neutrophils+ HK *S. aureus*+ anti-CD151 antibodies. Data obtained from single experiment.

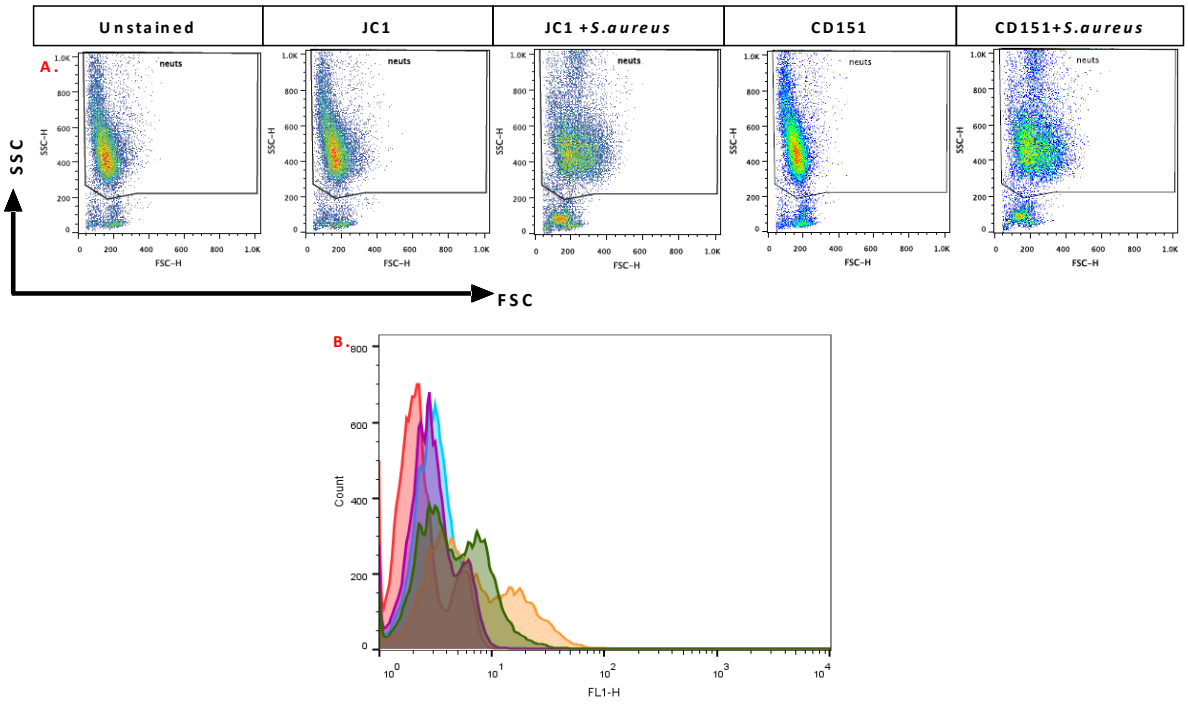


Figure 4.26: Flow cytometry analysis of CD151 expression on the surface of neutrophils using Alexa Fluor 488 conjugated anti-CD151 antibodies.

Neutrophils were incubated for 15 minutes with media or HK *S. aureus* at MOI10. The cells were then stained with conjugated Alexa Fluor 488 JC1 isotype control or anti-CD151 antibodies at 10µg/ml for 30 minutes. Unstained neutrophils were used as negative control. A) FSC/SSC dot plot of neutrophils under different conditions with the delimitations from contaminating cells. B) FL1-H histogram with a right shift in Alexa Fluor 488^{+} FL1-H fluorescence of neutrophils under different conditions: red-unstained neutrophils, blue-neutrophils+ JC1 isotype control, orange- neutrophils+ HK *S. aureus*+ JC1 isotype control, green- neutrophils+ anti-CD151 antibodies, purple- neutrophils+ HK *S. aureus*+ anti-CD151 antibodies. Data obtained from single experiment.

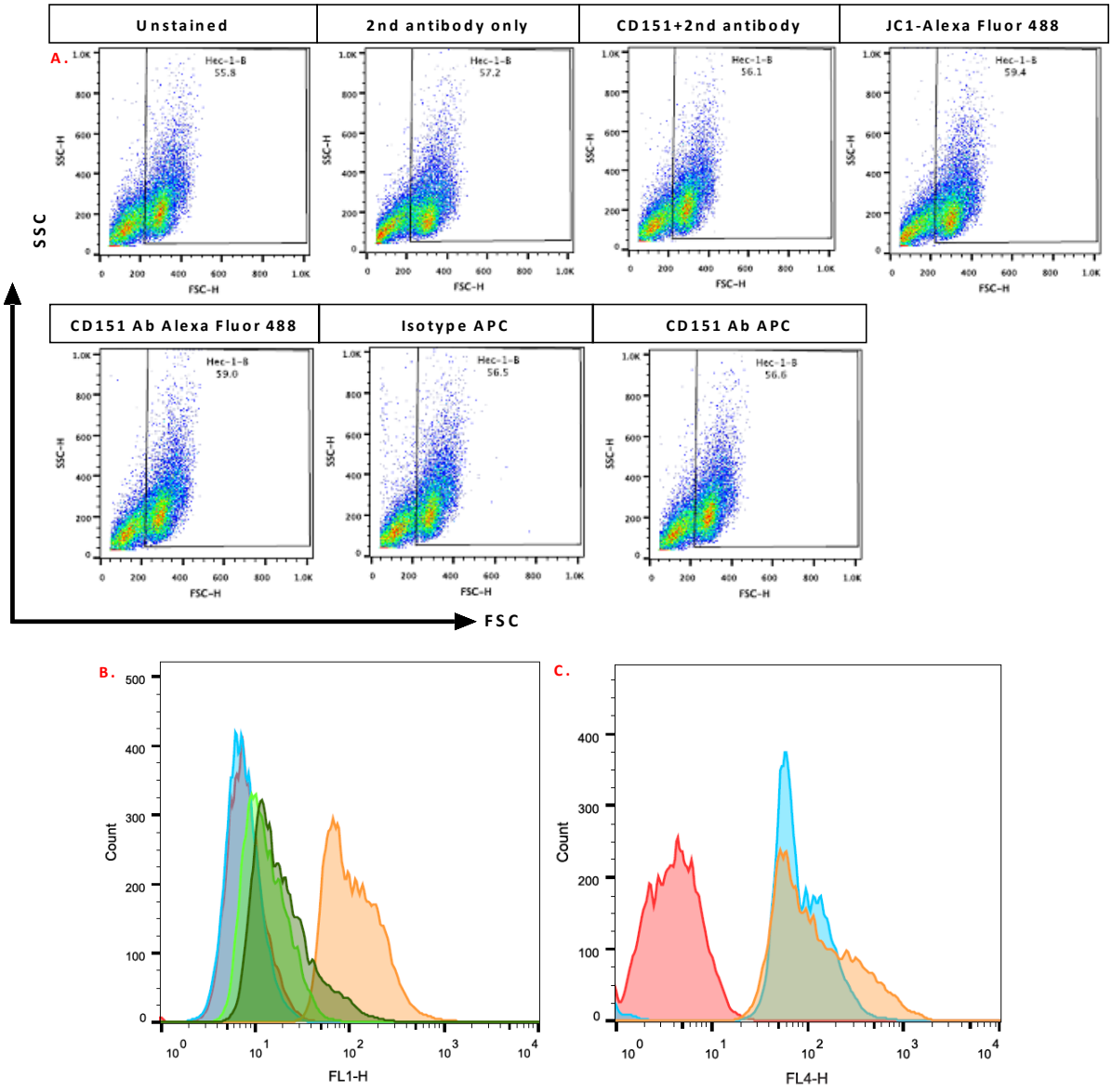


Figure 4.27: Flow cytometry analysis of CD151 expression on the surface of HEC-1-B using different anti-CD151 antibodies.

