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Investigation of the manipulation of phagocytes by *Staphylococcus aureus*.

Kate O'Keeffe

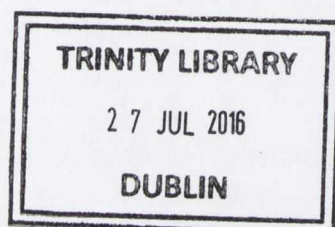
B.A (Mod) Biochemistry with Immunology



**A thesis submitted to
Trinity College Dublin
For the degree of
Doctor of Philosophy**

**Supervisor: Dr. Rachel M. McLoughlin
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September 2015



Thesis 11074

Declaration of Authorship

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Acknowledgements

First and foremost I would like to sincerely thank my supervisor Rachel McLoughlin for all of her support, patience and encouragement. Her door really has always been open and that is hugely appreciated.

To the rest of the McLoughlin lab- thank you for everything! I couldn't have asked for a better group to spend the last 4 years with! Belinda and Mieszko, thank you both for helping me find my feet in the world of science. Ali, I am not sure I would have made it through without you sitting beside me for most of it.... turned out you weren't quite the prude I thought! Thank you for listening to me whinge, providing chocolate when necessary and Disney sing-a-longs in the hood! Aisling, my Chicargo buddy, I think the evening of the fireplace-building will forever be one of my most productive in the lab! John (best-friend!), thank you for all the evening chats over a cuppa, very helpful with the thesis procrastination! Michelle, you have made sure my social media presence always has a nerdy side.... Keep it up! You have also given me many a pep talk on not stressing out.... It worked – and thank you for that! Stephen, you have been a fantastic addition to our lab, when you are a big PI remember me when I come looking for a job! Keenan, my travelling guide, thanks for all the tips so far.... No doubt there will be many more questions to come! Finally, a big thank you to Katie who did her undergrad project with us this year for the data included in this thesis.

Thank you to all the members of both the Dunne and the Stevenson labs, for the chats, craic and sweets!! Clare and Emma, you guys have helped keep me sane (or maybe the opposite!) over the last 4/6 years, thank you both for all the laughs!

To Rachel and Sarah, my roomies, thank you both so much for all the memories, good times were had in Kennedy court!! Can't wait to see where the next few years bring us all.

Thank you to all the Skerries girls who were always around for a cup of tea and a catchup, no matter how many times I wasn't! That means more than you know, In particular, a special thanks to Sophie and Graham for letting me set up shop in their new home!

To Rory, thank you for always being there and knowing exactly when I needed tough love and to be told to go and do some writing or when I just needed a hug! I can't wait for the next adventure on our cards!

And finally to my mental, noisy, wonderful family. Thank you for everything – the love of science (cultivated by a phenomenal Biology teacher – Mam!), the singing, the dancing. But mostly thank you for all of your unconditional encouragement, love and support.

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Plos Pathogens *In Press*.

Abbreviations

Agr	Accessory gene regulator
AIP	Autoinducing Peptide
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
Apaf1	Apoptotic protease activating factor 1
APC	Antigen Presenting Cell
Asp	Aspartate
ATG	Autophagy-related genes
ATP	Adenosine TriPhosphate
Bak1	BCL2-antagonist/killer 1
Bax	BCL2-associated protein X
Bcl-2	B-cell lymphoma 2
BID	BH3 interacting-domain
BMDC	Bone Marrow-derived Dendritic Cell
BMM	Bone Marrow-derived Macrophage
C	Celsius
CA	Community Acquired
CC	Clonal complex
CCL	CC chemokine ligands
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CGD	Chronic Granulomatous Disease
CHIPS	Chemotaxis Inhibitory Protein of <i>Staphylococcus aureus</i>
CHO	Chinese Hamster Ovary cells
ClfA	Clumping factor A
ClfB	Clumping factor B
CP	Capsular Polysaccharide
CRAMP	Cathelicidin-related antimicrobial peptide

CSA	Columbia Agar
CXCL	C-X-C motif ligand
d	Day
DC	Dendritic Cell
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
<i>E. coli</i>	<i>Escherichia coli</i>
Eap	Extracellular adherence protein
EB/AO	Ethyl Bromide / Acridine Orange
EfB	Extracellular fibrinogen binding protein
ELISA	Enzyme-linked Immunosorbant Assay
FACS	Fluorescence-activated cell sorting
FADD	FAS-associated protein with Death Domain
FAS	Apoptosis Stimulating Fragment
FLIPr	Formyl Peptide Receptor-like 1 Inhibitor
FMO	Fluorescence minus one
FPR	Formyl Peptide Receptor
g	Gravity
G-CSF	Granulocyte Colony Stimulating factor
G-CSF	Granulocyte-Colony Stimulating Factor
GAS	Group A Streptococcus
GFP	Green Fluorescence Protein
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
h	Hour
H ₂ O ₂	Hydrogen peroxide
HA	Hospital Acquired
Hla	α -toxin (haemolysin α)
HNP	Human Neutrophil Peptide

HOCl	Hypochlorous acid
i.p.	Intraperitoneal
iBMM	Immortalised bone marrow derived macrophage
ICAM-1	Intracellular Adhesion Molecule- 1
IFN γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
IsdB	Iron regulated surface determinant
KC	Keratinocyte chemoattractant
kDa	kilo Daltons
KO	Knockout
LAP	LC3-Associated Phagocytosis
LC3	Microtubule-associated protein light chain 3
LDH	Lactate Dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LTA	Lipotechoic Acid
LukAB	Leukotoxin AB
Mcl	Myeloid leukemia cell differentiation protein
MCP1	Monocyte chemotactic protein 1
MEF	Mouse Embryonic Fibroblasts
MFI	Mean Fluorescence Intensity
Mg	milligrams
MHC	Major Histocompatibility Complex
Mins	Minutes
MIP-2	Macrophage Inflammatory Protein 2
ml	Millilitres
MOI	Multiplicity of Infection

MPO	Myeloperoxidase
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
Mtb	<i>Mycobacterium tuberculosis</i>
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil Extracellular Trap
NK	Natural Killer
nm	nanometer
nM	nanoMolar
NO	Nitric Oxide
NOS2	Nitric Oxide Synthase 2
OD	Optical Density
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PBST	PBS-Tween
PCR	Polymerase Chain Reaction
PEC	Peritoneal Exudate Cells
PFA	Paraformaldehyde
PGN	Peptidoglycan
PI	Propidium Iodide
PMN	Polymorphonuclear Neutrophil
PMSF	Phenylmethanesulfonyl fluoride
PRR	Pattern Recognition Receptor
PSM	Phenol soluble modulins
PVDF	Polyvinylidene fluoride
PVL	Panton Valentine Leukocidin
RIP	Receptor-Interacting Protein
RNA	Ribonucleic Acid
Roryt	Retinoid-related orphan receptor γ t

ROS	Reactive Oxygen Species
RPM	Rotation Per Minute
RT	Real Time
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
s.c.	subcutaneous
SCIN	Staphylococcal Complement Inhibitor
SDS-PAGE	Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis
SLR	Sequestosome-like Receptor
SNP	Single nucleotide polymorphism
SPF	Specific Pathogen Free
STAT	Signal transducer and Activator of Transcription
T-bet	T-box Transcription Factor
tBID	truncated BH3 interacting-domain
TCD	Trinity College Dublin
TCR	T Cell Receptor
TGF β	Transforming Growth Factor beta
Th1	T helper cell 1
Th17	T helper cell 17
Th2	T helper cell 2
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
TRADD	TNF receptor type 1-associated Death Domain protein
Treg	Regulatory T cell
TSA	Tryptic Soy broth Agar
TSB	Tryptic Soy Broth
TSST1	Toxic Shock Syndrome Toxin 1
ULK1	Unc51-like kinase 1
VLT	Vesicle Lysis Test
WT	Wild Type

$\alpha\beta$	Alpha Beta
$\gamma\delta$	Gamma Delta
μg	microgram
μl	microliter
3-MA	3-Methyladenine

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Abstract

The increasing prevalence of antibiotic resistance in *Staphylococcus aureus* (*S. aureus*) heralds the advent of a “post-antibiotic era” that urgently necessitates the development of novel treatment methods such as vaccines and immunomodulatory therapies to provide an alternative to antibiotics. In order to facilitate development in these therapeutic areas, a greater understanding of the interactions between this bacterium and the host is required. *S. aureus*’ infective prowess stems from its vast capacity for immune evasion. In recent years, it has been established that this bacteria is even capable of survival within phagocytes, the archetypal killing cells of the immune system. It has been proposed that this ability of *S. aureus* to survive within phagocytes is likely a critical factor facilitating dissemination. This thesis proposes that *S. aureus* survives inside phagocytes by subversion of autophagy and manipulates apoptosis to prolong the availability of this niche.

This study demonstrates for the first time that dendritic cells (DCs) play a key role in the direct killing of *S. aureus*. The presented data show that *S. aureus*’ susceptibility to DC killing is strain dependent and evasion of killing relies upon the strain’s ability to subvert autophagy to provide an intracellular niche. The observed differences between strains are shown to be dependent upon the level of Agr activity, as only strains with high Agr activity are capable of subversion of the autophagic pathway resulting in accumulation of autophagosomes in which they can survive, thus providing an intracellular niche within professional phagocytes, which ultimately may facilitate dissemination.

While even brief survival within phagocytes is clearly advantageous to the bacterium, the brevity of the life span of phagocytes (in particular neutrophils) calls into question the value of expending a large amount of energy to gain this very transitory niche. This thesis presents the novel finding that *S. aureus* can directly manipulate neutrophil apoptosis in order to extend its survival period within the cell. Neutrophils containing viable *S. aureus* were found to be skewed towards a non-apoptotic state, suggesting that the ability to delay apoptosis facilitates the prolonged intracellular survival of the bacteria. Furthermore, IFN γ – which is produced upon T cell activation by *S. aureus* – was found to inhibit human neutrophil apoptosis both *in vitro* and *in vivo* resulting in the accumulation of neutrophils that provide an intracellular survival niche for the bacteria.

Taken together, these results illustrate that *S. aureus* are capable of manipulating autophagic and apoptotic pathways within the host cell to facilitate and prolong their intracellular survival.

Chapter 1.

Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a Gram-positive cocci bacterium. It is a facultative anaerobe and is distinguishable from other members of the *Staphylococcus* genus by its gold pigmentation (as described by Rosenbach in 1884), production of a thermostable DNase and its ability to ferment mannitol. *S. aureus* strains are predominantly coagulase-positive, which can also be used to distinguish it from other, coagulase-negative *Staphylococcal* species (1). Multilocus sequence typing (MLST) is used to classify *S. aureus* strains into distinct groups, known as clonal complexes (CC) based on allelic variation within seven different housekeeping genes (2). *S. aureus* strains can also be differentiated based on their expression of Capsular Polysaccharide (CP), which aids in immune evasion. The majority of *S. aureus* strains express serotype CP5 and CP8; these account for 25% and 50% of isolates respectively (3, 4). 20-25% of typed strains express no recognisable capsule (4).

S. aureus causes a wide range of infections in humans; from mild superficial infections (boils, impetigo) to more invasive and life threatening disease (osteomyelitis, septicemia). The mortality rate in patients with a *S. aureus* bloodstream infection ranges from 10-30% within 30 days, despite appropriate antibiotic treatment (5) and bloodstream infection with *S. aureus* can lead to metastatic, invasive infections such as endocarditis or pneumonia (6). Infection with *S. aureus* has become increasingly difficult to treat as the bacterium is capable of rapidly developing resistance to antibiotics. The first case of Methicillin Resistant *S. aureus* (MRSA) was reported in 1961 (7),

shortly after the introduction of Methicillin as a response to penicillin-resistant *S. aureus*. In Ireland, 20% of *S. aureus* bloodstream infections presenting in hospitals are resistant to methicillin, as reported by the European Antimicrobial Resistance Surveillance Network (EARS-Net) in 2014. Furthermore, *S. aureus* has continued to develop resistance to other antibiotics. Recently there have been cases reported of *S. aureus* resistance to glycopeptide antibiotics such as Vancomycin, which are widely considered to be the last class of antibiotic available to treat infections caused by this bacterium (8). A further cause for concern is the emergence of Community Associated (CA)- MRSA. Classically, MRSA was seen as a cause of nosocomial infections, particularly in patients with indwelling devices such as catheters (9). However since 2000 there has been an emergence of CA-MRSA among groups of healthy individuals with no healthcare-associated risk factors (10, 11). Strains of CA-MRSA causing infection in these patients are considered to be more virulent than the hospital-acquired MRSA strains, a fact originally attributed to the secretion of Panton-Valentine Leukocidine (PVL) by the CA-MRSA strains. However in recent years, this epidemiologic link has been refuted (12) and the cause of CA-MRSA's increased virulence now seems likely to be due to multiple factors.

In addition to its invasive infective potential, *S. aureus* is an important component of the human microbiome. It predominantly colonises the moist squamous epithelium of the anterior nares, but can colonise a host of other tissues including the skin and the gastrointestinal tract (13). Nasal colonisation is a multifactorial process involving multiple staphylococcal

surface proteins and cell wall factors. In particular it has been shown that the surface protein Clumping factor B (ClfB) interacts with the host squamous cell epithelial protein Loricrin and this interaction is critical for facilitating *S. aureus* colonisation of the anterior nares (14). 20% of the population is permanently colonised with *S. aureus* in the anterior nares, while the remainder are either intermittently colonised or not colonised at all (15, 16). Colonisation is a significant risk factor for invasive infection as *S. aureus* carriers have a higher rate of infection than non-carriers (17, 18), with infection usually attributable to the autologous strain (19). Conversely, there may be some protection conferred by colonisation as *S. aureus* bacteremia-attributable death is reduced in colonised patients compared to non-colonised patients (20). Thus it may be that *S. aureus* exposure through colonisation somehow modulates the immune system, incurring some degree of a protective response upon subsequent infection. The specifics of this protection and its mechanism of action have yet to be fully elucidated.

The increasing prevalence of *S. aureus* infections that are not treatable with available antibiotics is a profound cause for concern worldwide. In fact, there have been reports in recent years describing an emerging “post-antibiotic” era (21), where antibiotics may become obsolete due to the evolution of multi-antibiotic resistant forms of bacteria, such as *Mycobacteria tuberculosis* and *S. aureus*. To prevent the return of high mortality rates from these bacterial infections, significant efforts are being employed to develop treatment programs that represent an alternative to antibiotics, primarily the development of vaccines and immunomodulatory therapies that would

manipulate the immune system to increase bactericidal activity rather than targeting the bacteria directly.

To date, there has been limited success in the development of anti-*S. aureus* treatments. Despite significant efforts all attempts to develop an efficacious anti-*S. aureus* vaccine have failed in various stages of clinical trials. These vaccine strategies have primarily strived to induce a humoral response to *S. aureus* (22). An emerging school of thought in the field is that an antibody response alone is not sufficient to protect against *S. aureus* infection and that a cell mediated immune response must also be generated. A study reporting that B cell deficient mice are equally susceptible to *S. aureus* infection as wild-type mice, while mice deficient in T-cells have increased susceptibility to infection supports this hypothesis (23). Furthermore, a recent study demonstrated that protective responses can be attained using a vaccine against *S. aureus* surface protein Clumping Factor A (ClfA) in the complete absence of an antibody response (24). Evidently, the correlates of protection required for a protective memory response need to be more completely understood before an effective vaccine can be developed. Similarly, without a thorough understanding of all facets of the immune response to *S. aureus*, effective employment of therapeutic immunomodulation will be impossible.

There are two significant hurdles to the development of new therapies for the treatment of *S. aureus* infection. Firstly, as discussed above, there is a lack of understanding of what constitutes an effective anti-*S. aureus* immune

response. Secondly, due to the fact that the entire population is exposed to *S. aureus* at some stage in their lifetime, no one can be considered immunologically naïve. Furthermore, because *S. aureus* has evolved as a commensal organism it has an intricate relationship with the immune system, allowing the development of many, complex immune evasion strategies. *S. aureus* is capable of inhibiting phagocyte recruitment, preventing opsonification and directly killing immune cells (25), as discussed further in later sections. This ability to manipulate the immune response represents a key hurdle that is only beginning to be understood and that must be overcome in the design of new therapies. Clearly, in order to develop effective immunotherapies there needs to be a more lucid understanding of the interactions between *S. aureus* and the host immune system.

1.2 Host immune response to *S. aureus*

1.2.1 Innate immune response

The innate immune response is the first line of defense the host has against all invading bacteria. The innate immune system non-specifically targets pathogenic or foreign material in the body and can destroy it while also initiating an antigen-specific adaptive response.

Once *S. aureus* has breached the body's barriers (skin, mucosa), its pathogen associated molecular patterns (PAMPs) are recognised by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), expressed by cells of the immune system (26, 27), leading to the initiation of the innate immune response. Professional phagocytes (neutrophils, macrophages and dendritic cells (DCs)) are recruited, become activated and contain the infection through phagocytosis. The importance of this recognition by TLRs in *S. aureus* infection is seen in mice lacking TLR2. These deficient mice have increased mortality upon *S. aureus* infection compared to wild type mice (27). Humoral components of the innate immune system that are also important in the innate response to *S. aureus* include the complement system and antimicrobial peptides.

1.2.1.1 Complement

The complement system is an important feature in the innate immune response. It consists of a large number of plasma proteins, the majority of

which are zymogens. These inactive or pro-forms of the proteins are enzymatically cleaved to the active form as part of the complement cascade. Activation of the three complement pathways - the classical pathway, the alternative pathway and the lectin pathway - result similar outcomes; opsonisation of the pathogen by C3b, release of immunomodulatory molecules such as C3a and C5a that can increase inflammation and recruit phagocytes and other immune cells, and the formation of a membrane attack complex which can form pores in the membranes of gram-negative bacteria (28). Clearly, a system like this has the potential to cause significant damage to the host if left unchecked. Thus, the complement system is tightly controlled by several different inhibitory proteins to prevent unwarranted activation (28).

In the context of a *S. aureus* infection, opsonisation by C3b is an important step allowing for the recognition and clearance of the bacteria by monocytes and granulocytes (29, 30). It has also been shown that C3a, C4a and C5a - which are produced as a result of activation of C3, C4 and C5 - are generated in response to Protein A on the surface of *S. aureus* and act as a chemotactic signal to granulocytes (31).

S. aureus has evolved a multitude of techniques to evade complement. These include the secretion of molecules such as staphylococcus complement inhibitor (SCIN), which stabilises the C3 convertase on the surface of the pathogen, preventing further cleavage of C3 (32); extracellular fibrinogen-binding protein (EfB) which binds to C3, inhibiting its adherence to the cell

surface of the bacteria (33) and staphylokinase, which can bind host plasminogen (34) and activate it to form plasmin, a potent serine protease which cleaves surface bound C3b and IgG, thus reducing opsonification and phagocytosis (35).

1.2.1.2 Antimicrobial peptides

Antimicrobial peptides (AMPs) are small amphipathic molecules that are expressed by many different organisms as part of an innate immune system. In humans, the most prevalent AMPs are classed in groups known as cathelicidins and defensins, secreted both by cells of the immune system (e.g. Neutrophils and Macrophages) and by epithelial cells, as well as histatins, which are found in saliva. AMPs can act on many pathogenic organisms including gram-positive and gram-negative bacteria, viruses and fungi. There are several modes of action employed by AMPs to kill pathogens. These include membrane disruption and pore formation, metabolism interference and intracellular targeting of cytoplasmic components. Some AMPs have also been shown to act as a chemoattractant for T cells and dendritic cells (36, 37).