HEC-1-B cells were blocked for 15 minutes with 10% goat serum. A 30-minute incubation was then conducted with Alexa Fluor 488 JC1 isotype control, unstained anti-CD151 antibodies, Alexa Fluor 488 anti-CD151 antibodies at 10µg/ml or 5µl APC isotype and APC anti-CD151 antibodies. A further 30 minute incubation with FITC labelled secondary anti-mouse IgG antibodies was conducted where unstained anti-CD151 antibodies were used. A) FSC/SSC dot plot of HEC-1-B cells under different conditions with the delimitations from contaminating cells. B) FL1-H histogram of right shift in FITC/Alexa Fluor 488^{+} FL1-H

fluorescence: red-unstained HEC-1-B, blue-secondary FITC-labeled antibodies only stained HEC-1-B, orange- anti-CD151 antibodies+ secondary FITC labeled antibodies HEC-1-B, light green- Alexa Fluor 488 prestained JC1 isotype, dark green- Alexa Fluor 488 prestained anti-CD151 antibodies. C) FL4-H histogram of right shift in APC^{+} FL4-H fluorescence C) red-unstained HEC-1-B, blue-APC prestained isotype, orange- APC prestained anti-CD151 antibodies. Data obtained from single experiment.

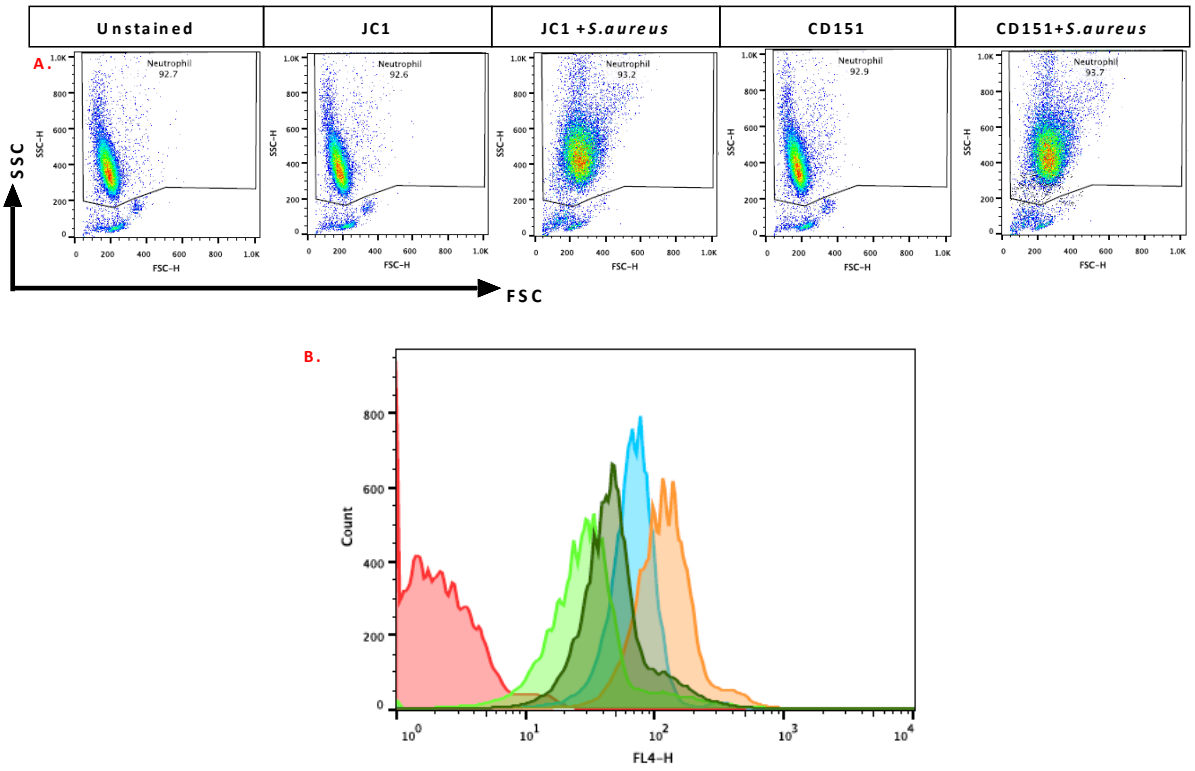


Figure 4.28: Flow cytometry analysis of CD151 neutrophil surface expression using commercially available conjugated anti-CD151 antibodies.

Neutrophils were incubated for 15 minutes with media or HK *S. aureus* at MOI10. A further 30-minute incubation was done with 5µl of commercially available, conjugated APC isotype control or anti-CD151 antibodies. Unstained neutrophils were used as negative control. A) FSC/SSC dot plot of neutrophils under different conditions B.) FL4-H histogram indicating right shift in APC^{+} FL4-H fluorescence of neutrophils under different conditions: red- unstained neutrophils, blue-neutrophils+ JC1 isotype control, orange- neutrophils+ HK *S. aureus*+ JC1 isotype control, light green- neutrophils+ anti-CD151 antibodies, dark green- neutrophils+ HK *S. aureus*+ anti-CD151 antibodies. Data representative of three experiments.

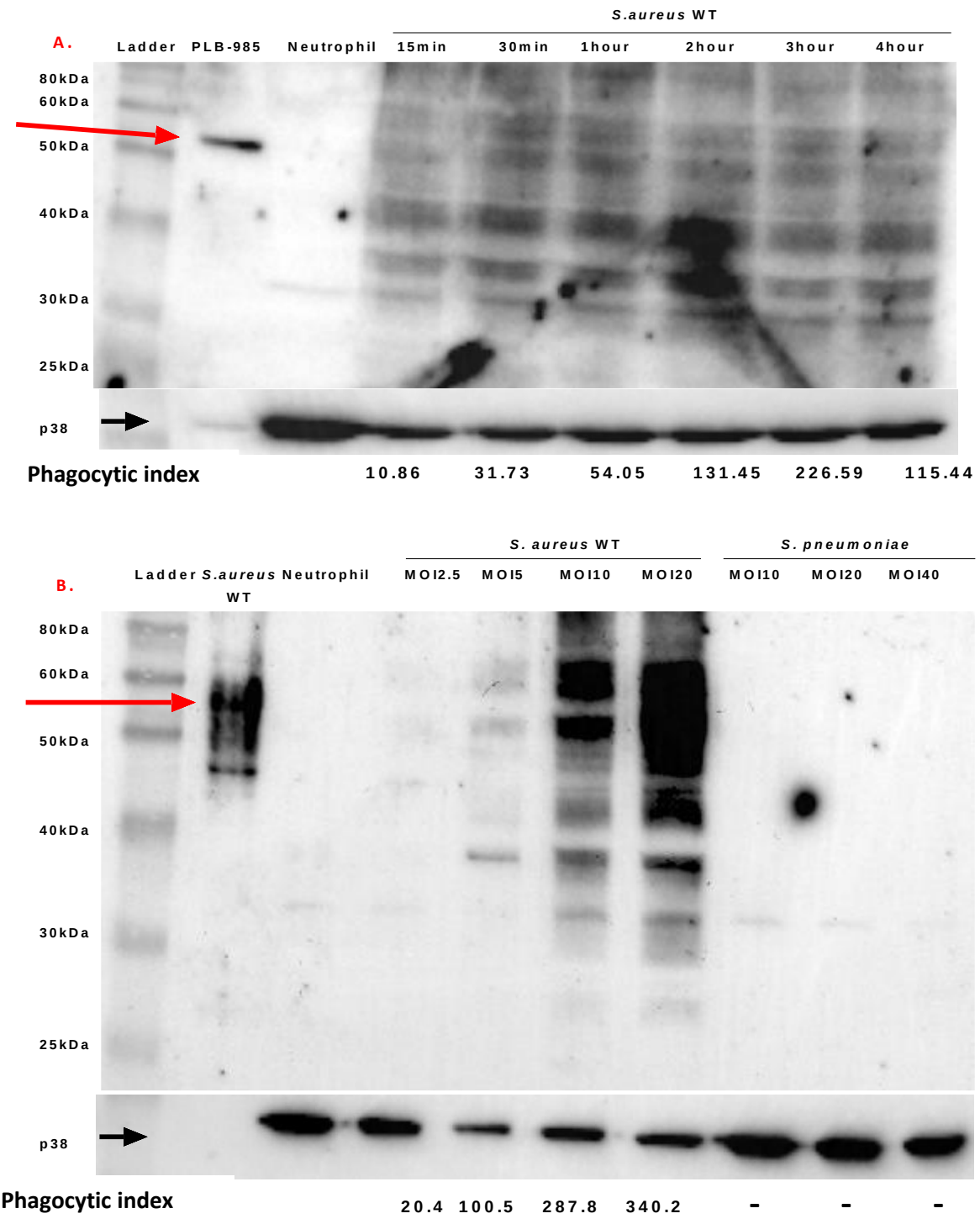


Figure 4.29: Determination of PI4-K type II protein expression in neutrophils incubated with HK *S. aureus* WT.

Neutrophils were incubated with HK *S. aureus* WT at MOI 20 for 15min, 30 min, 1 hour, 2 hours, 3 hours or 4 hours (A) or with HK *S. aureus* WT at MOI 2.5, 5, 10, 20 or opsonised *S. pneumoniae* at MOI 10, 20, 40 for 2 hours (B). Whole cell lysates from neutrophils incubated

as above, HK S*. aureus* WT (MOI20) or PLB-985 (gifted by Dr. Pranvera Sadiku) were run on 12% SDS-PAGE gel. Protein extracted from $2X10^6$ neutrophils, $40x10^6$ HK S*. aureus* WT and $1x10^6$ PLB-985 were loaded on each lane and probed for PI4-K type II (1:600). HK *S. aureus* WT lysed cells were used as control. Blot (A) was probed with a Sigma antibody while blot (B) was probed with an Abcam antibody. The Colorplus prestained protein ladder was used for the 2 blots. Red arrow is indicative of the expected 55kDa molecular weight of PI4-K type II in neutrophils. Probing for unphosphorylated p38 (1:2000) was used as control for even protein load. Where available, the phagocytic index from light microscopy analysed cytospins was indicated under the blots. Blots obtained from single experiments.

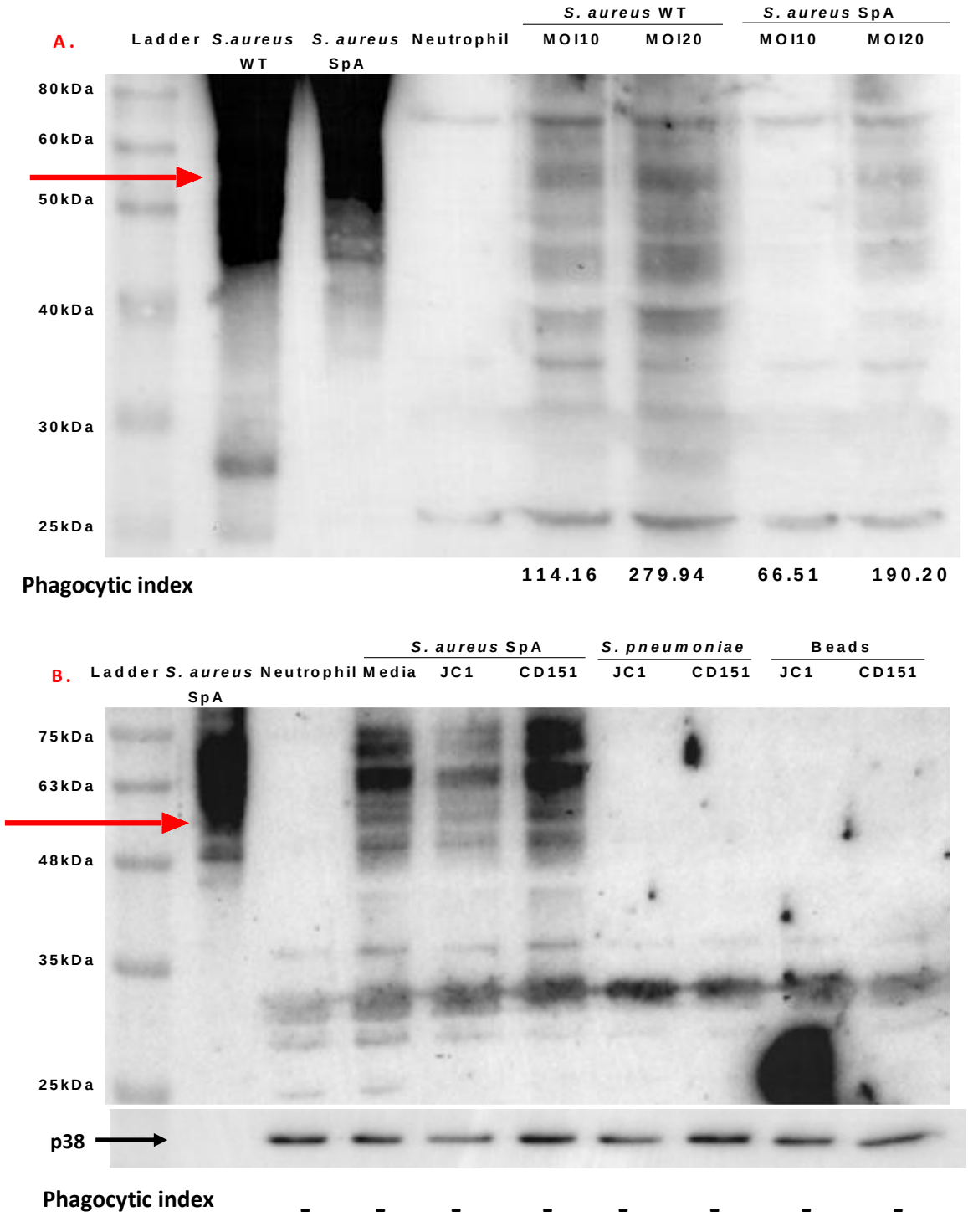


Figure 4.30: Determination of PI4-K type II protein expression in neutrophils incubated with HK *S. aureus* SpA.

Neutrophils were incubated with HK *S. aureus* WT or SpA at MOI10 or 20 for 2 hours (A) or pre-incubated for 1 hour with JC1 isotype control or anti-CD151 antibodies at 10µg/ml followed by a 2 hour incubation with HK *S. aureus* SpA or *S. pneumoniae* at MOI20 or the equivalent to the bacteria MOI20 of beads (B.). Whole cell lysates extracted from $2X10^6$ neutrophils, $40x10^6$ HK *S. aureus* WT or SpA were loaded on each lane on a 12% SDS-PAGE gel and probed for PI4-K type II (1:600). HK *S. aureus* WT or SpA mutant lysed cells were used as controls. Blot (A) was probed with Abcam antibody and the prestained Colorplus protein ladder was used while blot (B.) was probed with an Abgent antibody and the Geneflow prestained protein ladder was used. The red arrow indicates the expected 55kDa molecular weight of PI4-K type II in neutrophils. Probing for p38 (1:2000) was used as control for even protein load. Where available, phagocytic index obtained from each condition by light microscopy was indicated underneath. Blots obtained from single experiments.

4.2.24. Anti-CD151 antibodies do not significantly inhibit interaction of neutrophils with SRCO11 and SRCO12 *S. aureus* mutants

CD151 is known to interact with β1 integrins, proteins involved in the binding of the extracellular matrix protein-fibronectin (Jonsson et al., 1991; Yauch et al., 2000). This protein is regularly used by *S. aureus* to adhere to cells (Jonsson et al., 1991; Piroth et al., 2008). Considering this, I propose that CD151 modulates neutrophil interaction with *S. aureus* by supporting the formation of a putative CD151-integrin-fibronectin complex to facilitate *S. aureus* adhesion to neutrophils. To test this, two fibronectin binding mutants, SRCO11 and SRCO12 on the *S. aureus* WT background were used (See Section 2.4.1. for details on the strains). Neutrophils were incubated for 1 hour with media, JC1 isotype control or anti-CD151 antibodies at 10μg/ml followed by a 45 minute incubation with *S. aureus* WT or the fibronectin mutants SRCO11 and SRCO12 at MOI 5. Statistical analysis of the data showed that there is significant reduction of neutrophil interaction with *S. aureus* WT in the presence of anti-CD151 antibodies (Figure 4.31). Although there is reduction in neutrophil interaction with SRCO11 and SRCO12 mutants in the presence of anti-CD151 antibodies, this reduction is not significant.

4.2.25. Effect of anti-CD151 and anti-β1 antibodies on neutrophil interaction with *S. aureus*

As previously presented, a very stable interaction is known to be formed between α3β1 integrin and CD151 (Yauch et al., 1998). However, the CD151-α3β1 integrin role in the context of neutrophil interaction with *S. aureus* has not been investigated yet. To test this, neutrophils were incubated for 1 hour with media or with JC1 isotype control, anti-CD151 (10μg/ml) or β1 integrin (5μg/ml) antibodies. This was then followed by a 30 minute incubation with live *S. aureus* at MOI5. The 5μg/ml of anti- β1 integrin antibody was used since previous optimisation experiments showed better inhibition of neutrophil interaction with *S. aureus* compared to 10μg/ml of the antibody (Appendix IV). Statistical analysis of the data showed significant reduction of neutrophil interaction with live *S. aureus* when neutrophils were incubated separately with anti-CD151 or anti-β1 integrin antibodies but not when they were co-incubated with both antibodies (Figure 4.32).