There are multiple AMPs expressed in humans including HNPs1-4 (human neutrophil alpha-defensins 1-4), human β -defensin 3 and LL-37 (a cathelicidin) with demonstrated *S. aureus* killing ability (38-40). A murine homolog of cathelicidin, cathelin-related antimicrobial peptide (CRAMP) has

also been shown to kill *S. aureus* at various concentrations *in vitro* (41) and *in vivo*, although *S. aureus* has been shown to be capable of resisting this killing through the mechanisms of the *dlt* operon (42). These AMPs can kill *S. aureus* in a variety of ways. The α -defensins target the cell membrane of *S. aureus* (43) while human β -defensin 3 is thought to kill through perforation of the cell wall (40).

1.2.2 Phagocytic response to *S. aureus*.

While humoral components of the immune system such as complement and AMPs can opsonify, inhibit and in some cases directly kill bacteria, the key effector arm of the innate immune response responsible for the clearance of bacteria are the phagocytic cells. These short-lived cells respond quickly to infection, phagocytosing and clearing the bacteria from the site of infection. In the case of *S. aureus* infection (a typical extracellular bacteria), the key phagocytes in the host response are neutrophils, macrophages and DCs. Of these, neutrophils are classically considered the archetypal cell of the phagocytic immune response to *S. aureus*.

1.2.2.1 Neutrophils

Polymorphonuclear neutrophils (PMN) or neutrophils, are short-lived phagocytic cells of the innate immune system. They originate in the bone marrow and circulate in large numbers in the blood as mature or nearly mature cells that lack proliferative potential. Their lifespan in the circulation is typically very short (5-6 days in a human (44)). Neutrophils are identified

morphologically by their polylobed nucleus and granular appearance. In mice they are also recognised by the expression of the surface molecules CD11b and Ly6G.

The importance of neutrophils in *S. aureus* infection is exemplified by patients with deficiencies in neutrophil activity. These patients are susceptible to increased pyrogenic bacterial infections, often caused by *S. aureus*. For example, patients who suffer from Chronic Granulomatous Disease (CGD) are deficient in NADPH oxidase, a critical enzyme involved in the generation of reactive oxygen species for O₂-dependent killing in neutrophils. These patients suffer from recurrent *S. aureus* infections (45). Patients who suffer from Chédiak-Higashi syndrome have reduced neutrophil chemotaxis and phagolysosome activity, again resulting in increased occurrence of *S. aureus* infection. Furthermore, mice depleted of neutrophils had increased mortality in response to pulmonary challenge with *S. aureus* compared to immunocompetent mice, illustrating the critical requirement of an effective neutrophil response for resistance to *S. aureus* infection (46).

Neutrophils are a hallmark of acute infection due to their rapid recruitment to the site of infection. Upon recognition of infection, local cells e.g. endothelial cells and macrophages release the chemokine CXCL8 (IL-8). Both secreted and surface associated molecules of *S. aureus* have been shown to be sufficient to induce CXCL8 production. These include lipoteichoic acid (LTA) (47), capsular polysaccharide (48) and secreted toxins such as toxic shock

syndrome toxin 1 (TSST-1) and enterotoxin A and B (49). The production of CXCL8 stimulates granulopoiesis and release of neutrophils from the bone marrow, increasing the number of neutrophils in the circulation available to fight infection. It also creates a chemotactic gradient, which directs neutrophils to the site of infection and allows them to cross from the circulation into infected tissue through a process known as extravasation (28).

Once in the infected tissue the neutrophils recognise the pathogen through various receptors such as PRRs, Fc receptors and complement receptors. PAMPS are specific to pathogens and are recognised by PRRs (24). Fc and complement receptors recognise antibody and complement opsonins respectively. These opsonins have been shown to be vital for efficient clearance of *S. aureus* (50). They bind to the surface of the bacteria specifically, maximising recognition by the neutrophils and enhancing phagocytosis (51). Upon recognition, the neutrophil extends pseudopodia around the pathogen, engulfing it within a membrane-enclosed vesicle, called a phagosome. This phagosome then fuses with a lysosome to form the phagolysosome where bacterial killing takes place via both O_2 -dependent and independent mechanisms. Neutrophils undergo a respiratory burst within the phagolysosome, using NADPH oxidase to generate oxygen free radical species (O_2^- and H_2O_2)(52), which can directly kill the pathogen. Additionally, the neutrophil enzyme myeloperoxidase (MPO) can convert H_2O_2 to $HOCl$, a chemical that is capable of killing bacteria (53). Independently of oxygen, the bacteria can be killed through the actions of microbicidal proteins, such as AMPs and elastase. These are introduced to the bacteria in the

phagolysosome on fusion of the two vesicles, killing the pathogen (See Figure 1.1).

Neutrophils can also kill pathogens extracellularly, through various mechanisms. As previously discussed, they may secrete microbicidal AMPs. Neutrophils can also target extracellular bacteria through the formation of Neutrophil Extracellular Traps (NETs) that consist of chromatin and granule proteins that are released into the extracellular space. This traps the pathogen to limit spread of infection. It also exposes the pathogens to the high levels of antimicrobial molecules in the locality of the neutrophils. These NETs are triggered by many stimuli, including exposure to ROS, activated platelets or bacteria, including *S. aureus* (54, 55).

While critical for the clearance of pathogens, neutrophils may also pose a significant threat to the host as the toxic substances they secrete (such as HOCl and H₂O₂) can cause tissue damage if the cells are left unchecked. Thus, the neutrophil response must be tightly controlled. To prevent an accumulation of superfluous neutrophils, they are constitutively programmed to undergo a form of programmed cell death known as apoptosis (See Section 1.5.1), triggered upon recognition and phagocytosis of bacteria at the site of infection. Apoptotic neutrophils are cleared through phagocytosis by macrophages in a process known as efferocytosis.

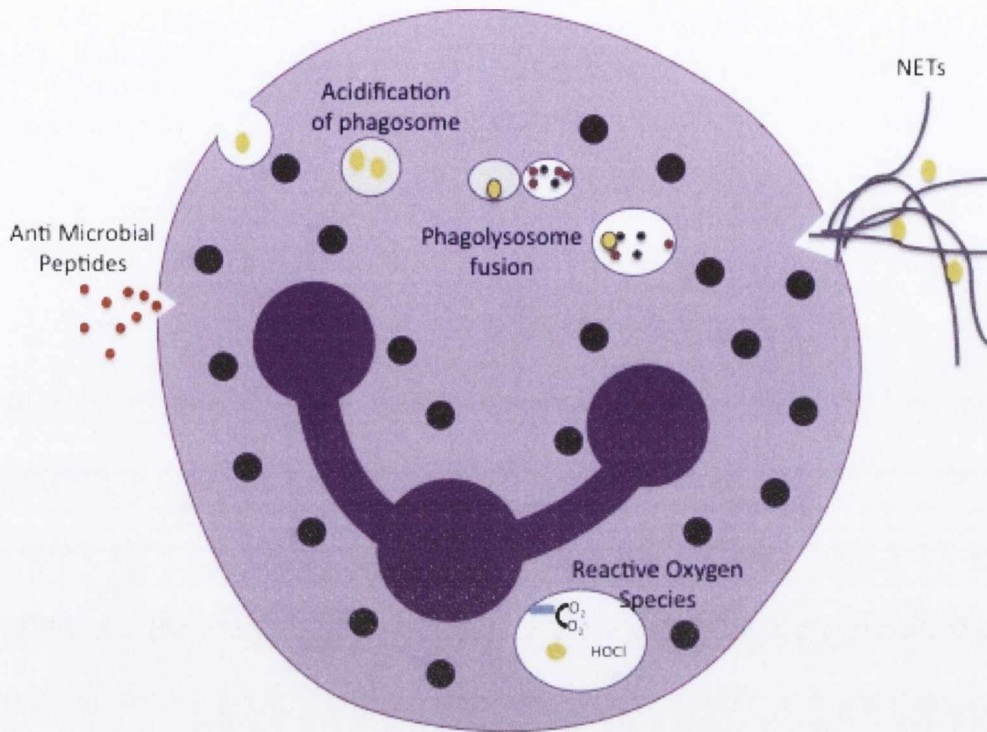


Figure 1.1 Antimicrobial properties of neutrophils.

Neutrophils have multiple mechanisms of killing bacteria. These include extracellular mechanisms such as release of NETs and AMPs and killing of phagocytosed bacteria through phagosome acidification, phagolysosomal fusion and generation of ROS that can directly kill bacteria.

1.2.2.2 Macrophages

Macrophages are mononuclear cells that circulate in the blood as monocytes. They are recruited to the site of infection by chemokines such as CCL2 (MCP-1) (56). When the monocyte encounters a chemotactic gradient or other signaling mechanism it passes through the endothelium into the tissue where it differentiates to become a macrophage (57, 58). Certain macrophages are resident and highly specialised in tissues such as alveolar macrophages

(lungs) and Kupffer cells (liver) (58). Macrophages are identified by the expression of the surface markers CD11b and F4/80 in mice and CD14 and CD16 in humans. Monocytes express high CD14 but low CD16 while mature macrophages express low CD14 and high CD16.

Macrophages recognise pathogens in a similar manner to neutrophils, through ligation of PRRs by PAMPS on the bacterial surface resulting in phagocytosis (59). They also express receptors such as scavenger, Fc and complement receptors that recognise opsonins or ligands expressed on the surface of bacteria (50, 60, 61). As in neutrophils, the phagocytosed pathogen is enclosed within a vesicle termed a phagosome, which then fuses with a lysosome to become a phagolysosome. Macrophages can also kill bacteria using similar mechanisms to neutrophils, through phagosome acidification and phagolysosome fusion (62). Macrophages generate ROS within the phagosome in order to kill pathogens. They also produce nitric oxide (NO) using the inducible form of NO synthase (iNOS), which has been shown to be a requirement for the killing of *S. aureus* by macrophages (63). These methods all contribute to the killing of intracellular bacteria, and bacteria that have been phagocytosed and internalised by the cell.

Macrophages also play an important role in initiating both the innate and the adaptive immune response. Once activated, macrophages secrete a range of immunostimulatory molecules, including AMPs, chemokines (such as CXCL8 which recruits neutrophils) and cytokines (such as IL-1 β which is highly pro-

inflammatory). Production of IL-1 β can act in a positive feedback loop to activate other macrophages in the surrounding area. Macrophages can also initiate the adaptive immune response through their role as professional antigen presenting cells (APCs). Following phagocytosis of the bacteria, macrophages present peptide fragments to the adaptive immune system (specifically CD4 T cells) via the Major Histocompatibility Complex II (MHCII) molecule. Upon activation, murine macrophages become polarised into one of two subsets. M1 macrophages are pro-inflammatory and secrete cytokines such as IL-12, which drives a Th1 type CD4 response (64). They represent the macrophage subset that is responsible for clearance of bacteria (61). In contrast, M2 macrophages have a more regulatory phenotype, secrete IL-10 and play an important role in resolution of infection and wound healing (65).

An important task in the resolution of infection is the removal of apoptotic cells. This is crucial to maintain normal tissue homeostasis by removing dead/dying cells from the tissue and preventing the build-up of debris. It is also a vital component of an effective immune response as the large numbers of neutrophils that are recruited to the site of infection, phagocytose bacteria and then apoptose, need to be cleared away in order to resolve the infection. Apoptotic neutrophils “flip” phosphatidylserine from the inner leaf of the phospholipid bilayer to the outer surface as an “eat-me” signal. This is then recognised by macrophages that phagocytose and degrade the apoptotic cell (66). Eventually, the macrophages themselves will undergo apoptosis and be cleared from the tissue by newly recruited macrophages.

The importance of macrophages in the immune response to *S. aureus* was demonstrated in mice that lack either TLR2 or MyD88. These are important components in the signaling pathway that recognises *S. aureus* peptidoglycan. Both TLR2^{-/-} and MyD88^{-/-} mice have increased susceptibility to infection by *S. aureus*, associated with reduced TNF α and IL-6 production by peritoneal macrophages *ex vivo* (27). The increased mortality and dissemination of bacteria to peripheral tissues observed in mice depleted of macrophages through treatment with clodronate liposomes is further proof that macrophages are vitally important cells in the context of *S. aureus* infection (67). Brown et al. show that macrophage depleted mice have increased bacterial burden both locally and systemically following intraperitoneal infection with *S. aureus* in comparison to mice treated with PBS-filled liposomes, and this was associated with significant mortality in the clodronate treated group (*In press*). Furthermore, inhibition of NOS activity is associated with increased severity of both *S. aureus* septic arthritis and bacteremia as well as reduced bactericidal activity by the macrophages *in vitro* (68).

The specific role of M2 macrophages in *S. aureus* infection has not been fully established, however it has been shown that M2 macrophages are present during chronic rhinosinitis that are associated with impaired phagocytosis (69). Macrophages that infiltrate *S. aureus* biofilms are polarised to an M2 phenotype (70), while colonisation of mice by *S. aureus* causes the induction

of M2 alveolar macrophages (71). These recently published data support an emerging theory that exposure to *S. aureus* in the context of a biofilm infection or perhaps colonisation may allow the bacteria to manipulate the polarisation process of macrophages to induce a regulatory, rather than inflammatory, immune response, thus enhancing its survival.

1.2.2.3 Dendritic Cells

Dendritic cells (DCs) are a third phagocytic effector cell of the innate immune system and act as a bridge linking the innate to the adaptive immune systems. They are found as resident cells in tissues that are exposed to the environment e.g. skin (Langerhans Cells) and gut. DCs can also circulate as immature cells in the blood stream that mature upon ligation of their PRRs by PAMPs (72) .

DCs are continuously sampling the environment through a process called macropinocytosis, where the DCs engulf the surrounding extracellular fluid. This allows them to recognise extracellular bacteria and viral particles. Under homeostatic conditions, these DCs are immature and do not respond to any innocuous antigens. Recognition of a pathogen induces the maturation of the DC, causing upregulation of MHC and accessory molecules, migration of the activated DC to the draining lymph node and presentation of the antigen to CD4 and CD8 T cells of the adaptive immune system.

In order to present antigen, DCs must process peptides and present them bound to MHC for recognition by the T cell receptor (TCR). All cells, including DCs, constitutively express MHCI and are capable of presenting intracellular bacterial or viral peptides on MHCI for recognition by CD8+ T cells. However, as professional APCs, DCs are also capable of phagocytosing pathogens and degrading them in order to present peptides on MHCII for recognition by CD4 T cells.

To then activate adaptive immune cells, DCs must express three different signals. Signal 1 is the interaction between the MHC molecule and the TCR. Signal 2 involves the interaction between costimulatory molecules CD80/86 and CD28 on the T cell. Finally, cytokines secreted by the DC modulate T cell differentiation into different subsets specific to the perceived threat, thereby providing the third signal. For example, the release of IL-12 skews the CD4 immune response towards a Th1 response, specific for cellular immunity against intracellular bacteria and viruses while the release of IL-1 β or IL-6 propagates a Th17 response, which is critical in host defense against extracellular bacteria (72, 73).

It is well established that DCs are important for survival in the context of a bacterial infection as mice depleted of CD11c+ DCs were shown to have increased mortality in response to polymicrobial sepsis induced by cecal ligation, although the mechanism of the protection conferred was not known as bacterial dissemination and cytokine profiles were similar between WT and

DC^{-/-} mice (74). Interestingly, in comparison to neutrophils and macrophages it has been suggested that mature DCs have a reduced ability to kill *S. aureus* as activation enhances antigen presenting functions and limits phagocytosis and bacterial killing. Immature DCs show comparable levels of phagocytosis to macrophages but did not kill *S. aureus* in this study (75). However, there has been limited research into the role of DCs as effector phagocytic cells, focus has instead been on their effects as APCs. In the context of *in vivo* *S. aureus* infection it has been demonstrated that DCs play an important role in coordinating the immune response as DC depleted mice were more susceptible to mortality from infection in a model of bacteremia (76). However, this study suggested that the mechanism of DC protection was not due to direct bactericidal effects by the DCs. Instead it implicated modulation of neutrophil recruitment through altered secretion of cytokines KC and MIP-2 as the mechanism of action of DC-mediated protection.

1.2.3 Adaptive immune response to *S. aureus*, and its downstream effects on phagocytes.

There are two arms of the adaptive immune response that are important in the response to *S. aureus* infection. The first is a humoral response with the production of antigen specific antibodies against *S. aureus*. The second is the cellular response, comprising the recruitment and activation of pathogen specific T cells.

1.2.3.1 Humoral immunity

Antibody mediated protection is an important facet of the immune response to *S. aureus*. Exposure to *S. aureus* through colonisation and infection has been shown to raise *S. aureus* specific antibody titres in the blood (77, 78). These antibodies are important for neutralising toxins (77), inhibiting bacterial adherence to host tissues (79) and facilitating opsonisation of the bacteria to aid in opsonophagocytic clearance (80). As discussed earlier (See Section 1.1), all candidate anti-*S. aureus* vaccines that have thus far failed in clinical trials have exclusively elicited antibody responses (22). These immunisation strategies have generated a strong antibody response against several antigens specific to *S. aureus* (22), however it has been found that these antibodies are not sufficient to confer protection (78, 81). One theory suggests that this may be due to the fact that antibodies to different antigens on the bacteria can neutralise each other, reducing their opsonic effects (82). An emerging school of thought is that antibodies are important in preventing toxin mediated disease as well as the facilitation of opsonophagocytosis of the bacteria but they may not be sufficient to induce protection against infection. This is due to the fact that in order for opsonophagocytic killing to be effective, sufficient numbers of phagocytes need to be recruited to the site of infection, to make use of the increased opsonification of bacteria. While the vaccines generated against *S. aureus* thus far have succeeded in eliciting a substantial antibody response, perhaps they did not induce appropriate phagocytic recruitment and need to modulate T cell cytokine production to facilitate this.

1.2.3.2 Cellular immunity

Recently, studies have identified three specific T cell subsets that are particularly important in protection against *S. aureus* infection in mice. Th1 cells and Th17 cells (which both express $\alpha\beta$ TCRs) and $\gamma\delta$ T cells have all been shown to modulate the phagocytic response to *S. aureus* infection. Mice lacking $\alpha\beta$ T cells have been shown to have a decreased bacterial burden in the surgical wound model of *S. aureus* infection (83). $\gamma\delta$ cells are unique T cells that have a TCR comprised of γ and δ chains instead of the conventional $\alpha\beta$ TCR. Mice lacking $\gamma\delta$ cells had increased morbidity in response to *S. aureus* than their wild type littermates in a model of subcutaneous abscess infection (84), as well as increased bacterial burden at the site of a *S. aureus* surgical wound infection (85). In both models described above, the T cells were shown to mediate their effects through phagocyte recruitment in response to secreted cytokines. IFN γ and IL-17 are the primary T cell cytokines that are known to regulate the phagocyte response to bacterial infection.