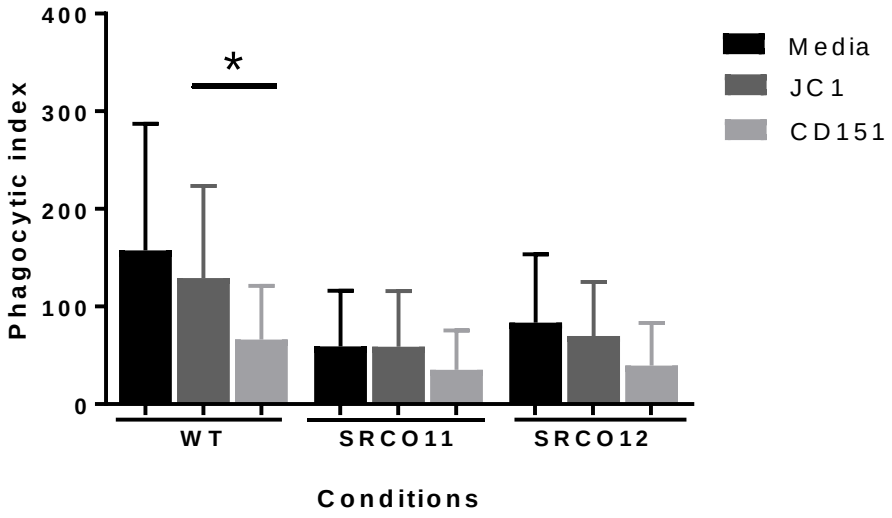


Figure 4.31: Effect of anti-CD151 antibodies on neutrophil interaction with live *S. aureus* WT and fibronectin mutants SRCO11 and SRCO12.

Plasma/Percoll-purified neutrophils from venous blood of healthy volunteers were incubated for 1 hour with media, JC1 isotype control or anti-CD151 antibodies at 10µg/ml followed by a 45 minute incubation with live *S. aureus* WT or fibronectin mutants SRCO11 and SRCO12 at MOI5. Neutrophil interaction with the different bacterial strains was assessed by light microscopy. Data from 7 independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures (*$p<0.5$).

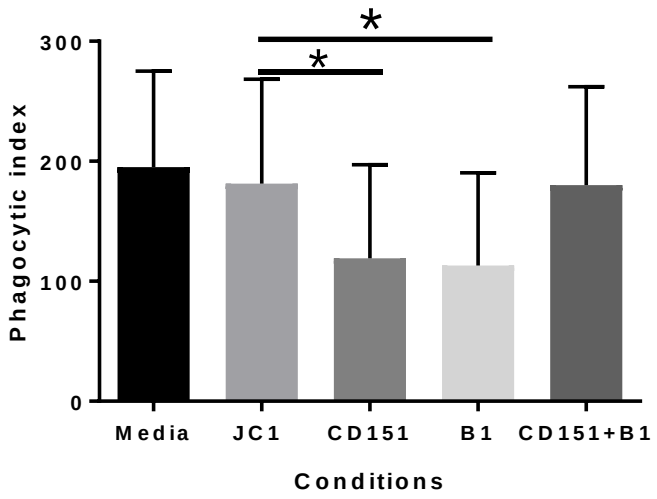


Figure 4.32: Effect of anti-CD151 and anti-β1 integrin antibodies on neutrophil interaction with *S. aureus*.

Plasma/Percoll isolated neutrophils from venous blood of healthy volunteers were incubated for 1 hour with media, JC1 isotype control, anti-CD151 antibodies at 10µg/ml or/and anti-B1 integrin at 5µg/ml followed by a 30minute incubation with live *S. aureus* at MOI5. Neutrophil interaction with live *S. aureus* was assessed by light microscopy. Data from 5 independent donors is expressed as mean±SD. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test with repeated measures (*p<0.5).

4.3. Discussion

In this chapter I have shown for the first time that tetraspanins have a potential role in neutrophil interaction with the important clinical pathogen *S. aureus*. Light microscopy indicated that CD151 may play a role in neutrophil interaction with different strains of *S. aureus*, but not with *S. pneumoniae* or NTHi. No role for CD151 was shown in MDMs interactions with *S. aureus*, although this was a single experiment and this hypothesis would need more rigorous testing. Gentamicin protection assays and fluorescent microscopy data further strengthened the findings by showing less *S. aureus* internalisation in anti-CD151 antibody treated neutrophils. Moreover, results indicate no cumulative role for anti-CD151 and anti-β1 antibodies in reducing neutrophil phagocytosis of *S. aureus*. The overall data suggest, therefore, a putative role for CD151 in modulating neutrophil *S. aureus* phagocytosis.

Anti-CD151 antibodies reduced neutrophil interactions with *S. aureus* WT and JE2 strains. No role was shown for CD9, CD63 and CD37. A collective role was shown for CD9, CD63 and CD151 in modulating *S. aureus* interactions in epithelial cells (Green et al., 2011). It is possible that the collective antibody use against the tetraspanins mentioned above, would result in a more profound decrease in neutrophil interaction with *S. aureus* compared to the single antibody use, though more research must be conducted. Furthermore, investigations in this thesis were conducted using only two strains of *S. aureus*. A comprehensive understanding of CD151 therapeutic implication in neutrophil-*S. aureus* interactions, would require, however, the use of more *S. aureus* clinical isolates and possibly other *Staphylococcus* strains.

Results in this chapter show no role for CD151 in neutrophil interaction with NTHi and *S. pneumoniae*. These results are not surprising. Unlike *S. aureus*, *S. pneumoniae* was opsonised prior to neutrophil incubation suggesting the use of phagocytic receptors by the bacteria to enter the cells. Neutrophils from mice poorly phagocytose *Haemophilus influentzae,* which is in accordance with the 4-5% NTHi internalisation rate by neutrophils seen in this thesis (Tay et al., 2015). Taken together, this suggests that *S. pneumoniae* and

NTHi are most probably not internalised by neutrophils using the same mechanism employed for *S. aureus* internalisation.

The MDMs gentamicin protection assay results showed no change in *S. aureus* internalisation in the presence of anti-CD151 antibodies. However, the variable results with anti-CD151 antibodies seen in MDMs killing of *S. aureus* indicate that perhaps CD151 may play a role in MDMs killing of the bacteria. To confirm this, more rigorous investigations would need to be conducted. Although to date it is known that monocytes express CD151 at the plasma membrane and cytoplasmic level, there is no evidence in the literature for CD151 involvement in macrophage/monocyte-bacterial interactions (Tohami et al., 2004).

The flow cytometry CD151 expression data indicates that CD151 expression on the neutrophil cell surface could not be quantified accurately. Incubation of neutrophils with pre-stained antibodies, obtained in house or commercially, showed a marked nonspecific binding of the JC1 isotype control, even after serum blocking of Fc receptors. When using the HEC-1-B positive control cell line, CD151 detection was possible with both the in house anti-CD151 antibody, probed with a secondary antibody, and with the pre-stained commercial antibody, although the effect was more moderate. Furthermore binding of the JC1 isotype or secondary antibody only controls was, unlike in neutrophils, similar to the unstained baseline control, which is possibly due to the high level of Fc receptors on the neutrophil surface (Hostoffer et al., 1993; Sengelov et al., 1994). In addition, the literature suggests that neutrophils have low CD151 expression, most of which being localised in the intracellular compartment in the endosomes, late endosomes and lysosomes with small amounts on cell surface (Sincock et al., 1999; Tohami et al., 2004). Considering this, high surface levels of Fc receptor could hinder accurate CD151 detection. Though in this thesis CD151 expression could not be detected on neutrophil surface, considering the literature neutrophils would be expected to have low levels of CD151 on their surface. It is possible that the experimental design was not sensitive enough to detect the tetraspanin or a different technique would need to be used to demonstrate CD151 presence on neutrophil cell surface. At the same time, considering this thesis results, the hypothesis that neutrophils do not have CD151 on their surface cannot be excluded. Future experiments could include human serum to attempt to reduce the JC1 nonspecific binding or employ a

different technique, such as fluorescent microscopy, to probe for CD151 presence on neutrophil surface.

To determine if the anti-CD151 antibodies affected the internalisation or adhesion of *S. aureus* to neutrophils, Trypan Blue was used to initially to distinguish between internalised and surface bound bacteria due to its ability to quench the external bacteria fluorescence. However, ToPro-3 staining indicated that it is an unsuitable method due to neutrophil lysis which most probably resulted in Trypan Blue cell entry. The unsuitability of the technique is contrasting previous findings, where the method was used successfully to discriminate between internalised and cell-surface bacteria (Nuutila and Lilius, 2005; Santos et al., 2015). One of the reasons for cell lysing is the high toxicity of Trypan Blue which may have affected cell viability. However, detrimental cell toxicity with Trypan Blue has only been determined 30 minutes post incubation, a much longer time compared to the experiments conducted in this thesis (Awad et al., 2011). Therefore, it can be speculated that *S. aureus* neutrophil incubation could have rendered cells more susceptible to lysis allowing Trypan Blue cell entry and internal bacteria fluorescence quench.

Consequently, fluorescent microscopy was used to investigate this hypothesis. Significantly less pHrodo stained live *S. aureus* was seen with anti-CD151 antibodies compared to the control. Since pHrodo fluoresces only at an acidic pH, the results indicate that less *S. aureus* is phagocytosed in the presence of anti-CD151 antibodies (Aziz et al., 2013). Attempts to co-stain *S. aureus* with both pHrodo and Alexa Fluor 647 dyes resulted in poor visualisation of Alexa Fluor 647 stained bacteria. Alexa Fluor 647 stained bacteria were found to be fluorescent by flow cytometry suggesting the staining procedure was successful. Co-visualusation of the two dyes is also possible, as indicated by *in vivo* studies conducted in zebrafish model (Ellett et al., 2015). It is possible that the microscope used could have photobleached quite rapidly the Alexa Fluor 647 dye, resulting in poor visualisation of the dye.

Gentamicin protection assays further confirm that less *S. aureus* is internalised by neutrophils with anti-CD151 antibodies. The results also show that the bacteria were not as effectively killed with the anti-CD151 antibody, as shown by the comparable bacterial load

between the two time points. The differential internalisation effectiveness was initially proposed to be due to differential cell lysis rates between conditions, a hypothesis disproved by similar %ToPro-3 cell staining between conditions. A role for CD151 in the endocytosis of a foreign pathogen was demonstrated in the context of cervical cancer (Scheffer et al., 2013). Human papillomavirus type 16 (HPV16)-CD151 complexes were seen to be endocytosed in endosomes in a clathrin-independent mechanism, in a process that required TEMs (Spoden et al., 2008). A similar mechanism could be seen in this context. CD151 may be involved in *S. aureus* internalisation by promoting fusion of late and early endosomes with the phagosome to aid in its sealing and maturation. Alternatively, CD151 can be endocytosed via the clatherin-dependent pathway, due to its tyrosine-based sorting motif, by interacting with AP-2 (Sincock et al., 1999; Winterwood et al., 2006). However, the close proximity of the motif to the plasma membrane can constrain stericaly the interaction with AP-2, suggesting a clatherin-independent internalisation of the protein being most probable (Rohrer et al., 1996). Fusion of CD151 with early and late endosomes can be probed, using fluorescent microscopy technique, by conducting co-localisation studies looking at *S. aureus* and CD151 co-localisation with Rab5, Rab7 stained early and late endosomes. This mechanism could also require TEMs formation to bring in close proximity other CD151 binding partners to facilitate this process. Integrins are known to be involved in pathogen uptake as a result of their ability to initiate signalling to promote actin rearrangement upon activation (Dupuy and Caron, 2008). CD151 can form stable interactions with many integrins to modulate cellular processes, including α3, α6 and β1 (Hong et al., 2012b; Lammerding et al., 2003; Nishiuchi et al., 2005; Puklin-Faucher and Sheetz, 2009; Takeda et al., 2007). In glioblastoma cells the CD151-integrin complex is required for AKT phosphorylation and downstream signalling following migration, while in epithelial cells CD151 promotes multimolecular complexes formation in the membrane by promoting α3β1/ α6β1 interaction with the GTPases Ras, Rac1, Cdc42 (Hong et al., 2012a; Tilghman et al., 2016). As described in Chapter 1, CD151-dependent signalling activation is dependent on the cellular context. Therefore, considering that GTPases are involved in neutrophil cytoskeletal rearrangements and AKT is involved in neutrophil response to *S. aureus*, the use of the anti-CD151 antibodies could have altered integrin interactions to

disrupt either of the pathways (Arbibe et al., 2000; Liu et al., 2013; Zhong et al., 2003). Kinase and GTPase activation can be probed using commercially available kits such as the LISA activation assay kits to detect GTPase activation. Moreover, the separate incubation of neutrophils with the anti-CD151 and β1 blocking antibodies resulted in significant reduction of neutrophil interaction with *S. aureus* while antibody co-incubation showed similar interaction rates to the control. This lack of the cumulative effect with the antibodies may be due to the steric antibody inhibition when CD151 and β1 integrin are in close proximity. This is highly probable since the anti-CD151 antibody used in this study, 14A2, is presumed to disrupt the tetraspanin interaction with the α3, β1 and β4 integrin chains (Geary et al., 2001; Yamada et al., 2008). This is due to the α3 region of the α3β1 integrin and the anti-CD151 antibody binding domains recognising two overlapping epitopes in the EC2 region of the tetraspanins (Berditchevski et al., 2001; Yamada et al., 2008; Yauch et al., 2000). Though disruption of integrin –CD151 interaction by the antibodies could explain the reduced neutrophil phagocytosis of *S. aureus*, more research must be conducted to confirm this.

Further investigations using light microscopy showed decreased, but non-significant, neutrophil interaction with fibronectin mutants in the presence of anti-CD151 antibodies when compared to the control. The fibronectin mutants (See section 2.4.1.) were used due to lack of ability to bind to fibronectin. This result possibly indicates that fibronectin may not be involved in mediating a CD151 dependent neutrophil *S. aureus* interaction. However, before this hypothesis is excluded I consider that more investigations need to be done by using a different technique. For instance, neutrophil, fibronectin and *S. aureus* WT and mutants triple staining can allow visualisation of the interaction and bacterial internalisation outcome with or without the anti-CD151 antibodies. It is possible that fibronectin may still be involved in this process, considering the lack of significant inhibition with anti-CD151 antibodies. Moreover, *S. aureus* adhesion to fibronectin may be also facilitated by other tetraspanins. TEMs association can orchestrate many cellular processes including cell migration, signalling in immune complexes and viral cell entry (Levy and Shoham, 2005b; Mantegazza et al., 2004; Spoden et al., 2008). CD151-CD9, CD151-CD81 homodimers can be found in cell plasma membrane (Kovalenko et al., 2004). For example, CD9 can interact with fibronectin binding integrins and Hsc70 receptor, exploited by *S.*

aureus to adhere and gain entry into host cells (Guerrero and Moreno, 2012; Wilkinson et al., 1995). Most recently, CD9 was shown as a potential therapeutic target against *S. aureus* adhesion to epithelial cells (Ventress et al., 2016). CD9 can therefore promote *S. aureus* adhesion to neutrophils and potentiate the phagocytic process. However, anti-CD9 antibodies were without effect suggesting that the tetraspanin may not be involved in the process. Nonetheless, CD81 is required for *Listeria monocytogenes* bacteria entry into epithelial cell (Tham et al., 2010). CD81 can form complexes with fibronectin binding integrins such as α4β1 found on neutrophil surface (Pereira et al., 2001; Serru et al., 1999; van den Berg et al., 2001). The function of this tetraspanin was not probed in our model system but evidence suggests investigative potential.

CD151 can interact with PI4-K type II and bring into close proximity the $\alpha_3\beta_1$ integrin to facilitate cell migration (Yauch et al., 1998). Therefore, activation of PI4-K type II upon neutrophil interaction with *S. aureus* was investigated by western blotting. Despite using different antibodies, time points, MOI and strains of *S. aureus*, change in PI4-K type II expression between conditions could not be seen in neutrophils due to the presence of nonspecific bands. PI4-K type II expression could not be seen in neutrophils incubated with *S. pneumoniae* or beads. Less nonspecific binding was seen when neutrophils were incubated with *S. aureus* SpaA mutant compared to WT. However, the high number of nonspecific bands seen with both *S. aureus* strains in the 55kDa expected region of PI4-K type II, indicates unsuitability of the technique to study the kinase expression. Alternative techniques could be used to study the interaction such as co-immunoprecipitation, although the high amount of nonspecific binding seen with the antibodies can lead to the precipitation of artefact proteins, as well as nuclear magnetic resonance (NMR). NMR would be a more desirable technique since it is highly sensitive and transient protein interactions can be detected.