1.2.3.2.1 IFN γ

Th1 cells are the primary producers of IFN γ , a cytokine that is critical in the activation of macrophages (86, 87). It is also produced by activated macrophages, acting in a positive feedback loop. IFN γ upregulates MHC expression on macrophages, enhances phagocytosis and primes intracellular bactericidal components such as NO and lysosomal enzymes (88). In terms of the neutrophil response, IFN γ plays a role in modulating recruitment to the site of infection (89) and activation (90). It has also been reported that IFN γ may play a role in modulating neutrophil apoptosis. IFN γ can delay apoptosis

through IL-6 signalling in a murine model of acute peritoneal inflammation (91). In a study of patients with acute coronary syndromes, elevated levels of IFN γ and other cytokines in the blood serum were associated with decreased neutrophil apoptosis. This study also found that IFN γ and other factors found in the sera of these patients such as GM-CSF and soluble mediators from platelets could inhibit neutrophil apoptosis *in vitro* (92).

The specific role of IFN γ in the clearance of *S. aureus* remains ill defined. It has been shown that IFN γ treatment can enhance bacterial killing in a whole blood model *in vitro* (93). This corroborates another report that showed enhanced phagocytosis and bacterial clearance upon IFN γ administration in an *in vivo* septicemia model (94). However, in the same study it was shown that IFN γ can be detrimental in the context of an *S. aureus* septic arthritis model, highlighting the complexities of the role of IFN γ . Yet another study found that IFN γ had no effect on the outcome of primary infection but that it was beneficial in the context of recurrent infection as IFN $\gamma^{-/-}$ mice failed to generate a protective memory response upon re-exposure, compared to WT mice (95). However, there are many studies in the literature that show that IFN γ has detrimental effects on infection outcome. IFN $\gamma^{-/-}$ mice had reduced bacterial burden at the site of a surgical wound infection in comparison to WT mice, and this was associated with reduced neutrophil accumulation, suggesting IFN γ -mediated neutrophil accumulation may be deleterious to the host (83, 89). Furthermore, mortality of IFN $\gamma^{-/-}$ mice was reduced compared to WT mice in the later stages of a *S. aureus* sepsis model, associated with a reduced bacterial burden and lower levels of proinflammatory cytokines (96).

This suggests that at the later stages of sepsis, IFN γ has a deleterious role, inhibiting the clearance of bacteria. Taken together these contrasting results highlight the pleiotropic effects of IFN γ in *S. aureus* infection, which may be dependent upon the nature and location of the infection.

1.2.3.2.2 IL-17

IL-17 is a cytokine that is particularly important in the immune response against extracellular bacteria and fungi. It is produced in an innate manner primarily by $\gamma\delta$ T cells and in response to recognition of cognate antigen by Th17 cells. IL-17 has a well-described role in regulating neutrophil recruitment during infection through its ability to induce CXC chemokines (97) and is also important in neutrophil survival via induction of Granulocyte-colony stimulating factor (G-CSF) (98). It has also been shown to enhance bacterial killing by neutrophils (99). Unlike IFN γ , the role of IL-17 in *S. aureus* infections is well defined. Patients with impaired Th17 responses (and therefore reduced IL-17) due to defects in STAT 3 function have increased incident of *S. aureus* infections (100). IL-17 is produced by both Th17 and $\gamma\delta$ T cells and has been shown to be protective in both the wound and the subcutaneous abscess models of *S. aureus* infection (84, 85). Furthermore, IL-17 production has been identified as the mechanism by which memory $\gamma\delta$ T cells protect against re-exposure to this bacteria (101). Cumulatively these data provide substantial evidence of the positive contribution made by IL-17 in the clearance of *S. aureus*.

The ability of T cells and the cytokines they produce to modulate phagocyte effector functions, and their capacity to induce apoptosis and clearance of phagocytes, indicate important roles for IFN γ and IL-17 in determining the outcome of *S. aureus* infections.

1.3 Immune evasion by *S. aureus*

S. aureus has an immense capacity for evading the immune system, perhaps its key attribute in becoming one of the most lethal infectious agents in Western medicine. It has a formidable repertoire of evasion strategies, targeting many different facets of the host immune response. *S. aureus* is part of the normal human microbiome, colonising the anterior nares and skin (as well as other body sites) of a large proportion of the population, and so has evolved concomitantly with the human immune system. Thus it has developed an array of mechanisms to target both the innate and adaptive arms of the immune response. This includes the inhibition of complement activity, antibody binding and all aspects of the phagocyte response, as discussed in more detail below. In addition, *S. aureus* has developed mechanisms to manipulate adaptive cells such as T and B cells discussion of which is beyond the scope of this thesis.

1.3.1 Evasion of phagocytes by *S. aureus*

S. aureus has become very adept at evading killing by phagocytes, and can inhibit almost every stage of the phagocyte response; recruitment, phagocytosis and killing, as well as being able to kill the phagocyte directly (See Figure 1.2).

As discussed previously, phagocytes are rapidly recruited to sites of *S. aureus* infection in response to chemoattractant molecules such as chemokines (e.g.

CXCL8, CCL2), components of the complement pathway (C5a) and formylated peptides expressed by the growing bacteria. 65% of *S. aureus* strains express the chemotaxis inhibitory protein of staphylococci (CHIPS) (102). This protein can bind to both the C5a receptor and the Formyl peptide receptor (FPR), preventing ligation of the receptor and subsequent signaling to the phagocyte (103). Another protein expressed on the surface of *S. aureus* is the extracellular adherence protein (Eap). This can bind to ICAM-1 on endothelial cells, blocking the binding of LFA-1, a crucial step for the extravasation and recruitment of phagocytes to the site of infection (104).

In addition to the inhibition of phagocyte recruitment, *S. aureus* has the ability to inhibit phagocytosis through multiple mechanisms. Surface-expressed staphylococcal protein A binds to the Fc portion of IgG molecules and orientates them in the wrong direction, preventing recognition by phagocytes (105). *S. aureus* Formyl Peptide Receptor-like 1 Inhibitor (FLIPr) binds to the Fcγ receptor, inhibiting phagocytosis and killing mediated by IgG (106). ClfA and ClfB are fibrinogen-binding proteins expressed on the surface of *S. aureus* that are also thought to be important in the inhibition of phagocytosis. These proteins are highly expressed at different stages of the bacterial growth cycle and are believed to bind to fibrinogen, coating the bacteria and preventing the deposition of opsonins (107, 108). Furthermore, the capsule layer of *S. aureus* has been proposed to be anti-opsonic as it may block access to the cell wall by opsonins and phagocyte receptors, thereby preventing recognition (109). The importance of the capsule in inhibiting phagocytosis remains unclear when compared to other encapsulated bacteria such as

Streptococcus pneumoniae. This bacteria expresses a very thick polysaccharide capsule that has been shown to inhibit phagocytosis (110). The biological importance of this is evident as all clinical isolates of *S. pneumoniae* causing invasive disease are encapsulated (110). In contrast, the most prevalent strain of CA-MRSA in the US, USA300 (111), has no detectable polysaccharide capsule (112), bringing into question the importance of the capsule in promoting *S. aureus*' evasion of the immune system.

Upon phagocytosis however, the bacteria possess the ability to resist the killing mechanisms of the phagocytes in a number of ways. *S. aureus* can secrete proteins that act to inhibit the activity of AMPs. These can act both within the phagosome and extracellularly. The staphylococcal membrane protein MprF and proteins encoded by the *dlt* operon can modulate the bacterial surface to reduce its affinity to the cationic AMPs. Mutants lacking the *mprF* gene or the *dlt* operon are more susceptible to defensin-mediated killing (113, 114). Secreted staphylokinase can bind defensins preventing them from attacking the bacteria (115), while aureolysin inactivates the cathelicidin LL-37 by cleavage (116).

The evasion mechanisms mentioned above, specifically evade oxygen-independent killing by phagocytes, but *S. aureus* is also capable of inhibiting oxygen dependent killing. The carotenoid pigment that gives *S. aureus* its golden colour can scavenge the oxygen free radicals formed during

respiratory burst. A mutant *S. aureus* strain with disrupted carotenoid biosynthesis was more susceptible to oxygen dependent killing and was less virulent in a subcutaneous infection model *in vivo* than a wild type strain (117). *S. aureus* also expresses two superoxide dismutases that remove O_2^- . They dismutate O_2^- to H_2O_2 and O_2 , preventing cell damage. Bacteria deficient in either superoxide dismutase were shown to have reduced virulence in an *in vivo* abscess model, in comparison to wild type strains (118).

S. aureus is capable of escaping NETs through the expression and secretion of nuclease. Bacteria expressing nuclease were found to have delayed clearance from the lung and associated increased mortality in an *in vivo* respiratory tract infection, as well as reducing entrapment by NETs *in vitro*, when compared to a mutant that does not express nuclease (119).

As well as being able to escape the killing mechanisms of phagocytes, *S. aureus* secretes several toxins that are capable of directly killing phagocytes and other leukocytes. There are six leukocidins with different expression profiles across *S. aureus* strains (120). These include leukocidin A/B (LukAB) which binds specifically to CD11b (121), and Panton-Valentine leukocidin (PVL), which can induce necrosis or apoptosis in neutrophils, depending on concentration of PVL present (122). Interestingly, it has been shown that leukocidins display species specificity. For example, human and rabbit neutrophils are highly sensitive to PVL and LukAB, while murine neutrophils are insensitive (123). This could be a factor in the relative insensitivity of mice

to *S. aureus* infection and may be an important factor to bear in mind when drawing conclusions from murine *S. aureus* infection experiments. PVL has recently generated a large amount of research interest following its association to the higher virulence of CA-MRSA (124).

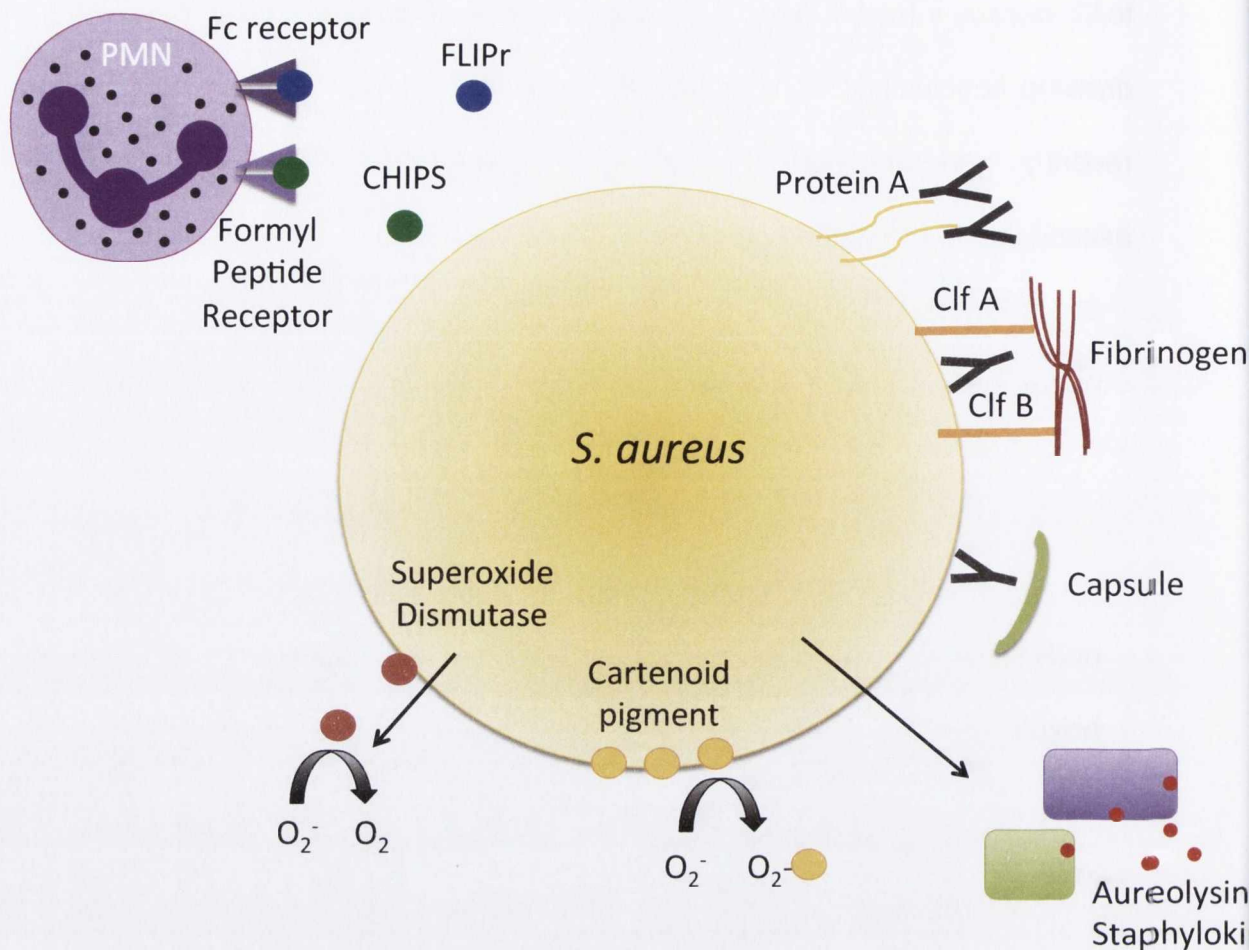


Figure 1.2 *S. aureus* evasion of phagocyte killing.

Mechanisms of phagocyte evasion by *S. aureus*. Phagocyte recruitment is inhibited through the secretion of CHIPS. Phagocytosis is prevented through the binding of antibody in the wrong orientation by Protein A, binding of Fcγ receptor by FLIPr and the shielding of opsonins from recognition by the capsule or by fibrinogen-binding proteins ClfA and ClfB. *S. aureus* can also inhibit phagocyte killing through scavenging oxygen free radicals, dismutating O_2^- and inhibiting the actions of AMPs.

Adapted from (125)

1.3.2 Intracellular survival of *S. aureus* within phagocytes.

It has been well established that *S. aureus* is capable of surviving in non-professional phagocytic cells such as endothelial cells and epithelial cells (126, 127). Furthermore, an increasing literature documents the survival of this bacterium within phagocytes, including monocytes, macrophages and even neutrophils (128-130). This stands to reason given *S. aureus*' proven capacity to evade phagocytic killing. *In vitro* and *in vivo* evidence confirms the survival of *S. aureus* both in the phagosome and free in the cytoplasm of neutrophils isolated from the peritoneal cavity of infected mice (130). These neutrophils contained viable intracellular bacteria when lysed, and transfer of these infected neutrophils was sufficient to establish infection in naïve mice. Intriguingly, the presence of large numbers of neutrophils at the site of *S. aureus* infection has been shown to be associated with increased pathogenesis. Gresham et al. demonstrated that administration of MIP-2 into the peritoneal cavity caused increased neutrophil infiltration associated with increased bacterial burden, while McLoughlin et al demonstrated a similar effect in a surgical model of *S. aureus* infection. Conversely, inhibition of neutrophil infiltration through administration of an anti-CXCR2 antibody resulted in a decrease in bacterial burden in this model (83). These studies suggest that although neutrophils are vital for the efficient clearance of *S. aureus*, elevated numbers of neutrophils present at the site of infection may be detrimental and may actually impede bacterial clearance.

There are also clinical observations to support this theory. In a clinical study that assessed bloodstream infections in cancer patients, patients with reduced

numbers of neutrophils (absolute neutrophil count of ≤ 500 cells/mm³) were found to suffer from significantly fewer *S. aureus* bacteremias than patients with a normal neutrophil count. Other bloodstream infections such as *Escherichia coli* and *Klebsiella pneumonia* were found to be more common in neutropenic patients (131). Neutropenic patients were also found to be less susceptible to metastatic infections than non-neutropenic patients (132). Based on these studies Thwaites et al. have proposed the “Trojan Horse” theory and postulated that the survival of *S. aureus* within neutrophils actually serves as a mechanism for bacterial dissemination around the body (133). It is clear that a balance in neutrophil numbers at the site of infection is key to controlling *S. aureus* infection as patients with no neutrophil functions e.g. CGD patients suffer severe and recurrent *S. aureus* infections. This difference between neutropenic patients and those with dysfunctional neutrophils suggest that while no neutrophil activity is evidently deleterious to the patient, in the context of *S. aureus* infection, reduced numbers of neutrophils seems to be beneficial. It is likely that a critical threshold of neutrophil numbers are required to contain infection, but continued neutrophil recruitment or defective neutrophil apoptosis could propagate infection by providing a survival niche for *S. aureus*.

Macrophages can also act as a reservoir for the bacteria, which replicate inside the cell before lysing the membrane to escape (128). The mechanism by which *S. aureus* directly evades macrophage killing has yet to be elucidated, although it has been shown to be reliant upon Sigma factor B (a regulatory locus that contributes to the control of expression of virulence

factors) and global regulator of *S. aureus* virulence, *agr* (134). *S. aureus* was also found to skew the polarisation of macrophages in biofilm infections. Typically there is little infiltration of phagocytes into the biofilm, however a small population of macrophages can infiltrate but then become polarised to an anti-inflammatory M2 phenotype. This may well be a direct manipulation by *S. aureus* to prevent the generation of an inflammatory milieu within the biofilm (135).

1.4 Apoptosis

1.4.1 Overview of apoptosis

Apoptosis is a mechanism of programmed cell death. It is an essential tool in the maintenance of homeostasis as it allows damaged or unneeded cells to be removed without eliciting an immune response. Apoptosis also plays a major role in the immune system. It is used to return the numbers of cells in circulation or at the site of infection to homeostatic levels following an infective challenge, to remove auto-reactive T cells (136), to kill cells that are cancerous or infected (28) and - crucially in the context innate immunity - to remove cells that have phagocytosed pathogens (137). These processes facilitate the resolution of inflammation.

There are considered to be two main pathways that lead to apoptosis; the intrinsic pathway and the extrinsic pathway (See Figure 1.3), however there is some evidence of “cross-talk” between the two pathways (138). The extrinsic pathway is activated by signals external to the cell, usually by the binding of a death receptor (e.g. FAS receptor binding FAS ligand, TNF- α binding TNFR1). This causes the receptors to cluster, activating the death domain. This then recruits other molecules containing a death domain such as FADD (FAS-associated death domain protein) or TRADD (Tumour necrosis factor receptor type1-associated death domain protein). TRADD then recruits FADD and RIP (Receptor-Interacting Protein). FADD then recruits pro-caspase 8, forming the DISC (death inducing signaling complex) that causes the

autocatalytic activation of caspase 8 from its inactive pro-form. Caspase 8 can then directly activate the effector caspases (139).

The intrinsic pathway is triggered by changes in the inner mitochondrial membrane, caused by positive signals such as hypoxia or toxin exposure. Negative stimuli such as lack of certain growth hormones can also trigger the intrinsic pathway. On receiving a stress signal, the pro-apoptotic molecules BAX, BAK and BID are recruited to the mitochondrial membrane and induce the release of cytochrome c. This binds to Apaf-1 and ATP to form a complex, which recruits caspase 9 and mediated its auto-activation, completing the formation of a complex known as the apoptosome that can activate effector caspases (140).

An example of crosstalk between the two pathways is the cleavage of BID. Caspase 8 is activated as part of the extrinsic pathway and cleaves BID to a truncated form (tBID). tBID can then translocate to the mitochondrial membrane where it induces the release of cytochrome c (138), a key step in the intrinsic apoptotic pathway.