As described in Chapter 3, the anti-tetraspanin antibodies could potentially alter neutrophil functions not only via their interaction with the target but also via their Fc region that could interact with the Fc receptors on adjacent neutrophils (Murphy, 2012; Porges et al., 1994). In this chapter only anti-CD151 antibodies were used to show a reduced neutrophil-*S. aureus* interaction. Therefore, to test in future experiments if the Fc region of the antibodies

alter neutrophil-*S. aureus* interactions by interacting with Fc receptors on adjacent cells, CD151 Fab fragments would need to be tested in parallel with the antibody. This experiment was not attempted in this thesis due to lack of availability of the CD151 Fab fragments. Moreover, reduced *S. aureus* interaction with neutrophils could be due to Staphylococcal SpA protein binding to the IgG region of the anti-CD151 antibody and preventing bacterial adhesion to the neutrophil and subsequent phagocytosis (Forsgren and Nordstro.K, 1974). Phagocytic index obtained from light microscopy samples (Figure 4.30A) showed less neutrophil interaction of *S. aureus* SpA mutant when compared to the WT strain. Although this is a single experiment and more thorough investigation would need to be conducted, this light microscopy data suggests that SpA protein-Fc anti-CD151 antibody region interaction does not take place or is not required to reduce neutrophil-*S. aureus* interactions.

Similar to Chapter 3 description, neutrophils are genetically intractable limiting the tools available to investigate CD151 function in mediating neutrophil interaction with *S. aureus*. Previous research conducted in our group showed, however, the suitability of lentivirus vectors to overexpress proteins in neutrophils (Dick et al., 2009). This technique can therefore be used to overexpress CD151 in neutrophils, generally seen at low levels, to investigate CD151 overexpression effects in neutrophil-*S. aureus* interactions. For further experiments, CD151 role in neutrophil-*S. aureus* interactions would need to be interrogated using a more amenable *in vitro* model or, for a more clinically-related context, using an *in vivo* system. PLB-985 cell line once differentiated have similar morphology and function to that of primary neutrophils (Pivot-Pajot et al., 2010). Therefore, PLB-985 cells are a good *in vitro* model to conduct CD151 knockdown to probe further *S. aureus*-neutrophil interactions.

The CD151 human and mouse homologs are 93% similar, the highest similarity of all tetraspanins, indicating that the mouse can be used as a suitable model to study the tetraspanin (Fitter et al., 1998). The four transmembrane regions, the N-glycosylation sites, the 15 cysteine residues as well as the EC2 domain are also highly conserved between the two species (98% and 89% identity) (Hasegawa et al., 1997). *In vivo* CD151 knockout mouse models were developed by different laboratories to investigate CD151 functions in different

illnesses (Sachs et al., 2006; Takeda et al., 2007; Wright et al., 2004). However, none of the groups looked at the role of CD151 in an infectious model. The use of mice to study the role of CD151 in neutrophil interaction with *S. aureus* could however give inaccurate results. According to the protein database bank, mice contain a protein of unknown function that has the extracellular domain 57% identical to the CD151 one, called BAB22942 (Hemler, 2001). What is more, the 11-amino-acid motif in the EC2 domain of CD151 is identical to the unknown protein one, differing by 2 amino acids (R*D*H*A*SNIYKVE- R*A*H*P*SNIYKVE) (Berditchevski et al., 2001). It is possible that this protein would have similar or complementary function to that of CD151. Furthermore tetraspanins are known to form TEMs where they often play redundant function, making it difficult for single tetraspanin function investigation (Huang et al., 2005). It is highly possible that CD151 knockout in the mouse would result in function compensation by other tetraspanins.

CD151 role in neutrophils have not yet been investigated using *in vivo* zebrafish knockout model. The Sanger institute initiative was to have a CD151 zebrafish mutant with a nonsense mutation by the end of 2016, however the fish are not available yet. A CD151 knockout zebrafish has been developed in the past two years by Katherine Marsay, a PhD student in Dr. Lynda Partridge group. The fish line is stable but it is yet to be phenotyped before CD151 implication in neutrophil interaction with *S. aureus* is investigated.

Using different molecular and biochemical methods, I showed for the first time a correlation between tetraspanin CD151 and neutrophil internalisation of *S. aureus*. Interestingly, no role for CD151 was determined for neutrophil interaction with NTHi or S. pneumoniae. These results confirm further the role of tetraspanins in pathogen cell interactions. However, the result needs to be understood further with additional experiments.

Chapter 5: Final Discussion

5.1. Thesis summary

In this thesis I have demonstrated for the first time that tetraspanins are involved in modulating two key neutrophil functions: survival and phagocytosis.

In chapter 3 I demonstrated using morphology and biochemical methods that anti-CD63 antibodies and Fab fragments inhibit constitutive neutrophil apoptosis. Interestingly, neutrophils from individual subjects varied in their responsiveness to anti-CD63 antibodies yet western blot analysis showed no variation between donors or time points. Contrary to the literature, flow cytometry analysis did not show a conclusive result of CD63 up-regulation on the neutrophil surface over time. My results also demonstrate that neutrophils from age matched healthy controls and COPD patients have similar apoptosis inhibition rates in the presence of anti-CD63 antibodies.

In chapter 4 I showed that anti-CD151 antibodies reduce neutrophil interactions with *S. aureus* but not with *S. pneumoniae* or NTHi. Fluorescent microscopy with pHrodo labelled bacteria suggest that anti-CD151 antibodies reduce phagocytosis of *S. aureus* as opposed to just adherence. Gentamicin protection assays confirmed a reduction in *S. aureus* internalisation. Attempts to investigate changes in CD151 expression on neutrophil surface, using a panel of in-house and commercially-available antibodies, failed to show any change in CD151 expression between the two conditions. Subsequent functional assays showed no cumulative role for anti-CD151 and β1 antibodies in reducing neutrophil interaction with *S. aureus* nor indicated a clear requirement for fibronectin in the process.

5.2. Major findings and impact

5.2.1. CD63 role in neutrophil survival

CD63 is a highly abundant lysosomal protein, often recruited to the plasma membrane upon neutrophil activation (Cham et al., 1994; Hatskelzon et al., 1993; Lopez et al., 1995; Nishibori et al., 1993). CD63 is part of the tetraspanin molecular facilitator group, with roles in diverse cellular functions (Horejsi and Vlcek, 1991; Yanez-Mo et al., 2009). In this thesis, I have

shown for the first time that CD63 is involved in modulating neutrophil apoptosis. Interestingly, CD63-mediated regulation of neutrophil apoptosis was proven to be donor dependent, with some donor cells experiencing profound apoptosis inhibition with anti-CD63 antibodies while others were without effect. Although no overall change in protein expression was seen between the two groups, the differential tetraspanin surface expression was not investigated and doing this may reveal clues about whether CD63 trafficking/recycling may play a role in mediating apoptosis. Age, gender, diet or environment can also potentially alter neutrophil survival results, but the study ethics did not allow us to collect and report on these characteristics (Spitzer and Zhang, 1996).

Neutrophils isolated from COPD patients also demonstrated a delay in apoptosis in the presence of anti-CD63 antibodies, which suggests this pathway is not dysregulated in this disease. These results are in contrast with the literature, where decreased CD63 expression was seen in the COPD cohort (Zhang et al., 2007). Age variability is often linked to changes in results outcome, being positively correlated to changes in neutrophil function in the elderly population (Hazeldine et al., 2014; Panda et al., 2009). Moreover, COPD prevalence is in 45+ age group (Doucet et al., 2016). Taken together, discrepancies between my current and Zhang et al. results may be due to the age matching of the healthy controls with COPD patients in this thesis while Zhang et al. used a much younger control group to match with the COPD patients.

The study adjoins another function to CD63 in neutrophils, to the already known one of cell migration and protein granule transport, suggesting that the tetraspanin may be more of a global regulator of neutrophil processes (Kallquist et al., 2008; Skubitz et al., 1996; Trikic et al., 2011).