The effector caspases mediate apoptosis through the cleavage of various cellular proteins. Caspase 3 primarily, but also caspases 6 and 7 are considered the effector caspases. These are *cysteine-aspartic proteases* that cleave proteins specifically at aspartic acid (Asp) residues. A feature of

caspases is that they are expressed as inactive zymogens that must be further processed themselves by cleavage at an Asp residue (141). Caspases may be activated by autoactivation, or activation by another caspase. This cascade style of activation allows for the rapid amplification of the signaling pathway. Once activated, these caspases can cleave many substrates including components of the cytoskeleton and nuclear proteins, causing the death of the cell (142). Features of apoptotic cells include translocation of phosphatidylserine to the outer leaflet of the membrane, membrane blebbing, nuclear condensation and DNA fragmentation (143). The dead cell is then phagocytosed and thus removed from the tissue. This is initiated through recognition of phosphatidylserine on the surface of apoptotic cells (144). Recognition of phosphatidylserine allows for the removal of the dead cell with virtually no inflammatory response. This is a vital process in the resolution of infection as neutrophils that have phagocytosed and killed the bacteria can then apoptose and be recognised and cleared by macrophages, without further propagating the immune response (145).

Other proteins in the cell tightly regulate caspase activation and the induction of apoptosis. The Bcl-2 protein family contains several anti-apoptotic proteins that prevent unwarranted activation of apoptosis. Examples of these include Bcl-2, Bcl-XL and Mcl-1 that bind to the pro-apoptotic BAX and BAK, preventing them inducing the release of cytochrome c from the mitochondria.

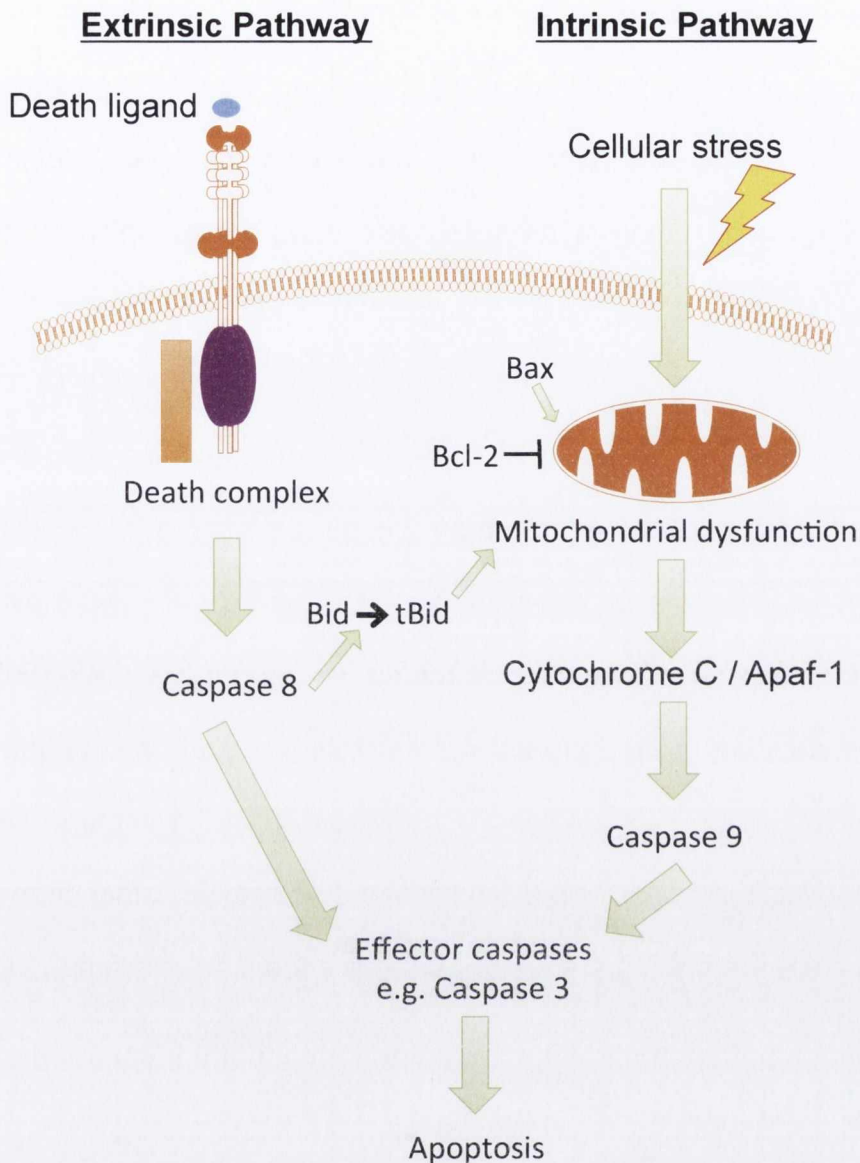


Figure 1.3 Apoptosis pathways.

There are two pathways that execute apoptosis. The first involves the ligation of death receptors leading to the formation of the death complex, which can activate caspase 8. Caspase 8 can then cleave the effector caspases. The second pathway is initiated by cellular stress, causing the release of cytochrome c from the mitochondria, which clusters with Apaf-1. This complex can activate caspase 9 that then joins to the complex to form the apoptosome, capable of activating the effector caspases. Cleavage of BID by Caspase 8 to tBID which induces cytochrome c release, illustrates crosstalk between the two pathways. **Adapted from (146)**

1.4.2 Manipulation of apoptosis by bacteria

It is clear that neutrophil apoptosis plays an important role in the clearance of infection and resolution of inflammation, however some species of bacteria are capable of manipulating neutrophil apoptosis as a mechanism of immune evasion. Kobayashi et al. have shown that some pathogenic bacteria, including *Borrelia hermsii*, *Listeria monocytogenes*, *Burkholderia cepacia* and *Streptococcus pyogenes* are capable of upregulating pro-apoptotic factors during infection of human neutrophils (147). Additionally, *Mycobacterium tuberculosis*, *Escheria coli* and *Candida albicans* have been shown to be capable of inducing apoptosis in neutrophils (148-150). This shrewd manipulation of the immune system kills the neutrophil before it can destroy the pathogen, thus eliminating the threat from the immune system's primary effector cell. Furthermore, by inducing apoptosis rather than simply lysing the cell, the bacteria engage a mechanism designed to limit inflammation and avoid the initiation of any immune response.

In contrast, rather than inducing apoptosis to destroy neutrophils, certain bacteria have evolved mechanisms to delay or inhibit apoptosis to maintain a survival niche within these cells. Bacterial lipoproteins (which are expressed by a diverse range of bacteria including *S. aureus*) were found to inhibit apoptosis in human neutrophils *in vitro* through the inhibition of Caspase 3 activities (151). *Anaplasma phagocytophilum* and *Wolbachia* are primarily intracellular pathogens that can induce expression of anti-apoptotic proteins in neutrophils, which inhibit Caspase 3 activity (152, 153). Inhibition of neutrophil

apoptosis prolongs the survival of the cell, conserving an intracellular niche for the pathogen.

S. aureus also has the potential to manipulate the apoptosis pathway. It has been shown that this pathogen can induce apoptosis in several cell types, including epithelial and endothelial cells through upregulation of pro-apoptotic factors and activation of Caspase 3 and 8 (126, 154). PVL, the pore forming toxin expressed by many strains of *S. aureus*, can induce apoptosis in neutrophils at low concentrations (122). Heat-killed *S. aureus* has also been described as capable of inducing apoptosis in neutrophils (155). Conversely, one study has shown *in vitro* that at low Multiplicities of Infection (MOIs), *S. aureus* is also capable of inhibiting neutrophil apoptosis, although this study did not see an effect at any MOI higher than 1, nor did it examine any *in vivo* model of infection (156). Similarly, *S. aureus* has been shown to inhibit apoptosis in macrophages in an *in vitro* model of infection. This inhibition was mediated through upregulation of anti-apoptotic factors including Bcl-2 and Mcl-1 (129, 157).

1.5 Autophagy

1.5.1 Overview of Autophagy

Autophagy is an important homeostatic process in all cell types. It is responsible for the turnover of cellular components by targeting organelles and constituents of the cytoplasm for degradation by the lysosome.

Three types of autophagy have been identified. Microautophagy occurs when the lysosome itself samples small amounts of cytosol (158). Chaperone-mediated autophagy refers to the degradation of proteins that have been targeted through binding of cytosolic chaperone proteins (159). The third, and key mechanism of autophagy is macroautophagy (hereafter referred to as autophagy). This process involves the formation of an autophagosome. An isolation membrane (also called the phagophore) forms around the cytoplasmic constituent to be sequestered and elongates, eventually closing to form the double membrane vesicle termed the autophagosome, containing the component to be degraded (160). The outer membrane of the autophagosome then fuses with the lysosome forming the autolysosome, allowing for the degradation of the autophagosomal components by the lysosomal enzymes (161). Several proteins are useful markers of autophagy including Beclin 1 (a signaling protein) and LC3 (a protein associated with the autophagosomal membrane)(162). This form of autophagy occurs at a persistent basal level, but is upregulated in response to stress conditions such as starvation.

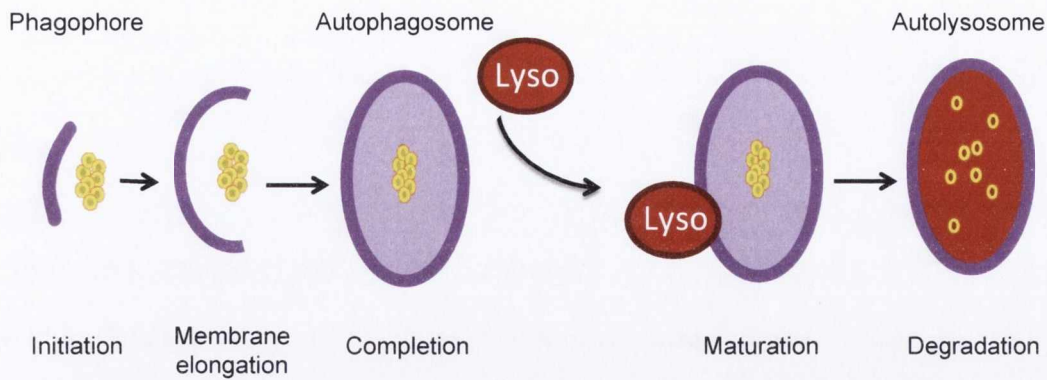


Figure 1.4 Initiation of autophagy in response to intracellular bacteria.

Initially, a phagophore forms around the cytoplasmic component to undergo autophagy (e.g. intracellular bacterium). It then elongates and fuses to enclose the bacterium within an autophagosome. This can fuse with a lysosome to form an autolysosome in which the bacterium is degraded. **Adapted from (163)**

Cells also use the autophagic pathway to destroy intracellular bacteria in a process known as xenophagy (161). Xenophagy is triggered as a defense response to bacteria that invade the cytosol of the cell. It can also be considered a safety mechanism that acts when first defenses (i.e. phagocytosis and phagolysosome fusion) fail. If bacteria escape from within the phagosome or damage the phagosomal membrane, an autophagic membrane forms around the bacteria, enclosing it within an autophagosome to fuse with a lysosome and destroy the invading pathogen. Most studies that focus on xenophagy have been conducted in macrophages, while there has been very little research into the role played by xenophagy in the response to bacteria by neutrophils. Neutrophils have been shown to induce autophagy in

response to both *E. coli* (164) and *B. pseudomallei* (165) infections as a means of killing the bacteria.

Killing of many bacterial species by autophagy in macrophages and non-phagocytic cells has been demonstrated. Group A *streptococcus* (GAS), while not a classical intracellular bacteria, is able to escape into the cytosol of the cell once it has been internalised. It is then trapped within the autophagosome, preventing replication and eventually killing the bacteria (166). *Mycobacterium tuberculosis* (*Mtb*) is also killed through interaction with the autophagic machinery. This mycobacterium prevents the fusion of the phagosome to the lysosome once it has been phagocytosed, and can survive and replicate within the phagosome (167). Autophagy is triggered in response to recognition of damage to the phagosome and engulfs both the bacteria and the phagosome, trapping it within the autophagosome, which can in turn fuse to the lysosome to kill the bacteria (168).

1.5.2 Manipulation of autophagy by bacteria

Bacteria have evolved many mechanisms of evasion of autophagy. Both *Shigella* and *L. monocytogenes* are capable of evading recognition by proteins that target them to the autophagosome. *L. monocytogenes* achieves this by camouflaging itself with its surface protein InlK while *Shigella* masks itself using the secreted bacterial protein IcsB (169-171). Other bacteria, such as *Legionella pneumophila* have been shown to inhibit autophagy entirely, through the expression of a protein known as RavZ, which is capable of

disrupting the autophagic pathway (172). Yet other bacterial strains including *Brucella abortus* and *Coxiella burnetii* can halt autophagosome/lysosome fusion at various stages throughout this pathway in order to replicate within the autophagosome (173).

Recent studies have shown that *S. aureus* is capable of subverting autophagy. α -haemolysin (Hla) produced by *S. aureus* has been shown to induce autophagy in rodent cells *in vitro* (174). Autophagy can also be induced in HeLa cells infected with *S. aureus*. Treating the cells with wortmannin to inhibit autophagy, or using *atg5*^{-/-} immortalised macrophages showed that inhibiting autophagy reduced the replication of the bacteria. This suggests that *S. aureus* is able to persist and multiply within the autophagosome (175). This engagement with autophagy results in the creation of an intracellular niche in which *S. aureus* can survive.

1.5.3 Crosstalk between apoptosis and autophagy.

Apoptosis and autophagy are two dynamic pathways and there has been a significant effort to understand the complex relationship between them. These distinct signaling processes are often activated sequentially within the same cell. Upon exposure to a stressor, autophagy is initiated to promote survival of the cell and this inhibits the induction of apoptosis. However, if the stress exceeds a critical threshold, apoptosis is then induced which can inhibit the autophagic pathway through caspase cleavage of autophagic proteins (176). In rare circumstances, autophagy has been shown to induce apoptosis. Autophagosome formation may provide a platform that allows for the cleavage of caspase 8, which cleaves effector caspase 3 to induce apoptosis (177).

Two molecules that play a key role in the cross talk between the apoptotic and autophagic pathways are ATG5 and Beclin 1. ATG5 is required for the formation of the autophagosome and thus is a pro-autophagic protein. However, upon exposure to a stressor, ATG5 is cleaved by calpain to generate a 24-kDa product. This can translocate to the mitochondria to bind with the anti-apoptotic BCL-x_L and to induce mitochondrial outer membrane permeabilisation (178), thus switching from pro-autophagic to pro-apoptotic.

Beclin 1 is another protein that is essential for the induction of autophagy. Phosphorylation of Beclin 1 by ULK1 protein kinase allows for the formation of a protein complex that initiates the generation of the phagophore. Interestingly, it has recently been shown that apoptosis can damage Beclin 1,

inhibiting the induction of autophagy. Cleavage of Beclin 1 by caspases results in products that cannot induce autophagy. Furthermore, a cleaved portion of Beclin 1 is capable of translocating into the mitochondria to act as a pro-apoptosis stimulus, thus switching from pro-autophagic to pro-apoptotic (179). Anti-apoptosis proteins of the Bcl-2 family bind and inhibit Beclin 1 adding a further level of cross talk between these two pathways. Inhibition of autophagy or apoptosis seems to be organelle-specific, with Bcl-2 proteins binding Beclin 1 to inhibit autophagy at the endoplasmic reticulum, while they exert their anti-apoptotic effects at the mitochondrial membrane (180).

1.6 Project aims

The immense immune evasion capacity of *S. aureus* causes untold difficulties in the search for new immunomodulatory therapies to treat this prevalent infection. While phagocytic cells are the immune system's key mechanism of defense against this bacterium, *S. aureus* has evolved an incredible array of evasion strategies against these cells. Neutrophils and macrophages are considered the archetypal phagocytic cells in the response to bacterial infection and the role of other phagocytes beyond neutrophils and macrophages has been largely unexplored in the context of *S. aureus* infection. Furthermore, the ability of *S. aureus* to survive within neutrophils is now well established, but the benefits of survival within such a short-lived cell seem negligible if *S. aureus* cannot modulate its death.

This project aims to establish whether *S. aureus* can parasitise phagocytic cells to provide a survival niche through manipulation of both the autophagic and the apoptotic pathways, or both . The project consists of two specific aims:

Aim 1: Investigate the role of dendritic cells in the immune response to *S. aureus* and determine if manipulation of autophagy can promotes intracellular survival within these phagocytes.

Specifically this section will:

1. Establish a role for dendritic cells in the direct killing of bacteria.

2. Identify autophagy as means by which *S. aureus* can evade phagocytic killing intracellularly and ascertain the relevance of this engagement with autophagy *in vivo*.

Aim 2: Establish if modulation of neutrophil cell death propagates *S. aureus*' intracellular survival.

Specifically this section will:

1. Determine whether *S. aureus* can modulate the apoptosis of human neutrophils.
2. Ascertain whether modulation of neutrophil apoptosis promotes intracellular survival of the bacterium
3. Assess whether cytokines produced by T cells in the course of *S. aureus* infection play a role in the manipulation of neutrophil apoptosis.

Chapter 2.

Materials and Methods.

2.1 Materials

Table 2.1 General Reagents

Name	Recipe
Tryptic Soy Agar (TSA)	30g Tryptic Soy (TS) Broth (Sigma Aldrich) 15g Agar (Sigma Aldrich) 1L dH ₂ O
Columbia Soy Agar (CSA)	35g Columbia Soy (CS) Broth (Sigma Aldrich) 15g Agar 20g NaCl (Sigma Aldrich) 1L dH ₂ O
cRPMI Media	500ml RPMI (Sigma Aldrich) 10% (v/v) Fetal Calf Serum (FCS; Biosera) 100mM L-Glutamine (Sigma Aldrich) 100µg/ml penicillin/streptomycin (Sigma Aldrich)
cDMEM Media	500ml DMEM (Sigma Aldrich) 10% (v/v) FCS 100mM L-Glutamine 100µg/ml penicillin/streptomycin
Ammonium Chloride Lysis Buffer	8.7g NaH ₄ Cl (Sigma Aldrich)

	1L dH ₂ O
10X Transfer Buffer	30g Tris (Sigma Aldrich) 144g Glycine (Sigma Aldrich) 1L dH ₂ O
10X PBS	80g NaCl (Sigma Aldrich) 7.62g Na ₂ HPO ₄ (Sigma Aldrich) 0.77g KH ₂ PO ₄ (Sigma Aldrich) 2g KCl (Sigma Aldrich) 1L dH ₂ O pH 7.2-7.4
FACS Buffer	.1% (w/v) BSA (Sigma Aldrich) 1X PBS
1M NaOH	40g NaOH (Sigma Aldrich) 1L dH ₂ O
PBS-Tween	1L 10x PBS 9L dH ₂ O 5ml Tween-20 (Sigma Aldrich)

10X Running Buffer	30.3g Tris 144g Glycine 10g SDS (Sigma Aldrich) 1L dH ₂ O pH 8.3
Permeabilisation Solution	50mg Sodium Citrate (Sigma Aldrich) 50 µl Triton-X 100 50ml PBS
EB/AO	2.5g Ethidium Bromide (Sigma) 2.5g Acridine Orange (Sigma) 50ml 1x PBS
20X MES Buffer	213.2g MES (Melford Labs) 121.2g Tris 20g SDS 6g EDTA (Sigma Aldrich) 1L dH ₂ O

Table 2.2 Additional Reagents

Reagent	Supplier
PVDF Membrane	Millipore
PBS	Sigma Aldrich
Poly-L-lysine	Sigma Aldrich
16% Paraformaldehyde	Thermo Fisher
Dextran	Sigma Aldrich
Saline	Baxter
Ficoll	Fisher
Trypan Blue	Sigma Aldrich
Kiovig (Human IVIG)	Baxter
Guinea Pig Complement	vhbio
Ketamine	Supplied by TBSI Comparative Medicine
Xylazine	Supplied by TBSI Comparative Medicine
Puromycin	Sigma Aldrich
Gentamicin	Sigma Aldrich

Collagenase IV	Sigma Aldrich
Protease Inhibitor Cocktail	Sigma Aldrich
Phenylmethanesulfonyl fluoride (PMSF)	Sigma Aldrich
Bradford reagent	Sigma Aldrich
Histopaque 1083	Sigma Aldrich
Western Blot Standards (SeeBlue)	Invitrogen

Table 2.3 Recipe for 15% gels

	15% Resolving gel (makes 2)	Stacking gel (makes 2)
30% Acrylamide (Fisher)	7.5ml	0.67ml
dH₂O	3.4ml	2.7ml
1.5M Tris	3.8ml	0
1M Tris	0	0.5ml
10% SDS	150µl	40µl
10% APS (Sigma Aldrich)	150µl	40µl
Temed (Sigma Aldrich)	6µl	6µl

Table 2.4 Recombinant Cytokines

Cytokine	Supplier	Concentration
IL-17	Peprotech	100ng/ml
IFN γ	Peprotech	100ng/ml

Table 2.5 FACS antibodies

Antibody	Fluorochrome	Clone	Company
CD11b	APC-eFluor780	M1/70	eBiosciences
Ly6G	Brilliant Violet 421	1A8	BioLegend
Ly6G	PE	1A8	BD
F4/80	PeCy7	BM8	eBiosciences
CD11c	FITC	N418	eBiosciences
CD11c	eFluor450	N418	eBiosciences

Table 2.6 Kits

Kit	Supplier
In situ cell death detection kit (TUNEL)	Roche
Annexin V staining kit	BD
LDH cytotoxicity reaction kit	Thermo Fisher

Table 2.7 Western Blotting Antibodies

Antibody	Species Reactivity	Source	Supplier
Caspase 3	Mouse/Human	Rabbit	Cell Signalling
LC3	Mouse/Human	Rabbit	Cell Signalling
β-actin HRP	Mouse/Human	Mouse	Sigma Aldrich
Anti-rabbit IgG HRP	Rabbit	Goat	Jackson Immune

2.2 Mice

Wild type C57BL/6 mice were purchased from Harlan at 6-8 weeks old. They were housed 5 per cage under specific pathogen free (SPF) conditions in the TCD Comparative Medicines facility. IFN γ ^{-/-} mice were bought from Jackson Laboratory and bred in-house. All animal experiments were conducted in accordance with the recommendations and guidelines of the Health Products Regulatory Authority (HPRA), the competent authority in Ireland, and in accordance with protocols approved by Trinity College Animal Research Ethics Committee.

2.3 Bacteria

All bacterial work was done in a Class II hood

2.3.1 Bacterial strains

S. aureus strains SH1000 (Clonal complex (CC) 8, a-capsular) and PS80 (CC30, Capsular Polysaccharide (CP) 8) have been previously described (181, 182). *S. aureus* clinical isolates were from blood culture bottles of patients diagnosed with *S. aureus* bacteremia at St George's Healthcare NHS Trust, London. Two isolates were used repeatedly throughout this study; Sa68 and Sa279. Both of these strains are methicillin sensitive and belong to the lineage CC1.

2.3.2 Preparation of bacteria.

All strains of bacteria were grown to stationary phase from frozen stocks on agar plates overnight. PS80, PS80 Δ *agr* and RMS-1 were grown on CSA plates, while all other strains were grown on TSA. Following overnight growth, bacteria were suspended in 6mls of PBS and vortexed thoroughly. The optical density (OD) of the bacterial solution at 600nm was determined using a spectrophotometer. The OD_{600nm} of the solution was then adjusted to 0.45 with PBS, which has been shown to be $\sim 4 \times 10^8$ colony forming units (CFU)/ml (83). The solution was then diluted with PBS to the

desired concentration (CFU/ml). The inoculum was confirmed by streaking serial dilutions onto TSA plates and counting the number of colonies after 24 h incubation.

In the case of PS80-GFP, log phase growth was required for optimal GFP expression. A single colony was inoculated into TSB and incubated with shaking overnight and subcultured into fresh TSB the following morning and allowed to grow until it reached an OD_{600nm} of 0.45nm. At this point, the bacteria were in log phase growth and were at $\sim 4 \times 10^8$ CFU/ml. Again, the solution was diluted with PBS to the desired concentration (CFU/ml) and the innoculum was confirmed by streaking serial dilutions onto TSA plates.

For immunoflourescent analysis, bacteria were stained with Cell Trace Violet (CTV, Life Technologies). Stationary phase bacteria were diluted to the aporopriate concentration in PBS then, incubated with CTV for 20 min at 37°C under rotation. They were then washed and resuspended in PBS prior to infection of cells.

2.4 Cell culture

2.4.1 Generation of Bone Marrow-derived Dendritic Cells (BMDCs)

BMDCs were generated by culturing bone marrow cells from wild-type C57BL/6 mice as described previously (183). Briefly, mice were euthanised and their hind legs dissected from the muscle. Bone marrow was flushed out with cRPMI (complete Roswell Park Memorial Institute media) using a 27 guage needle and 5ml syringe. Cells were centrifuged (1200rpm, 5 min) then resuspended in 5ml 0.87% ammonium chloride at RT for 2 min to lyse the erythrocytes before being centrifuged again, resuspended in 10ml of cRPMI and counted in Trypan blue. Cells were seeded in

T175 flasks at 1×10^6 cells/ml in cRPMI containing 20ng/ml GM-CSF (supernatant from GM-CSF secreting J558 cell line). After 3 days in culture, 15ml of media containing 20ng/ml GM-CSF was added to each flask. On day 6 of culture, the media was gently removed and discarded and 15ml of fresh cRPMI with 20ng/ml of GM-CSF added, taking care not to disrupt the cells that were adhered to the bottom of the flask. On day 8, another 15ml of cRPMI with 20ng/ml GM-CSF was added to each flask. On day 10, loosely adherent cells were removed by gentle pipetting of the supernatant across the base of the flask and the collected media was centrifuged at 1200rpm for 5 min. These BMDCs were counted using Trypan Blue staining and resuspended in antibiotic-free RPMI with 10ng/ml GM-CSF. Cells were plated at 2×10^5 cells/well in 96-well plates for killing assays, 1×10^6 cells/well on poly-L-lysine coated 19mm coverslips in 12-well plates for confocal microscopy or 3×10^6 cells/well in 6-well plates for western blotting and incubated overnight to rest.

2.4.2 Generation of Bone Marrow-derived Macrophages (BMMs)

BMMs were generated by culturing bone marrow cells from wild-type C57BL/6 mice as described previously (184). Briefly, mice were euthanised and their hind legs dissected from the muscle. Bone marrow was flushed out with complete Dolbecco's Modified Eagle Medium (cDMEM) using a 27 gauge needle and 5ml syringe. Cells were centrifuged (1200rpm, 5 min) then resuspended in 5ml 0.87% ammonium chloride at RT for 2 min to lyse the erythrocytes before being centrifuged again, resuspended in 10ml of cDMEM and counted in Trypan blue. Cells were seeded in petri dishes at 1×10^6 cells/ml in 10ml of cDMEM containing 20% L929-conditioned medium (which contains M-CSF). After 3 days in culture, 1 ml of cDMEM containing 20% L929-conditioned medium was added to each dish. On day 6, the media was replaced with fresh DMEM, the cells were scraped to remove them from the bottom of the dish, centrifuged (300g, 5 min) and resuspended in cDMEM containing 10%

L929-conditioned medium. These BMMs were counted in Trypan Blue and plated at 2×10^5 cells/well in 96 well plates. After overnight resting, the media was replaced with DMEM containing no antibiotics.

2.4.3 Culture of iBMMs

Immortalised BMMs (iBMMs) stably expressing EGFP-LC3 (GFP-LC3) were cultured in cRPMI under constant selection with 10µg/ml puromycin as previously described (185). Cells were seeded at 1×10^6 cells/well on poly-L-lysine coated 19mm coverslips in 12 well plates.

2.4.4 Isolation of human neutrophils

Blood was obtained from healthy volunteers following informed consent and in accordance with local institutional policy and human neutrophils isolated. Briefly, 30ml of blood was collected into heparinised tubes and the neutrophils isolated by sequential sedimentation. Initially, cells were sedimented in 15ml of 3% dextran in saline for 30 min at RT, then layered onto 10ml Ficoll and centrifuged (1400rpm, 20 min with no brake). Remaining erythrocytes were removed from the pellet by lysis with 0.87% ammonium chloride for 11 min. Neutrophils were then counted in Trypan Blue and resuspended to 1×10^7 cells/ml in antibiotic free DMEM.

2.5 Infection of phagocytes

2.5.1 BMDCs and BMMs

2.5.1.1 Infection of BMDCs or BMMs

Cells were infected with live *S. aureus* at multiplicities of infection (MOI) of 10 or 100 for the indicated times. As BMDCs and BMMs express many PRRs (in particular TLRs) with which they can recognise bacteria (186), no opsonins were added. In some cases, prior to infection cells were incubated with 10mM 3-Methyladenine (3-MA, Sigma) for 30 min. At 2 h post infection media was replaced with fresh media containing gentamicin (200 µg/ml) for 1 h to kill extracellular bacteria. This media was replaced with fresh media containing no antibiotics and this was considered time 0.

2.5.1.2 Assessment of bacterial killing by BMDCs or BMMs

For assessment of total killing, cells were infected with live *S. aureus* at MOI 10 or 100 for the indicated time points and were not gentamicin treated. At the indicated time point, infected cells were spun down, the supernatant removed and cells lysed by the addition of 20µl 0.1% Triton-X 100. The supernatant was then re-introduced into the well and mixed with the cell lysate. Serial dilutions of the suspension were prepared in PBS and plated on TSA to determine the CFU/ml. Bacterial killing was determined as the % reduction of CFU in wells containing bacteria and phagocytes as compared to wells containing bacteria only.

2.5.1.3 Assessment of bacterial escape

Cells were infected with *S. aureus* as described above (Section 2.5.1.1). At specific time points the cell free supernatants were collected, serially diluted in PBS and

plated on TSA to determine the number of bacteria that had escaped into the media. This was then expressed as the fold increase in Log CFU/well from time 0.

2.5.2 Human neutrophils

2.5.2.1 Infection of human neutrophils

Cells were seeded at 2.5×10^6 cells/eppendorf in DMEM. In some cases, prior to infection cells were incubated with 10mM 3-MA for 30 min. Directly before infection, cells were treated with 100ng/ml cytokines if required. Cells were then infected with *S. aureus* at an MOI of 10. As neutrophils target bacteria for phagocytosis through ligation of Fc and complement receptors (187), the bacteria were opsonised by the addition of human IVIG (1:10 dilution in PBS) and Guinea Pig complement (1:10 dilution in media) to the reaction mixture and the total volume in the eppendorf was equalised to 500 μ l. The cells were then incubated at 37°C, 5% CO₂ under rotation for the appropriate length of time.

2.5.2.2 Assessment of bacterial killing by neutrophils

At specified time points, 20 μ l aliquots were removed from the reaction mixture, serially diluted in PBS and plated on TSA to determine the CFU/ml. Bacterial killing was determined as the % reduction of CFU in eppendorfs containing bacteria and neutrophils as compared to eppendorfs containing bacteria only.

2.5.3 Analysis of viability

2.5.3.1 LDH assay

To assess *S. aureus* induced cytotoxicity, BMDCs, BMMs or human neutrophils were infected as described above (See Section 2.5.1.1 and 2.5.2.1 respectively). At

specified timepoints, the plates were centrifuged (300g, 5 min) and the cell-free supernatant collected. LDH release into the supernatant was measured using the Pierce LDH Cytotoxicity Assay kit according to the manufacturer's instructions.

2.5.3.2 PI staining

Propidium Iodine (PI) staining was also used to assess viability. BMDCs were infected as described above (Section 2.5.1.1). At specified timepoints, cells were removed from the plate by gentle pipetteing and resuspended in PBS in FACS tubes. PI (1µg/ml) was added and the sample was immediately assessed by flow cytometry.

2.5.3.3 EB/AO staining

To determine the viability of human neutrophils, 10µl aliquots were removed from the reaction mixture, diluted 1:20 in EBAO, and % of the total cells that were alive determined by comparing the number of green cells (live) to those that stained red (dead).

2.6 Intra-peritoneal (i.p.) infection model

Mice were infected with *S. aureus* (5×10^8 CFU) via i.p. injection. At specified time points post-infection the peritoneal cavity was lavaged with 2ml sterile PBS. For FACS analysis, the total peritoneal lavage was centrifuged (300g, 5min) and the cells resuspended in Fcy block for counting and surface staining.

To determine the total bacterial load in the peritoneal cavity, an aliquot of lavage fluid was serially diluted in PBS and plated on TSA. Alternatively, to assess the intracellular bacterial burden, the peritoneal exudate cells (PEC) were isolated by

centrifuging the lavage fluid (300g, 5 min) and resuspending the cells in 1ml RPMI containing 200µg gentamicin for 1 h. Cells were then washed, lysed in sterile H₂O, plated on TSA to determine the number of viable bacteria.

To determine the bacterial dissemination around the host, spleens and livers were isolated and homogenised in 2ml of sterile PBS using a homogeniser (Kinematica Polytron PT 2500E) with a 7mm blade. The homogeniser was cleaned between samples using 1M NaOH, sterile H₂O, 70% (v/v) EtOH and sterile PBS. Tissue homogenates were then serially diluted in PBS and plated on TSA to determine the tissue bacterial burden. Blood was collected by cardiac puncture with a 27-gauge needle and a heparinised 1ml syringe. The CFU/ml of blood was determined by serial dilution and plating on TSA plates.

To isolate blood leukocytes, blood was layered onto Histopaque-1083 (Sigma) for density gradient centrifugation at 400g for 30 min. Leukocytes were collected between the plasma layer and the pellet containing red blood cells (RBCs) and extracellular bacteria. As density gradient centrifugation separates cells based on their densities, the extracellular bacteria collect in the pellet with the RBCs (188). Isolated leukocytes were then washed well and resuspended in Fcy block for flow cytometric analysis or lysed in sterile water to quantify cell-associated CFU.

2.7 Surgical Wound model

2.7.1 Surgery

Surgical wound infection was established in mice as previously described (83). Briefly, mice were anaesthetised with ketamine (approx 3mg/mouse) and xylazine (approx 0.3mg/mouse), their hind leg shaved and disinfected with Iodine. The thigh

muscle was exposed and an incision made to the depth of the bone. The incision was closed with one silk suture and 10µl of the bacterial solution introduced under the suture. The skin was then closed with three prolene sutures. At appropriate timepoints the mice were euthanised with CO₂ and cervical dislocation and the infected muscle tissue excised.

2.7.2 Isolating cells from surgical wound for FACS

After excision, the wound was chopped into several pieces, collected in media containing Collagenase IV (0.5mg/ml) and incubated at 37°C under rotation for one hour, then filtered through 40µM cell filters and resuspended in Fcy block. The cell suspension was then counted and stained for flow cytometric analysis.

2.7.3 Determination of CFUs at wound

After excision, the muscle tissue was weighed and homogenised in 1 ml TSB. Serial dilutions were plated on TSA and left to grow at 37°C for 24 hours. Colonies were counted and adjusted to represent CFU/g of tissue.

2.8 Western Blotting

2.8.1 Preparing cells lysates

For analysis of protein expression by BMDCs, the cells were plated in a 6-well plate, with one well per condition. At the indicated timepoint, 50 µl of NP-40 lysis buffer containing Protease Inhibitor Cocktail and PMSF was added to each well and the cells scraped and removed to an eppendorf. Cells were incubated on ice for 10 min

to lyse and then the lysates were centrifuged (14000rpm, 10 min) at 4°C to remove debris.

2.8.2 Bradford Assays

Bradford Assays were used to determine the protein concentration of the samples. 1µl of sample was added to a well of a 96-well plate (in duplicate). A standard curve was generated using 1mg/ml BSA in NP-40 lysis buffer, with 0,1,2,3,4 and 5µl added to the well. 150µl of Bradford reagent was added to each well and incubated for 10 min in the dark. The plate was then read using an ELISA plate reader. The concentration of each sample was then known and could be used to standardise the samples. At least 30µg of protein was added to each well of the electrophoresis gel.

2.8.3 SDS-PAGE

Prior to running the gel, the BMDC lysates were diluted 1:2 with loading buffer containing 5% β-Mercaptoethanol. They were then boiled at 100°C for 5 min and centrifuged for 10 seconds. 15% gels were cast using the Bio-Rad system (See Table 2.3). Gels were inserted into the gel dock and the dock was filled with running buffer. 15µl of molecular weight marker was loaded into the first lane of each gel and then 24µl (~30ug protein) of each sample loaded. The gels were run at 120 volts until the samples were beginning to run off the gel.

2.8.4 Transfer to polyvinylidene difluoride (PVDF) membrane

The PVDF membrane was activated by soaking in methanol for ~20 seconds. It was then soaked in transfer buffer, as were two sheets of filter paper. The gel was removed from the dock and the gel itself removed from the glass cast. It was soaked

in transfer buffer before the “transfer sandwich” was assembled and inserted into the transfer dock which was filled with transfer buffer. The transfer was run at 200mA for 1 h 15 min.

2.8.5 Blocking and probing the membrane

After transfer, the membrane was removed and blocked to prevent non-specific binding of antibody. PBS-Tween (PBST) was used as a wash buffer throughout probing. The membrane was blocked in 5% dried milk solution in PBST for 45 min, then incubated with primary antibody (Rabbit anti-Caspase 3 or Rabbit anti-LC3) 1:1,000 in 5% milk-PBST, overnight at 4°C under constant agitation. The membrane was then washed 3 times in PBST, before incubation with secondary antibody (Goat anti-rabbit) 1:10,000 in PBST for 2 h at RT. The membrane was again washed with PBST then developed with ECL on a Bio-Rad GelDock.

2.9 Confocal Microscopy

19mm cover slips were coated by incubation in Poly-L-lysine for 5 min in the bottom of 12 well plates then washed in PBS. As described in section 2.5.1.1, cells were seeded at 1×10^6 cells/well. BMDCs were infected as described above and Monodansylcadaverine (MDC, 50µM) added 15 min prior to cell fixation. Cells were then fixed in 2% PFA.

Alternatively, GFP+LC3-BMMs were plated on coated coverslips in 12-well plates and infected as described above. At specific time points post-infection, cells were fixed in 2% PFA and permeabilised in Triton-X100 (0.1% in PBS). Non-specific binding was blocked by incubation in 5% BSA before cells were incubated with Alexa Fluor 555 - conjugated phalloidin (1/100 (Life Technologies)) for 1 h to stain actin.