5.2.2. CD151 role in neutrophil *S. aureus* interactions

Neutrophils are fundamentally important in the defence against *S. aureus* (Odell and Segal, 1991; Quie et al., 1967). It is well documented that tetraspanins are involved in pathogen trafficking and adhesion to different cell types (Monk and Partridge, 2012). In this thesis I have shown for the first time that CD151 is involved in neutrophil interaction with *S. aureus*. This adds to the current knowledge that *S. aureus* uses tetraspanins for attachment to cells

and induce cytotoxicity, as shown by studies conducted on epithelial cells (Green et al., 2011; Ventress et al., 2016; Virreira Winter et al., 2016). CD151 was indeed part of the TEMs involved in *S. aureus* adhesion to epithelial cells, along with CD9. However, our results did not indicate a role for CD9 in this context, suggesting perhaps that tetraspanin function is cell type dependent. CD151 has not been widely studied in bacterial interactions with host cells, however work conducted using HPV16 indicated a role for CD151 in viral endocytosis (Scheffer et al., 2013). Taken together, this indicates that CD151 has putative adhesion and trafficking abilities in this context, although more studies must be conducted to fully understand this in the neutrophil. Interestingly, CD151 had a specific role for S. *aureus* internalisation but not for *S. pneumoniae* and NTHi. This finding suggests a potential specificity of the tetraspanin for *S. aureus,* making it a good therapeutic target for this pathogen, albeit much research using different bacteria is needed to confirm this. Moreover, it may also be speculated that CD151 may act as a gateway for *S. aureus* infection. Although this hypothesis needs further research, there is evidence that *S. aureus* α-hemolysin uses TSPAN14 on epithelial cells to induce cytotoxicity (Virreira Winter et al., 2016). Taken together, the study presents for the first time CD151 role in specific neutrophil phagocytosis of *S. aureus*.

5.3. Therapeutic potential

Persistence of neutrophils in the lung, concomitant with a delay in apoptosis and an inability to clear intruding pathogens, has long been associated with increased COPD severity (Damiano et al., 1986; Hoenderdos and Condliffe, 2013; Nadel, 1991; Redinbo, 2014; Zhang et al., 2012). Current therapies in COPD inflammation mostly treat symptoms and fail to stop disease progression. These treatments often include bronchodilators such as β2-adrenoreceptor agonists and glucocorticoids. Both bronchodilators encourage an inflammatory phenotype with β2-adrenoreceptor agonists failing to inhibit neutrophil survival while glucocorticoids have been shown to promote increased neutrophil lifespan (Anderson et al., 2014; Burgon et al., 2014; Liles et al., 1995; Meagher et al., 1996).

Tetraspanins have already been proposed as therapeutic targets, mainly in infections and cancer (Cozens and Read, 2012; Haeuw et al., 2011; Hassuna et al., 2009; Hemler, 2008;

Murayama et al., 2015; Ventress et al., 2016). Antibody targeting of the two highly expressed tetraspanins in cancer, CD9 and CD151, was demonstrated to reduce tumour cell growth (Haeuw et al., 2011; Murayama et al., 2015). At the same time, anti-CD81 antibodies can prevent hepatitis C virus entry in hepatocytes by stopping adhesion of its E2 protein to the tetraspanin (Bertaux and Dragic, 2006). Most importantly, anti- *S. aureus* adhesion therapies are showing great preliminary promise in the context of S. *aureus* adhesion to epithelial cells (Ventress et al., 2016). The study showed that nanomolar levels of short peptides that were derived from the sequence of CD9 EC2 domain are effective in reducing *S. aureus* adhesion to epithelial cells and 3D tissue skin model (Ventress et al., 2016). This demonstrates therapeutic targeting potential for these tetraspanins.

Before consideration is given for tetraspanins as therapeutic targets to modulate neutrophil functions, I consider there are some questions that need to be answered. Neutrophils are required for an effective defence against invading microorganisms, and compromising immunity by inducing apoptosis may have deleterious effects for the host. This is particularly important for patients with chronic lung disease, who are frequently colonised with pathogens. Therefore, the choice of therapeutic treatment and delivery must take into account these factors. My data suggests that the use of anti-CD63 antibodies inhibit neutrophil apoptosis in COPD patients, suggesting the unsuitability of these antibodies to use as therapeutics in this context. However, CD63 ability to internalise antibodies makes it an useful target to deliver drugs or RNAi therapeutics, when conjugated with the antibodies, to alter cellular functions including targeting neutrophil apoptosis through a different pathway (Barrio et al., 1998). This approach has already been proposed in cancer therapy (Barrio et al., 1998). Moreover, if CD63 is demonstrated to associate with TEMs to modulate neutrophil survival, physical disruption of these association can be conducted to alter cell function. Acyl transferases are required for protein palmitoylation, an important process for protein assembly into TEMs, therefore targeting the relevant acyl transferases could disrupt TEMs interactions (Charrin et al., 2002; Mitchell et al., 2006).

Although more research needs to be conducted, the results obtained so far suggest that CD151 appears to be a good therapeutic target since its function seems to be restricted only to neutrophil phagocytosis of *S. aureus*. Neutrophils are professional phagocytes, and unlike

epithelial cells, *S. aureus* phagocytosis and killing is their main function. Thus a natural question we may need to address in future work is if we want to inhibit neutrophil phagocytosis of *S. aureus* and what would be the overall effects. As discussed in chapter 1, neutrophil can kill *S. aureus* with a vast array of proteolytic enzymes and ROS production released within the phagolysosome. Equally, *S. aureus* has evolved many mechanisms to evade killing by neutrophils including the ability to use neutrophils as a gateway for infection by surviving and replicating within the cytoplasm (Fraunholz and Sinha, 2012; Gresham et al., 2000; Grosz et al., 2014). The next step would thus be to understand if CD151 promotes *S. aureus* entry via the phagolysosomal pathway or if it is exploited by the bacteria to gain entry, survive and replicate within neutrophils. These are questions that require further understanding before tetraspanin targeting for therapeutics is considered.

The last thing that needs to be considered is the effect that tetraspanin targeting on neutrophils would have on the surrounding tissues. Tetraspanins are ubiquitously expressed in many tissues, aiding to conduct a multitude of processes, therefore targeting one cell may have negative effect on the surrounding tissues (Charrin et al., 2014). CD151 has an important role in the normal function of alveolar epithelial cells by maintaining adhesion strength of the basement membrane, its loss resulting in pulmonary fibrosis in mice (Tsujino et al., 2012). Therefore, if a CD151 containing spray therapeutic would be used in the lungs, in addition to reducing neutrophil S. aureus load, it could also promote bacterial entry in the organism by reducing basement membrane strength in the surrounding tissues. Consequently, experiments using in vivo models, such as mice, would play a vital role in determining the therapeutic potential and toxicological drawbacks of targeting tetraspanins on neutrophils.

5.4. Study limitations

A confounding variable of this study is the method employed to attain a purified neutrophil population from whole blood. The Percoll purification process can result in cell activation, although historically it has been shown to be less activating compared to other methods (Venaille et al., 1994). Additional variation that could result as well in cell activation can be introduced by different phlebotomists and scientists handling the blood (Wehlin et al.,

1998). However, a switch from round to an elongated cell morphology as well as neutrophil aggregation, as seen in a haemocytometer chamber, is usually indicative of cell activation. Moreover, as described above, donor age variability is often linked to variation in the results outcome due to the neutrophil differential response to stimuli (Hazeldine et al., 2014; Panda et al., 2009).

Another limiting factor in this study is that neutrophils are short lived in culture and genetically intractable. Thus, the experimental techniques used to probe their functions in this thesis were limited. Ideally I would have wanted to knockdown CD63 and CD151 in neutrophils to confirm the antibodies functions.

5.5. Future work

5.5.1. Studies on CD63 effect on neutrophil survival

There is a body of evidence that shows TEMs modulate various cell functions, including cell apoptosis (Kuhn et al., 2007). Future work will involve investigating CD63 TEMs formation to see which co-factors localise with CD63 and whether TEMs formation is required for neutrophil apoptosis to take place. CD63 association in TEMs can be initially investigated using a panel of anti-tetraspanin antibodies, followed by high resolution fluorescent microscopy co-localisation studies of these complexes, due to their small size of only several nm (Grove, 2014; Zuidscherwoude et al., 2015). Cholesterol depletion assays with methyl-β-cyclodextrin would also be a good technique to investigate CD63-partner protein interactions (Pierini et al., 2003; Yang et al., 2012). Methyl-β-cyclodextrin can disrupt, however, cellular actin having the potential to disrupt additional cellular functions (Kwik et al., 2003).