Coverslips were mounted onto glass slides with fluorescent Mounting medium (DakoCytomation) and analysed on an Olympus FV1000 laser scanning confocal microscope.

2.10 FACS

All FACS staining was done in FACS tubes. Cell suspensions from wound tissue or peritoneal lavage were transferred into FACS tubes after cell counts. 3 tubes of neutrophil reaction mixture, or 3 wells of a 96-well plate from a BMDC infection were pooled together into a FACS tube for each sample. Cells were removed from the 96-well plate by gentle pipetteing. Non-specific antibody binding was prevented by incubating cells with Fcy block (1µg/ml) for 15 min at 4°C. Cells were then stained for specific cell surface markers using different antibodies (see Table 2.5). They were incubated at a final antibody concentration of 1:100 in FACS buffer for 30 min at 4°C. They were then washed, centrifuged and fixed in 2% PFA, either for reading on the FACS machine or for TUNEL staining.

To assess apoptosis levels, cells were stained using the Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) kit. This assay consists of an enzyme that catalyses the binding of fluorescently labelled nucleotides to free 3'-OH DNA nicks in the fragmented DNA that is characteristic of cells in late-stage apoptosis. To stain cells with TUNEL, they were fixed for 15 min at room temperature with gentle shaking. After washing, cells were then resuspended in Permeabilisation Solution for 2 min on ice. Again, the cells were washed, before being incubated with TUNEL reaction mixture (containing enzyme and labelled nucleotides as per TUNEL staining kit's instructions) for 1 h in a humidified environment at 37°C and 5% CO₂.

After 1 h, the cells were centrifuged and resuspended in FACS buffer. Flow cytometric analysis was performed with BD FACSCanto II using FACS DIVA and FloJo software. Gating for analysis was set on respective Fluorescence Minus Ones (FMOs).

2.10 Statistics

Statistical analysis was performed using GraphPad Prism statistical analysis software. Differences in the means were assessed using one-way ANOVA with Tukey posttest, two-way ANOVA with a Bonferroni post-test or unpaired t-tests. Repeated measures were taken into account where necessary. A p value ≤ 0.05 was considered significant.

Chapter 3.

Manipulation of autophagy in phagocytes facilitates *Staphylococcus aureus* Bloodstream Infection.

3.1 Introduction

S. aureus is classically considered an extracellular bacterium, however it is capable of invading and persisting within non-phagocytic cells (189) in addition to professional phagocytic cells such as monocytes, macrophages and even neutrophils (128-130, 190). It has been proposed that *S. aureus* may be capable of subverting phagocytes to facilitate its dissemination by surviving within these motile cells (191). Despite the fact that the environment inside phagocytes is less than hospitable, gaining an intracellular niche, even briefly, within these cells affords a window of opportunity for extended survival and potential dissemination. Critical to survival is the ability to avoid destruction within phagolysosomes and *S. aureus* is equipped with a number of strategies to resist phagolysosomal killing (117, 192, 193). Having circumvented these killing mechanisms the bacterium can then escape into the cytoplasm, which in most cases eventually leads to host cell death, releasing the bacteria into the extracellular space where it has the opportunity to replicate and infect other host cells. Phagosomal escape by *S. aureus* has been shown to depend upon the regulatory system encoded by the *agr* locus (128, 194, 195), which controls expression of a number of virulence factors essential in intracellular *S. aureus* survival including α -toxin (Hla) (128) and Phenol-soluble modulins (PSMs) α (196, 197).

Autophagy plays an important role in host defense against invasive or intracellular pathogens such as *Streptococcus pyogenes* (166) or *Mycobacterium tuberculosis* (168). These organisms are sequestered in autophagosomes, which then deliver the bacteria to the lysosomes for destruction. Perhaps unsurprisingly, it has been discovered in recent years that many bacteria have evolved mechanisms that allow for the evasion or subversion of the autophagic pathway. These manipulations of autophagy can occur at any stage during the autophagic process. Certain bacteria

such as *Shigella* and *L. monocytogenes* have been shown to be capable of evading recognition by proteins that target them to the autophagosome (169, 170), while others such as *Legionella pneumophila*, inhibit the initiation of autophagy entirely (172). Furthermore, some bacteria (e.g. *Coxiella burnetti* and *Porphyromonas gingivalis*) are known to exploit the autophagic machinery for their own benefit; delaying autophagosomal maturation and lysosomal fusion in order to create a survival and replicative niche within the cell (198).

The interaction between *S. aureus* and autophagy is an emerging field of study. In 2007, Schnaith et al. identified that *S. aureus* causes accumulation of autophagosomes in HeLa cells in a manner dependent upon the expression of the global virulence regulator *agr*. Accumulation was found to have been as a result of a block in autophagic flux, as there was no colocalisation between the LC3+ autophagosomes and the lysosomal marker LAMP1. In this study, proliferation of *S. aureus* was impaired within fibroblasts deficient in the autophagy protein Atg5 (175) indicating an essential role for the autophagy pathway in facilitating replication of *S. aureus* within non-professional phagocytic cells. More recent work has shown specifically that Hla – which is under the control of the *agr* system - was sufficient to induce autophagy in Chinese Hamster Ovary (CHO) and Mouse Embryonic Fibroblasts (MEF) cell line. Furthermore, mutant strains of *S. aureus* that did not express Hla were not capable of inducing autophagy and were unable to replicate within the cells (174). *S. aureus* has also been shown to trigger the autophagic process in the mouse macrophage cell line RAW 264.7 through TLR2, but the effects of engagement of autophagy in this phagocytic cell line on bacterial survival was not examined (199). Maurer et al. have recently shown that autophagy confers protection against *S. aureus* lethality by mediating tolerance towards Hla. The protection conferred is not due to reduction in bacterial load as mice that are deficient in

autophagy did not display a reduction in bacterial burden of Hla-expressing *S. aureus* *in vivo*. However, in the absence of Hla expression by *S. aureus*, mice that have defective autophagic pathways have reduced bacterial burden *in vivo* compared to WT, suggesting that the survival of *S. aureus* in the autophagosome is independent of Hla expression (200).

The bulk of the research conducted into the survival within or killing of *S. aureus* by phagocytes has focused on neutrophils and, to a lesser extent macrophages. To date, the contribution of dendritic cells to direct killing of *S. aureus*, and the capacity of *S. aureus* to manipulate these particular phagocytes remains to be fully explored. It has been shown that DCs are important for survival in the context of *S. aureus* infection as CD11c+ DC depleted mice were more susceptible to mortality in a model of *S. aureus* bacteremia (76). However, it was suggested that the effects of DCs were not due to direct killing of the bacteria, rather Schindler et al. implicated modulation of neutrophil recruitment as the mechanism of action of DC-mediated protection.

Whether or not *S. aureus* can manipulate the autophagic process in professional phagocytes as a means to enhance survival remains to be established. Consequently this study initially aims to prove that DCs are important cells in the direct clearance of *S. aureus*. Furthermore, it presents data supporting the hypothesis that *S. aureus* is capable of subverting autophagy in phagocytic cells to generate an intracellular niche in which it can survive and potentially disseminate.

3.2 Results

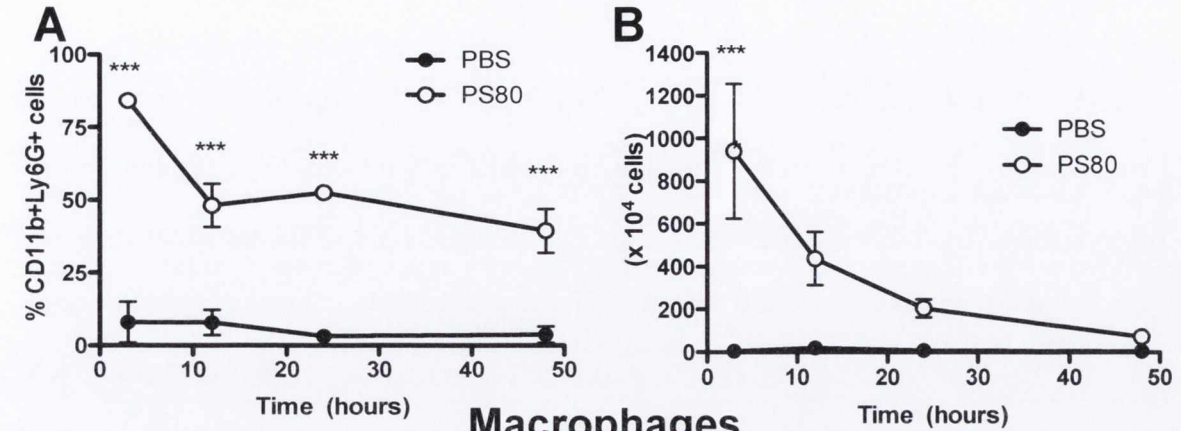
3.2.1 Exposure to *S. aureus* results in phagocyte influx to the site of infection

While the vital role of phagocytes in the clearance of *S. aureus* infection is undisputed, it has predominantly focused on neutrophils, as they are historically considered the key cell in the immune response to *S. aureus*. It has also been shown that macrophages play a significant part in the anti-*S. aureus* immune response. However, the bactericidal capacities of DCs have not been extensively explored. In order to determine whether these cells play an appreciable role in the immune response to *S. aureus* infection, mice were injected intraperitoneally (i.p.) with 5×10^8 CFU of *S. aureus* strain PS80, or PBS as a control, and phagocyte influx monitored over time.

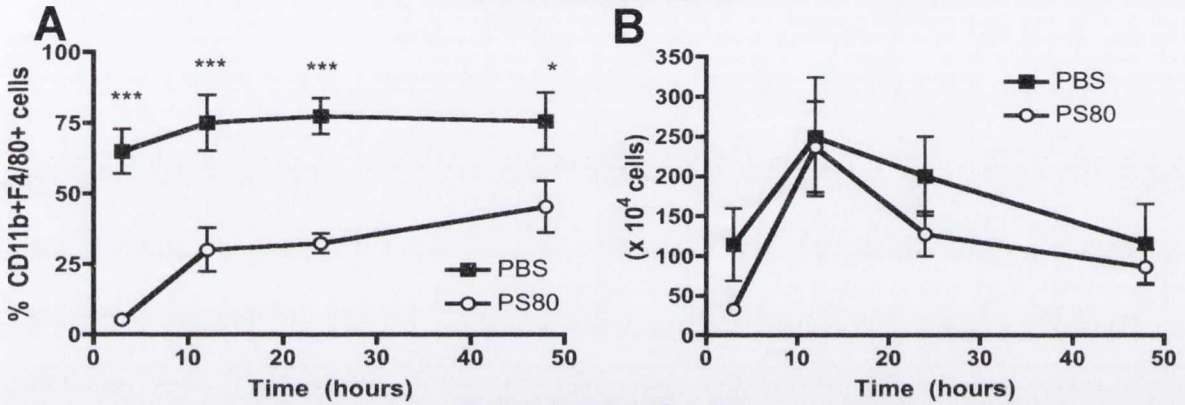
Within 3 hours, significant influx of CD11b+Ly6G+ neutrophils to the peritoneal cavity was observed (Figure 3.1), that are then rapidly cleared from the site of infection. This rapid recruitment of neutrophils highlights the importance of these cells in the initial innate response to *S. aureus*. In contrast, the proportions of CD11b+F4/80+ macrophages in the peritoneal cavity of infected mice were significantly lower than in uninfected controls. This is presumably due to the huge influx of neutrophils in these infected mice, and when absolute numbers are referred to, it is evident that infection with *S. aureus* has no impact on macrophage recruitment to the peritoneal cavity (Figure 3.1). Finally, the influx of CD11c+ dendritic cells in response to *S. aureus* challenge in the peritoneal cavity was also assessed. The proportion of cells that were CD11c+ DCs in the peritoneal cavity was significantly increased in infected mice by 24 hours post-infection in comparison to uninfected controls, suggesting that they may indeed play a role in the innate response to the PS80 strain of *S. aureus*.

(Figure 3.1). Interestingly, given that no increase in the recruitment of macrophages to the infection site was observed, these data suggest that in this model of infection, DCs may play an equal if not a more important role in controlling *S. aureus*.

Neutrophils



Macrophages



Dendritic Cells

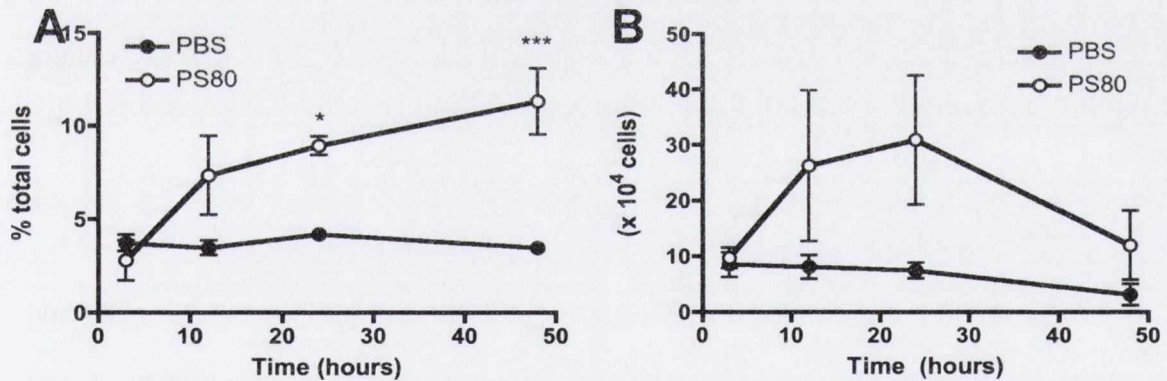


Figure 3.1: Analysis of phagocyte recruitment to the site of infection.

Groups of mice were exposed to *S. aureus* (5×10^8 CFU) or PBS via i.p. injection. At indicated time points post challenge, proportions (A) and absolute numbers (B) of neutrophils (CD11b+Ly6G+) macrophages (CD11b+F4/80+) and dendritic cells (CD11c+) were assessed in the peritoneal cavity by flow cytometry. Results expressed as mean $\pm$ SEM, n=6, *p<0.05, ***p<0.001.

3.2.2 Both BMDCs and BMMs vary in their ability to kill different strains of *S. aureus*

DCs have previously been shown to be involved in the immune response to *S. aureus* infection, although their contribution is classically thought to be predominantly through the activation of the adaptive immune response with little significance given to whether they can directly kill the bacteria (76, 201). Thus, the bactericidal capacities of these phagocytic cells were compared to that of macrophages which have a more clearly defined role in direct killing of *S. aureus* (202).

In the peritoneal model discussed above, *S. aureus* strain PS80 was the infecting bacteria. The infecting strain of *S. aureus* plays a significant role in determining the outcome of infection as different strains can elicit different responses from the immune system. In fact, previously published work shows PS80 and another laboratory strain of *S. aureus*, SH1000 induce different levels of activation of BMDCs, as measured by IL-1 β activity (85). Thus both of these strains were used to compare the bactericidal capacities of BMDCs and BMMs.

Primary BMDCs were infected with PS80 or SH1000 at MOI 10 (Figure 3.2A) and MOI 100 (Figure 3.2B) and bacterial killing was monitored over time. Within 6 hours of infection, approximately 70% of SH1000 was killed and by 16 hours almost 100% of SH1000 had been killed by the BMDCs at either MOI. In contrast, the BMDCs were unable to kill *S. aureus* strain PS80.

To establish whether the inability to kill *S. aureus* strain PS80 was specific to BMDCs, BMMs were infected with both strains of *S. aureus* at MOI 10 (Figure 3.2C)

and MOI 100 (Figure 3.2D). Similar to that observed with the BMDCs, BMMs efficiently killed SH1000 but were unable to kill PS80. Interestingly, BMDCs and BMMs demonstrated a similar capacity to kill *S. aureus* strain SH1000 (the % killing of MOI 100 at 16 hours was $90\pm6.8\%$ in BMDCs compared to $98.8\pm1.99\%$ in macrophages).

To confirm that the survival of PS80 was not as a result of the artificial *in vitro* growth of the BMMs and the BMDCs, macrophages were isolated from the peritoneal cavity of uninfected mice and infected with either strain of *S. aureus* at MOI 100. As was observed in both BMMs and BMDCs, there was significant killing of SH1000 within 3 hours, but PS80 was not killed by the peritoneal macrophages (Figure 3.2E).

Taken together these results suggest that BMDCs are capable of killing *S. aureus* to at least the same degree as BMMs and peritoneal macrophages, highlighting DCs as a noteworthy phagocyte to study in terms of direct killing of *S. aureus*. Interestingly, strain-dependent differences may impact upon the ability of both macrophages and dendritic cells to kill the bacterium. As similar results were obtained at both MOI 10 and MOI 100, all further experiments were conducted using MOI 100 only.

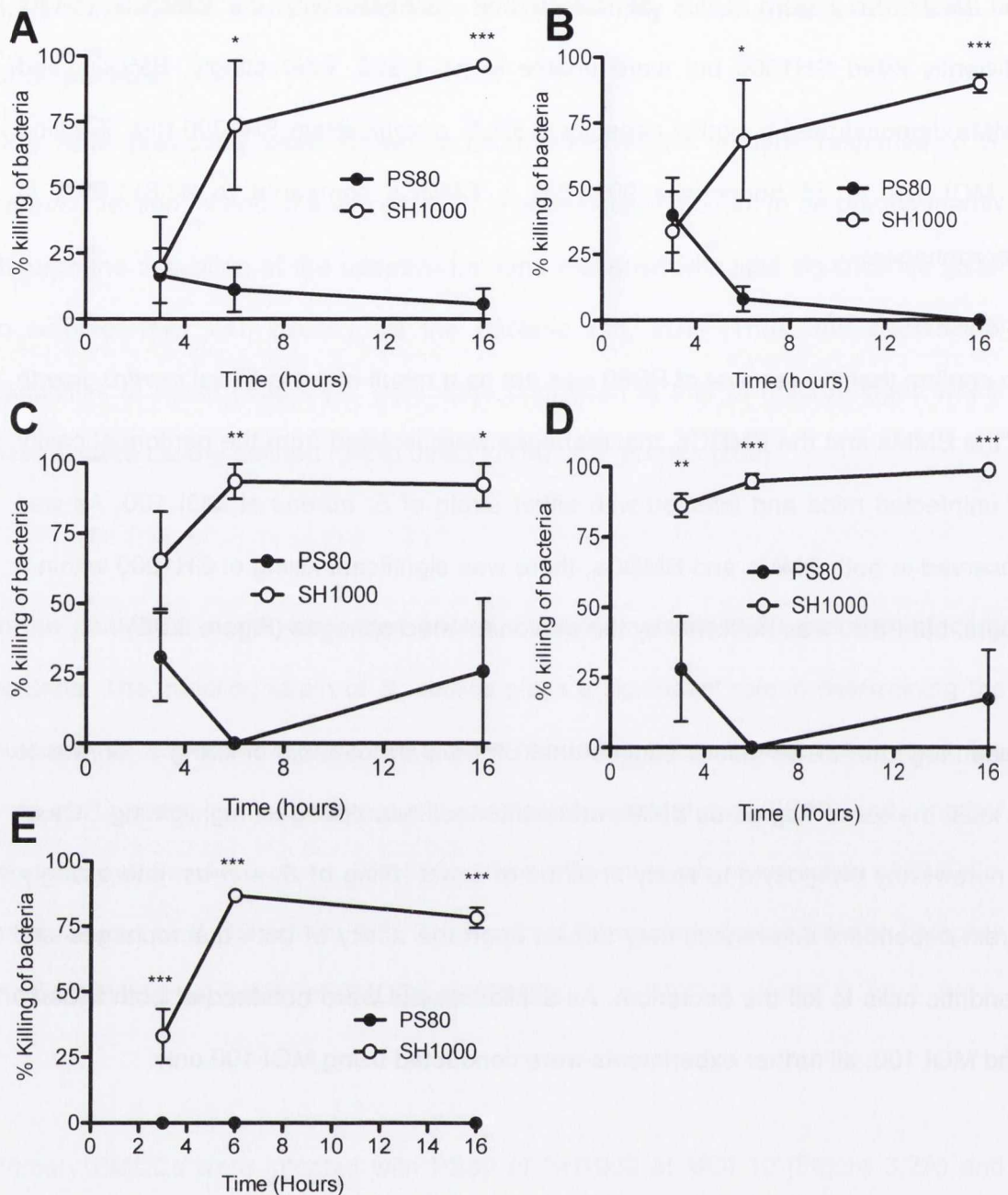


Figure 3.2: BMDCs and BMMs are able to kill certain strains of *S. aureus*.