As described in chapter 3 discussion, CD63 could regulate neutrophil survival by potentially disrupting intracytoplasmic signalling by affecting kinases, such as PKCδ and PI4-K type II, trafficking to the plasma membrane. Alternatively, the tetraspanin can regulate one of the Bcl-2 family members protein expression by promoting a signalling pathway activation, as seen in acute myelogenous leukemia cells where CD82 can regulate the expression of BCL2L12 via STAT5/AKT signalling (Nishioka et al., 2015). A next step in this process would

be to interrogate CD63 function in the above processes. Western blot can be used to determine signalling molecule activation, such as PKCδ, PI4-K type II and AKT, or by conducting fluorescent microscopy co-localisation studies or pull-down assays to probe CD63 interaction with the signalling molecules.

Here, I showed that neutrophil apoptosis reduction rates are similar in both healthy control and COPD populations with anti-CD63 antibodies, irrespective of overall CD63 protein expression. This is an interesting result considering it has been demonstrated that CD63 mRNA and plasma membrane protein level is reduced in COPD patients compared to HC (Zhang et al., 2007). Alternatively, neutrophil survival, from cells isolated from the sputum of COPD patients, can be investigated in the presence of anti-CD63 antibodies to see if the effect is different to circulating cells.

5.5.2. Further investigating the role of CD151 in phagocytosis of *S. aureus*.

As described above, it is possible that *S. aureus* uses CD151 to gain entry into neutrophils. While *S. aureus* is not strictly an intracellular pathogen, there is emerging evidence that this pathogen is able to survive and even replicate within the neutrophil cytoplasm and therefore may use the cell as a niche (Fraunholz and Sinha, 2012; Gresham et al., 2000; Grosz et al., 2014). To determine the function of the tetraspanin, CD151-*S. aureus* interactions could be investigated in the presence of the anti-tetraspanin antibodies using gentamicin protection assay or flow cytometry to screen a library of *S. aureus* mutants lacking candidate adhesion proteins or virulence factors, such as sar global regulator (Cheung et al., 1992). Moreover, time lapse super-resolution microscopy techniques could allow visualisation of *S. aureus* entering the neutrophil, from which it can be identified whether CD151 is required for bacteria binding or/and internalisation by neutrophils during this process. Moreover, CD151 TEMs formation to modulate neutrophil interaction with *S. aureus* can be investigated as described in the section above.

5.6. Conclusion

Chronic inflammatory diseases such as COPD are an increasing medical and economic burden, and are still poorly treated. Neutrophils are key cells in the pathophysiology of

chronic inflammation and I have investigated two elements of neutrophil behaviour, apoptosis and phagocytosis, because these form the foundation from which neutrophils perform important immune functions and contribute to inflammation. I have described previously undefined roles for the tetraspanins, CD63 and CD151 in apoptosis and phagocytosis, which not only adds to our knowledge of the mechanisms underpinning key neutrophil functions, but may also open up potential opportunities for therapeutic strategies in the future.

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Appendix I:

1mg/ml Hemin

Reagents	Quantities
Hemin powder	0.05g
NaOH	5ml

Appendix II:

Phosphate lysis buffer (500µl)

Reagents	Volume (µl)
Phosphatase inhibitor cocktail (PI) (1:100 dilution) (Merckmillipore, Watford, UK)	5
Phenylmethanesulfonyl fluoride (PMSF) (100mM) (Sigma-Aldrich, Poole, UK)	5
dH_2O (Baxter, Newbury, UK)	490

2X SDS-PAGE loading dye (10ml)

Reagents	Concentration in buffer	Volume
1M Dithiothreitol (DTT) (Sigma-Aldrich, Poole, UK)	0.1M	1ml
20% Sodium dodecyl sulfate (SDS) (Fisher Scientific, Loughborough, UK)	4%	2ml
100% Glycerol (Fisher Scientific, Loughborough, UK)	20%	2ml
0.5M Tris HCl (pH6.8) (Fisher Scientific, Loughborough, UK)	0.0625M	1250µl
0.2% Bromophenol Blue (Sigma-Aldrich, Poole, UK)	0.004%	200µl
dH_2O	-	3.55ml

Stacking gel (1.5mm plates)

Reagents	1 gel	2 gel	3 gel	4 gel
Water	3ml	6ml	9ml	12ml
40% Acrylamide	620µl	1240µl	1860µl	2480µl
0.5M Tris pH 6.8 (Bio Rad Laboratories, Hercules, California)	1260µl	2520µl	3780µl	5040µl
20% SDS	25µl	50µl	75µl	100µl
20% APS	50µl	100µl	15µl	200µl
TEMED	5µl	10µl	15µl	20µl

10X Running Buffer

Reagents	Quantities
Glycine (Fisher Scientific, Loughborough, UK)	190g
Tris Base (Fisher Scientific, Loughborough, UK)	30.3g
20% SDS	50ml
dH2O	to 1 litre

Resolving gel (1.5mm plates)

12% Resolving gel reagents	1 gel	2 gels	3 gels	4 gels
Water	6.6ml	9.9ml	13.2ml	16.5ml
40% Acrylamide (Geneflow, Lichfield, UK)	4.4ml	6.8ml	9ml	11.3ml
1.5M Tris pH 8 (Bio Rad Laboratories, Hercules, California)	3.8ml	5.7ml	7.6ml	9.5ml
20% SDS	75µl	112.5µl	150µl	187.5µl
20% APS (Sigma-Aldrich, Poole, UK)	150µl	225µl	300µl	375µl
TEMED (Geneflow, Lichfield, UK)	6µl	9µl	12µl	15µl

Transfer Buffer

Reagents	Quantities
Glycine	145g
Tris Base	29g
dH2O	to 800ml

Note: For experimental use, the Transfer buffer was diluted using a 1:2:7. ratio of Transfer buffer:methanol:water.

10X TBS

Reagents	Quantities
Tris-HCl 1M pH 8.0	100ml
Sodium Chloride (NaCl) (Fisher Scientific, Loughborough, UK)	97.3g
Water	to 1000ml

10 X TBS-Tween

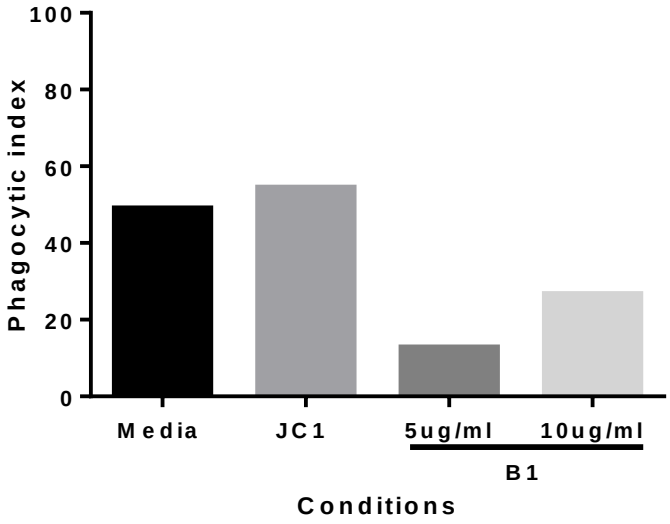
Reagents	Quantities
Tris-HCl 1M pH 8.0	100ml
NaCl	97.3g
Tween-20 (VWR chemical, Fontenay-sous-bois, France)	5ml
Water	to 1000ml

Primary and secondary antibody dilutions

Antibody	Dilutions
Primary JC1 isotype	1:600
Primary anti-CD63	1:600
Primary anti-CD151	1:600
Primary anti-phosphatidylinositol 4-kinase (PI4-K) type II (Sigma-Aldrich, Poole, UK; Abcam, Cambridge, UK; Abgent, San Diego, CA)	1:600
Primary anti-unphosphorylated P38 (Cambridge Bioscience, Cambridge, UK)	1:2000 and 1:4000
Secondary polyclonal goat anti-mouse (Dako, Cambridge, UK)	1:2000
Secondary polyclonal goat anti-rabbit (Dako, Cambridge, UK)	1:1000

Appendix III:

To make 4%PFA, 5g PFA was dissolved in 50 ml water, followed by the addition of 1ml of 1M NaOH. The mixture was stirred at 65°C until dissolved. It was then topped with 10ml of 10x PBS. The pH was adjusted to 7.4 by adding 1M hydrochloric acid (HCl). The 4%PFA was made by our in house technician Mrs. Catherine J. Cooke.



Appendix IV: Plasma/Percoll pure neutrophils were incubated for 20 hours with media JC1 IgG1 isotype control (10µg/ml) and anti-B1 antibodies at 5 and 10 µg/ml. Phagocytic index was assessed using light microscopy. Data obtained from single experiment.