BMDCs (A&B) or BMMs (C&D) were infected with PS80 or SH1000 at an MOI of 10 (A&C) or 100 (B&D). Alternatively, peritoneal macrophages were infected with either strain at MOI 100 (E). % killing of bacteria was determined by comparing the total CFU in the presence of phagocytes to bacteria in media only. Results expressed as mean $\pm$ SEM at each time point, $n=3/4$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to other strain.

3.2.3 PS80 and SH1000 are phagocytosed by BMDCs at the same rate

Given that BMDCs had a different capacity to kill *S. aureus* strains PS80 and SH1000, it was important to confirm that both strains were phagocytosed at the same rate. BMDCs were infected with CTV-labelled *S. aureus* and the uptake of bacteria into the BMDCs assessed after 30 minutes and 2 hours. Before FACS analysis, cells were incubated with gentamicin for one hour to kill any bacteria that had not been phagocytosed. At 30 min post infection PS80 and SH1000 were phagocytosed by BMDCs to the same extent, with ~30% of BMDCs staining positively for CTV-labelled PS80 or SH1000, indicating that there is no difference in the rate of phagocytosis of the two strains (Figure 3.3). Interestingly, by 2 hours post infection the % cells that were CTV-PS80+ had increased from 30 minutes, alluding to the survival of this strain inside the cells, with continued phagocytosis. In contrast, the % cells that were CTV-SH1000+ remained at a comparable level across both timepoints, hinting at the possibility that the cells were killing the bacteria as they were phagocytosed.

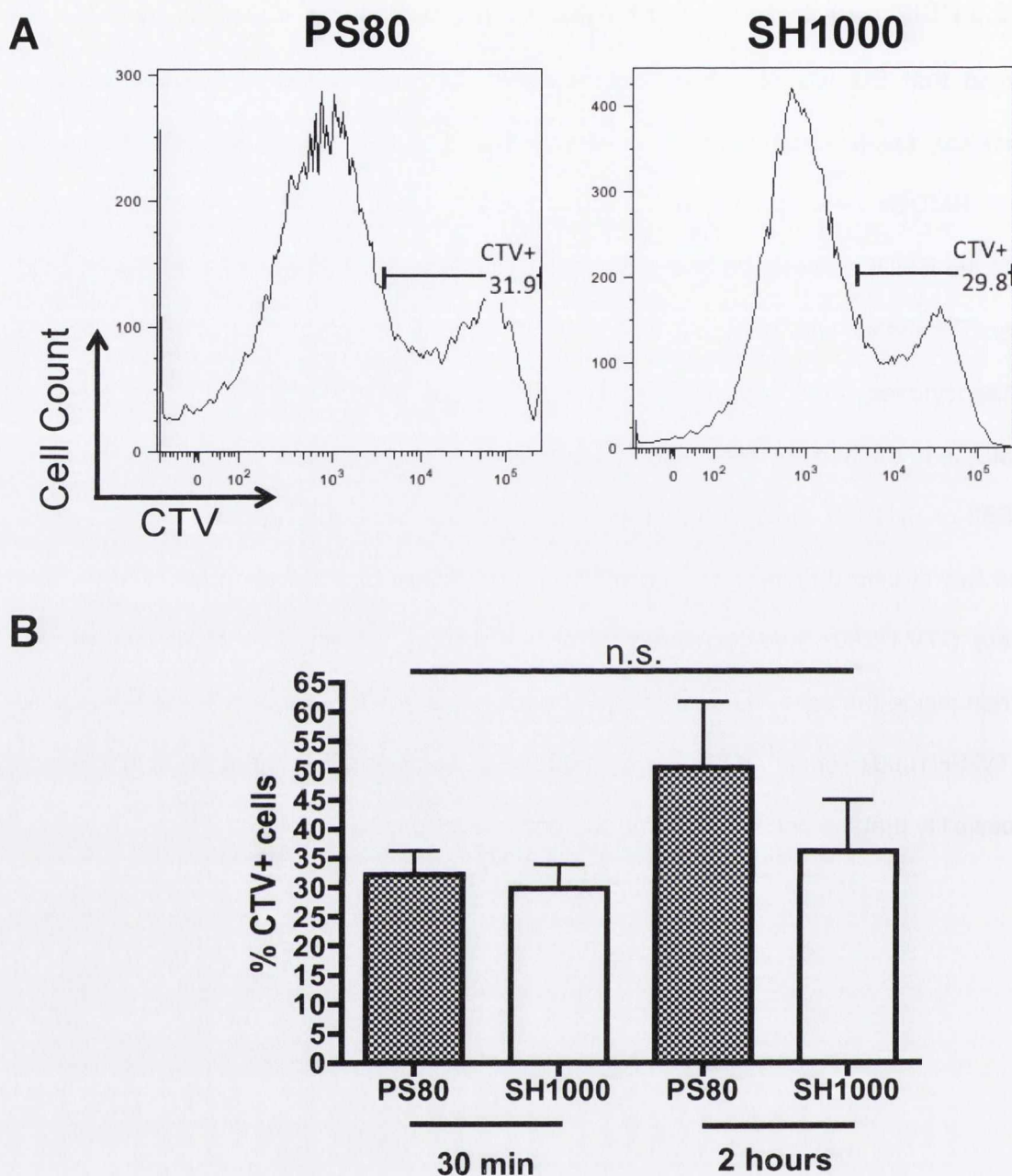


Figure 3.3: DCs phagocytose *S. aureus* strains at the same rate.

BMDCs were infected with either CTV-labelled PS80 or SH1000 at MOI 100 for 30min or 2 hours, then incubated with gentamicin for 1 hour. % uptake of bacteria was measured by flow cytometry. Representative FACS plots at 30min are shown (A). Results expressed as mean±SEM (B), n=3, n.s.= results are not significant.

3.2.4 PS80 can escape from BMMs and BMDCs while SH1000 cannot

S. aureus strains SH1000 and PS80 were both phagocytosed by BMDCs to the same extent but following phagocytosis PS80 was not killed (Fig 3.2 A&B). To establish whether PS80 escaped from the BMDCs, an assay was developed whereby cells were allowed to phagocytose the bacteria for 2 hours and any extracellular bacteria were killed by the addition of the bactericidal antibiotic gentamicin. Cells were washed after one hour in culture with the antibiotic and incubated in fresh medium (without antibiotics) – this point was considered time 0. As the escape assay relies on treating cells with gentamicin to kill all extracellular bacteria, it was imperative to initially ensure that the antibiotic treatment was not having a detrimental effect on the cells themselves. To test this, uninfected cells were incubated for 1 hour in the presence or absence of 200µg/ml gentamicin and then PI staining was assessed by flow cytometry to determine cell viability. There was no significant difference in the percentage of PI+ cells between antibiotic treated and untreated BMDCs (Figure 3.4), proving that the gentamicin did not affect the viability of the BMDCs.

Upon infection and gentamicin treatment, the escape of viable bacteria into the supernatant was measured at 6 and 12 hours. By 6 hours there was evidence of PS80 but not SH1000 escaping from the BMDCs. By 12 hours the level of PS80 in the cell culture supernatant was significantly higher than SH1000 (Figure 3.5A). A similar result was obtained in BMMs, with significantly higher escape of PS80 observed 12 hours post infection in comparison to those infected by SH1000 (Figure 3.5B).

To establish whether the escape of *S. aureus* from BMDCs was associated with cytotoxicity, LDH release from the infected BMDCs was measured. LDH activity was similar in uninfected BMDCs and BMDCs infected with SH1000 at both 6 h and 12 h

post-infection, indicating that SH1000 had no effect on the viability of the infected cells. In contrast, BMDCs infected with PS80 had significantly higher levels of LDH in the supernatant compared to cells infected with SH1000 or uninfected BMDCs at both time points (Figure 3.6A), indicating significant cytotoxicity. Again, the same trend was observed in infected BMMs, with PS80 eliciting significantly higher LDH release from the macrophages than SH1000 or uninfected controls (Figure 3.6B). These experiments conclusively support the hypothesis that BMDCs are capable of responding to *S. aureus* in the same manner as BMMs. Thus, BMDCs are used as a representative phagocyte throughout the remainder of this study.

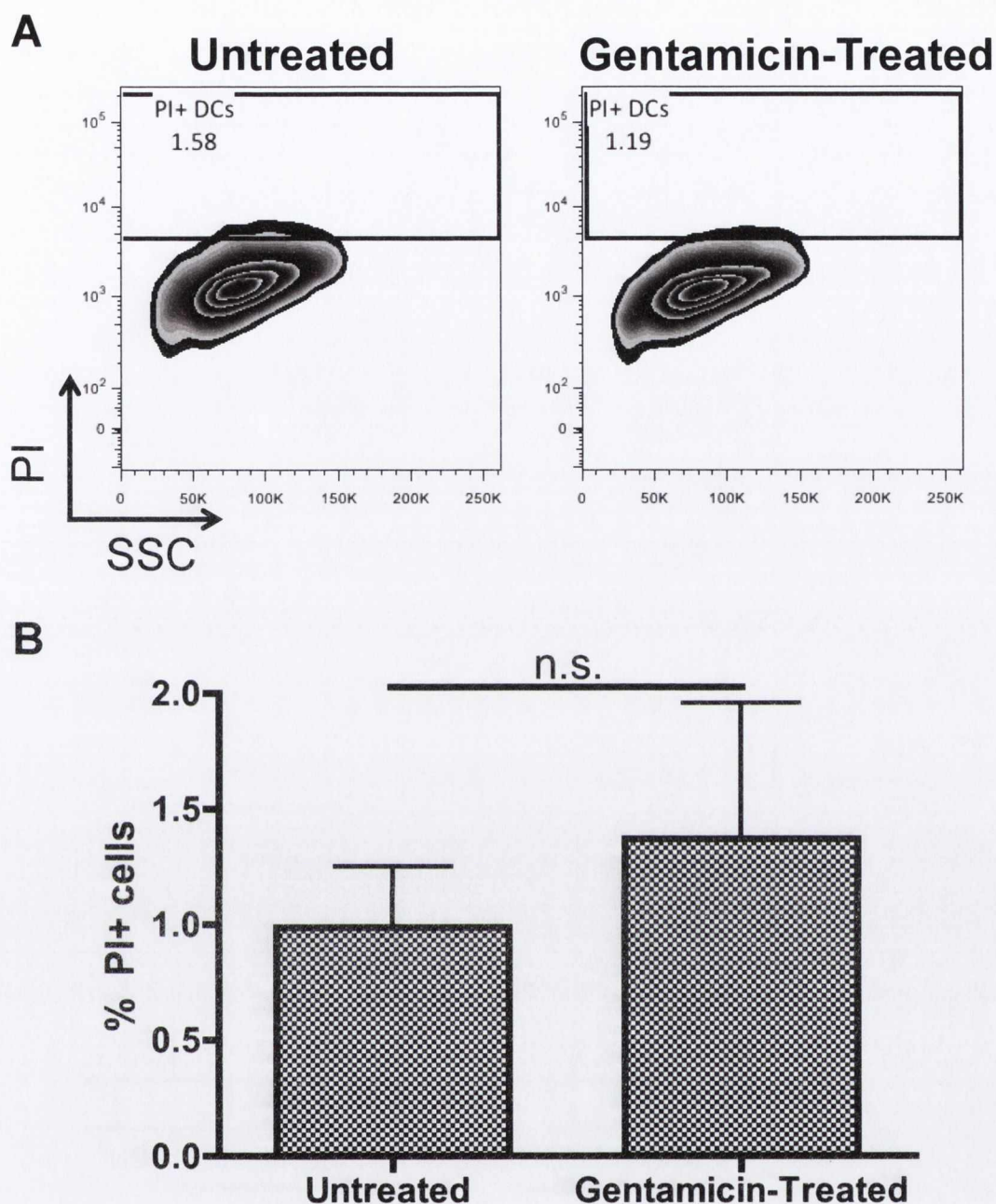


Figure 3.4: Antibiotic treatment has no effect on BMDC viability.

BMDCs were seeded at 2×10^5 cells/well and incubated in media only or media containing 200 μ g/ml gentamicin for 1 hour. Cells were then stained with PI and analysed by flow cytometry. Representative FACS plots are shown (A). Results expressed as mean $\pm$ SEM (B), n=3, n.s. = results are not significant.

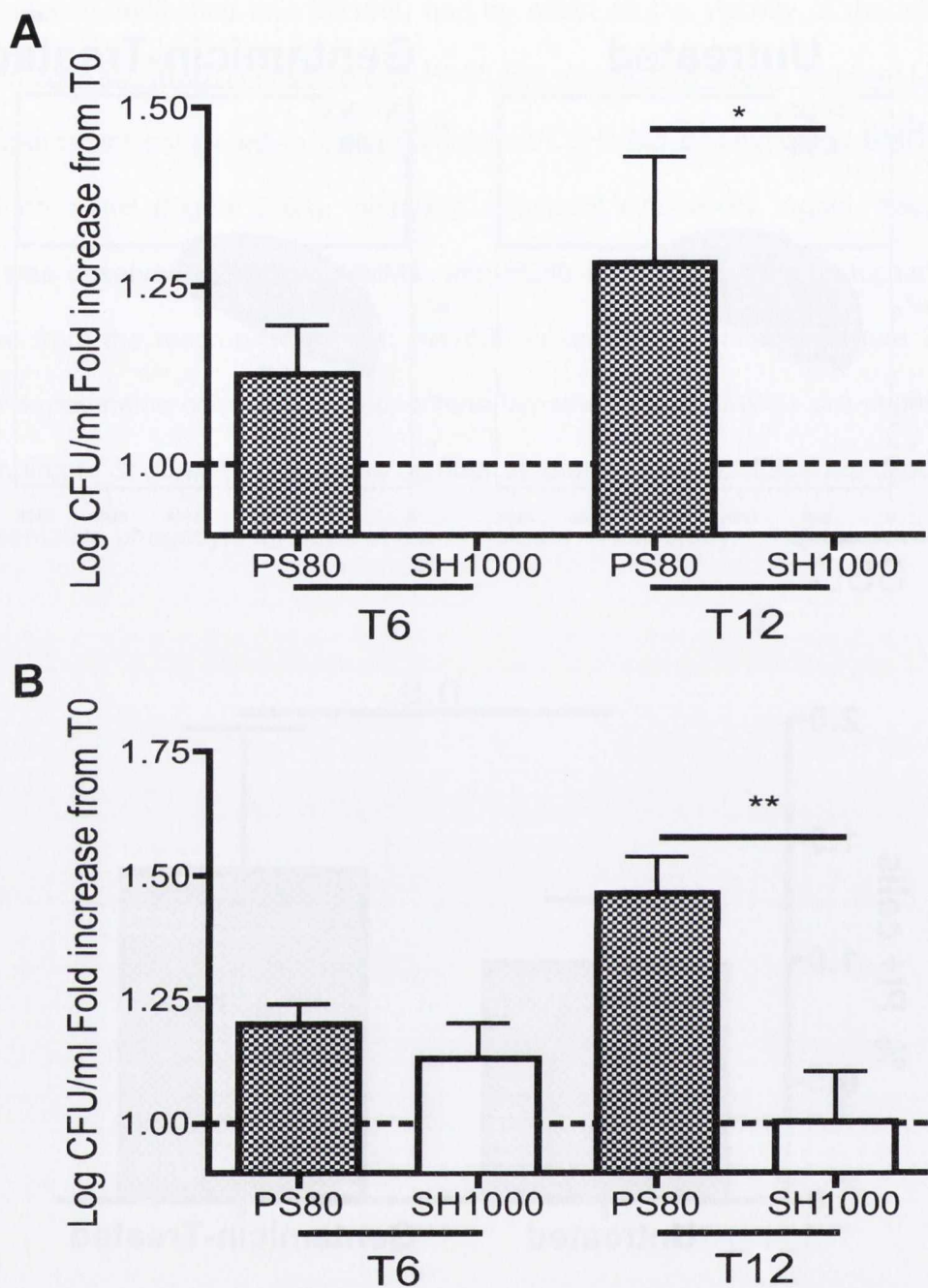
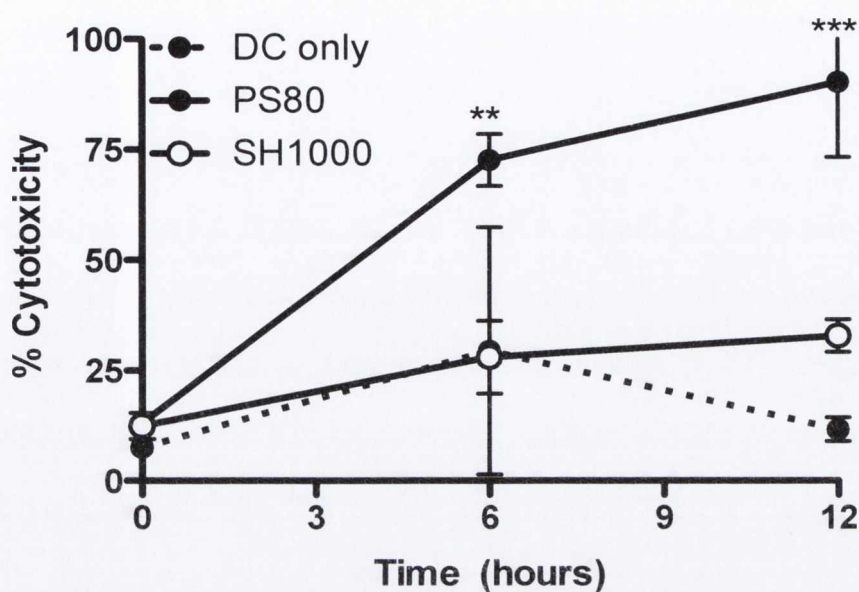


Figure 3.5: *S. aureus* strain PS80 but not SH1000 can escape from BMDCs and BMMs.

Following infection with PS80 or SH1000 at MOI 100, escape of each strain into the cell culture media was assessed at 6 hours and 12 hours post gentamicin treatment in BMDCs (A) and BMMs (B), expressed as fold increase from Log T0 (Dotted Line). Results expressed as mean $\pm$ SEM, n=3/4, *p<0.05, **p<0.01.

A



B

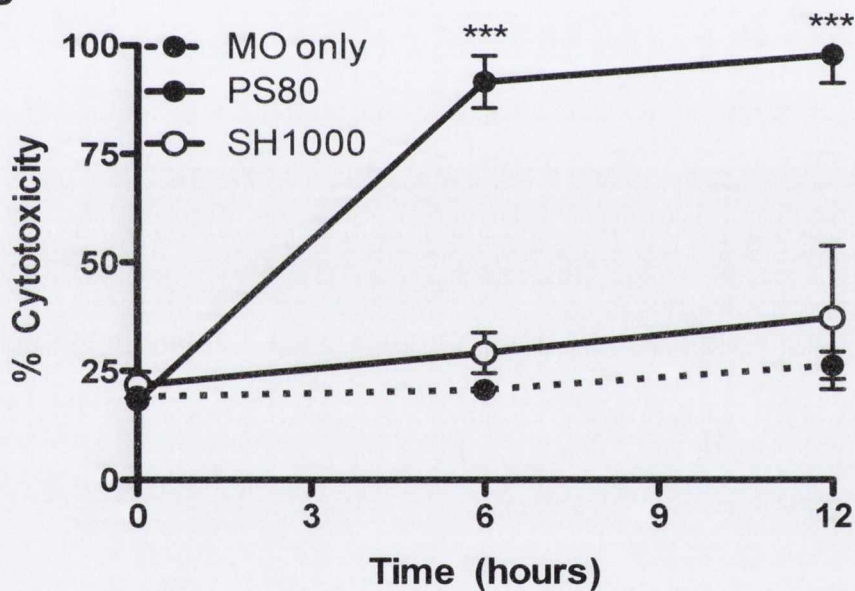


Figure 3.6: *S. aureus* strain PS80 but not SH1000 can kill BMDCs and BMMs.

Following infection with PS80 or SH1000 at MOI 100, LDH levels were assessed in the supernatant of both infected and uninfected BMDCs (A) or BMMs (B) post gentamicin treatment. Results expressed as mean $\pm$ SD. Representative of 3 independent experiments, ** p <0.01 *** p <0.001.

3.2.5 Identification of clinical isolates of *S. aureus* with similar phenotypes to laboratory strains

S. aureus PS80 and SH1000 are both well-characterised laboratory strains. However, their relevance to clinical isolates may be limited. Accordingly, *S. aureus* clinical isolates were obtained from Prof. Jodi Lindsay, St George's Healthcare NHS Trust, London. These isolates had been collected from blood culture bottles of patients diagnosed with *S. aureus* bacteremia. The isolates were screened for their cytotoxic effects on BMDCs. BMDCs were infected with each isolate and the viability of the infected DCs was assessed after 24 hours by staining with PI. The clinical isolates (labelled as Sa) clustered together into one group that was cytotoxic to BMDCs in a similar manner to PS80, a second group that did not exert any cytotoxic effects, akin to SH1000 and a third, intermediate group (Figure 3.7). A representative isolate from both the "PS80-like" group and the "SH1000-like" group were selected for further analysis; *S. aureus* 68 (Sa68) and *S. aureus* 279 (Sa279).

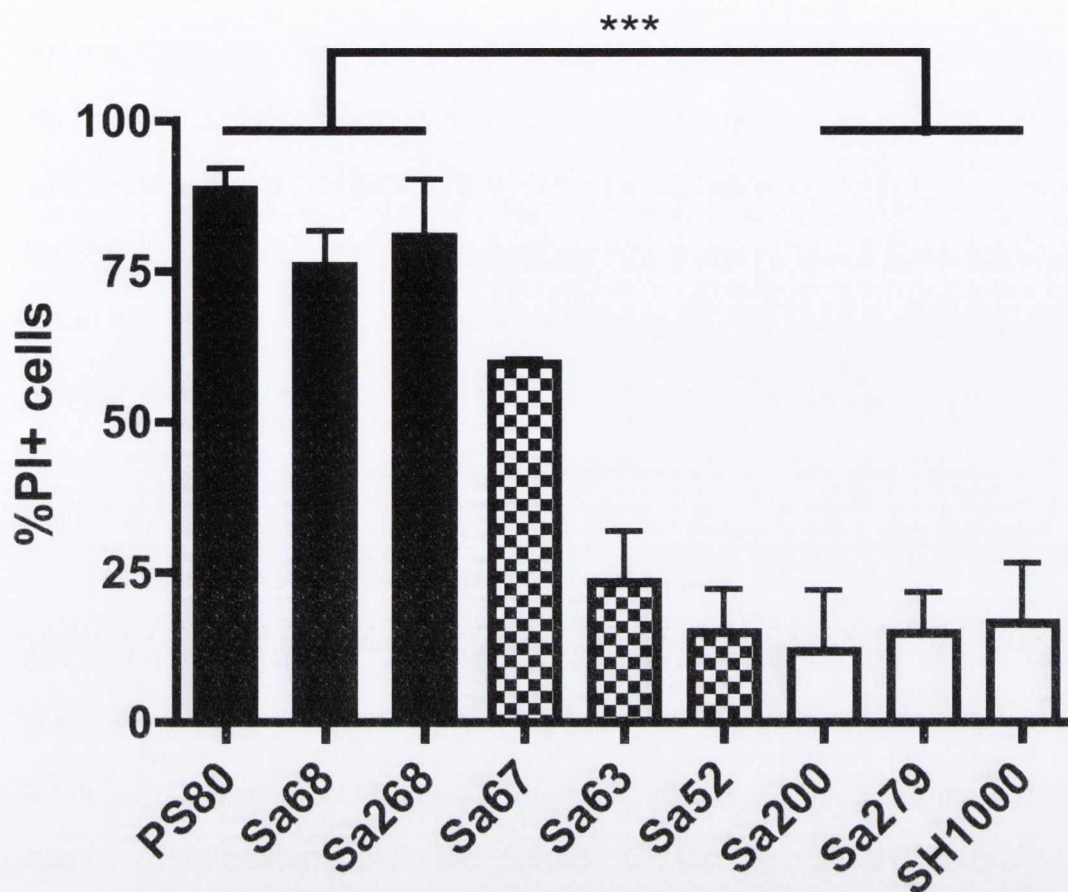


Figure 3.7: Screening of clinical isolates for cytotoxicity to BMDCs.

BMDCs were infected with a panel of *S. aureus* clinical isolates for 24 hours, then viability was assessed by staining the cells with PI and analysing by flow cytometry, identifying “PS80-like” strains (black), “SH1000-like” strains (white) and “intermediate” strains (checked). Results expressed as mean±SEM, n=3, ***p<0.001.

3.2.6 *S. aureus* strain Sa68 can escape BMDC killing while Sa279 cannot

In order to determine whether the clinically isolated strains Sa68 and Sa279 showed the same differences in phenotype as the laboratory strains PS80 and SH1000, BMDCs were infected with either Sa68 or Sa279 and bacterial killing monitored over time. As observed during infection with the laboratory strains, BMDCs had different responses to the two clinical strains; they were capable of killing strain Sa279 but were unable to kill strain Sa68 (Figure 3.8A). These data suggest that Sa68 is similar to PS80 and may be capable of escaping from phagocytes. It was confirmed that both clinical strains were phagocytosed by BMDCs at a similar rate by CTV-labelling the bacteria and infecting BMDCs as described above (Section 3.2.3). Similar to the uptake of PS80 and SH1000, approximately 30% of BMDCs were associated with CTV+ Sa279 or Sa68 by 30 min post infection (Figure 3.8 B&C), with the amount of Sa68 -but not Sa279- in the cells increasing by 2 hours, alluding to its survival in the cell. The ability of Sa68 to escape from the BMDCs was then assessed. By 6 hours there was a substantially higher number of Sa68 bacteria in the supernatant than Sa279 and after 12 hours this difference was significant (Figure 3.9A).

To establish if the ability of Sa68 to escape from the BMDCs correlated with cytotoxicity, cells were infected with Sa68, Sa279 or left uninfected. Following gentamicin killing of extracellular non-phagocytosed bacteria, the LDH release was monitored at 6 h and 12 h. Again, similar to the trend seen between PS80 and SH1000, the level of cytotoxicity (LDH release) associated with Sa68-infected cells was significantly higher than that of Sa279-infected cells or the uninfected control cells both 6 and 12 hours after infection (Figure 3.9B).

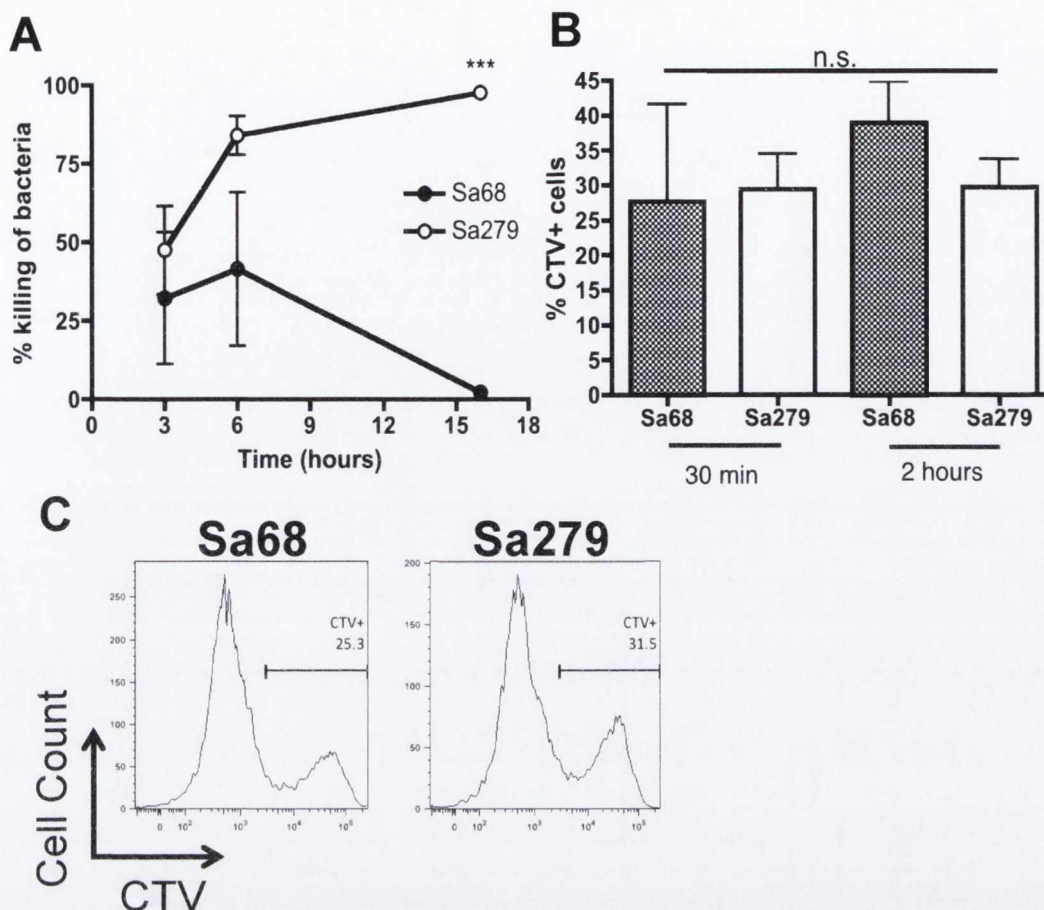


Figure 3.8: Clinical isolate Sa279 - but not Sa68 - is killed by DCs although uptake of both strains is equal.

BMDCs were infected with Sa68 or Sa279 and % killing of bacteria was determined by comparing total CFU in the presence of phagocytes to bacteria in media only (A). BMDCs were infected with either CTV-labelled Sa68 or Sa279 at MOI 100 for 30min or 2 hours, then incubated with gentamicin for 1 hour. % uptake of bacteria was measured by flow cytometry (B). Representative FACS plots at 30min are shown (C). Results expressed as mean $\pm$ SEM, n=3/4, ***p<0.001, n.s.= results are not significant.

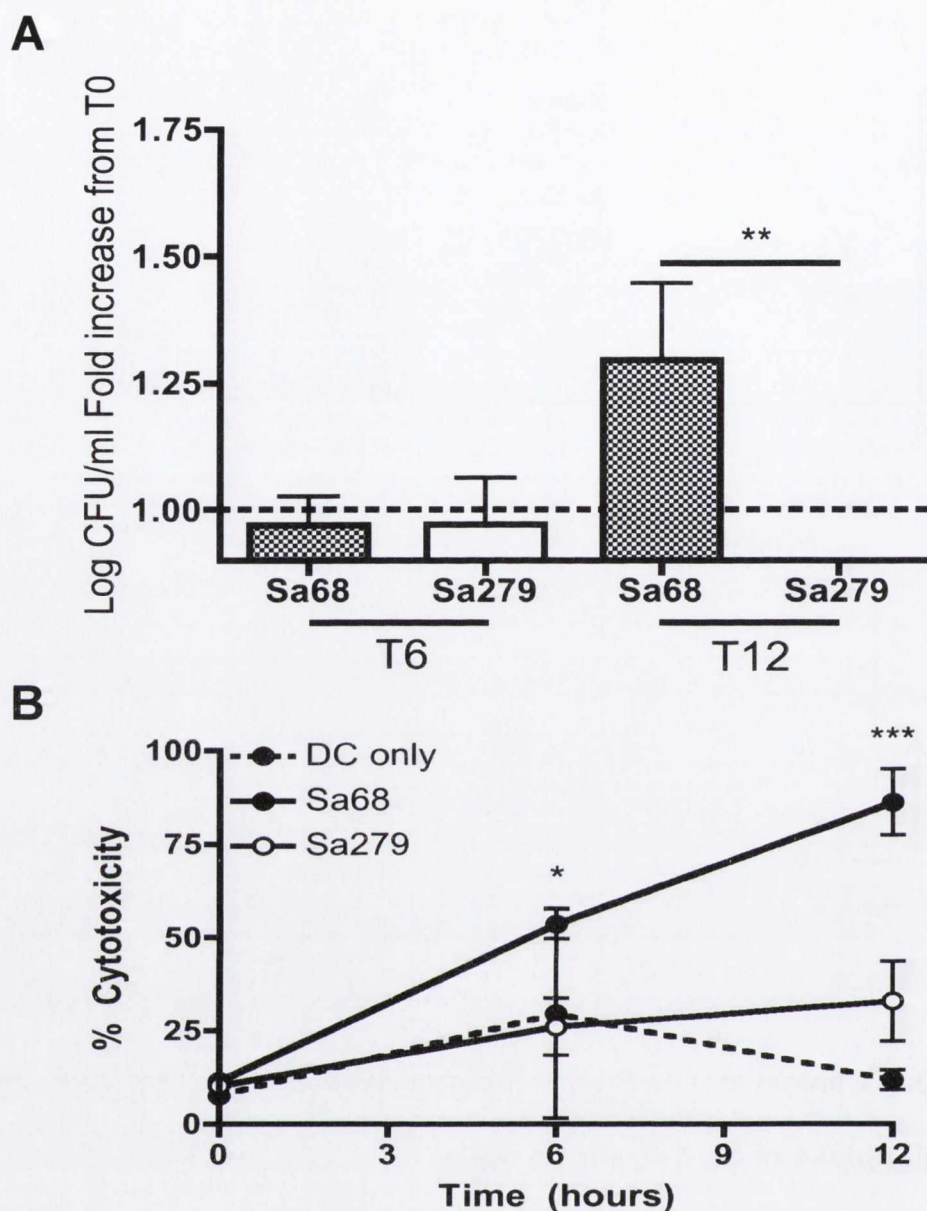


Figure 3.9: Sa68 but not Sa279 escapes from BMDCs causing associated cytotoxicity.

Following infection of BMDCs with Sa68 or Sa279 at MOI 100, escape of each strain into the cell culture media was assessed 6 hours and 12 hours post gentamicin treatment and expressed as fold increase from Log T0 (Dotted Line) (A). LDH levels were assessed in the supernatant of both infected and uninfected BMDCs post antibiotic treatment (B). Results expressed as mean $\pm$ SEM (A) or mean $\pm$ SD (B). n=4 (A), representative of 3 independent experiments (B), *p<0.05, ***p<0.001

3.2.7 Subversion of the autophagic machinery by certain strains of *S. aureus* is used as a means of intracellular survival

S. aureus has previously been shown to associate with autophagosomes in non-professional phagocytic cells. This provides a niche for the intracellular survival of *S. aureus* where it can replicate and eventually escape into the cytoplasm, ultimately leading to host cell death (174, 175). It was therefore postulated that *S. aureus* strain PS80 might use autophagosomes as a intracellular niche in BMDCs to evade killing. To assess whether *S. aureus* infection induced autophagy in BMDCs, cells were infected for 2 hours, then gentamicin treated for one hour, washed and cultured in fresh, antibiotic-free media. Cell lysates were then prepared at intervals up to 6 hours and processing of the autophagic marker LC3 was assessed by Western immunoblotting (203).

As autophagy is a homeostatic process that occurs constitutively in cells, there is always a degree of LC3-II processing detectable that does not change over time, as seen in uninfected BMDCs (Figure 3.10). Infection of BMDCs with *S. aureus* strain SH1000 resulted in a transient increase in LC3-II processing which was reduced by 2 hours post infection presumably due to autosome-lysosome fusion and subsequent degradation of autophagosomes. In contrast, infection of BMDCs with PS80 resulted in the persistence of substantial levels of LC3-II for at least 6 hours, indicating that normal autophagic flux was blocked, allowing for the accumulation of autophagosomes (Figure 3.10 A&B).

To confirm that PS80 was associating with autophagosomes, BMDCs were infected with CTV-labelled *S. aureus* strains PS80 or SH1000 and then treated with gentamicin to kill any extracellular bacteria. Staining with MDC (a fluorescent compound which accumulates specifically in autophagic vacuoles (204)) revealed

colocalisation between PS80 and the autophagosome. SH1000 was not seen to colocalise with MDC+ autophagosomes (Figure 3.11).

As further confirmation that PS80 interacts with the autophagosome, a RAW 264 macrophage cell line that had been stably transfected with GFP+LC3 (205) was infected with PS80 or SH1000 and gentamicin treated as above. Again, CTV-labelled PS80 was seen to colocalise with GFP+LC3 punctae at 3 hours post infection. In comparison, SH1000 did not show the same level of association with GFP+LC3 punctae (Figure 3.12).

To establish whether clinical isolates could also manipulate the autophagic process, BMDCs were infected with PS80, SH1000, Sa68 or Sa279 and lysates prepared after 6 hours. Processing of LC3 was assessed by Western immunoblotting. Similar to PS80, Sa68 infected cells had considerable levels of LC3-II present indicating a delay in the degradation of the autophagosomes. In addition, the level of LC3-II in Sa279 infected cells was similar to SH1000 infected cells or uninfected BMDCs, suggesting that these cells had normal autophagic flux (Figure 3.13).

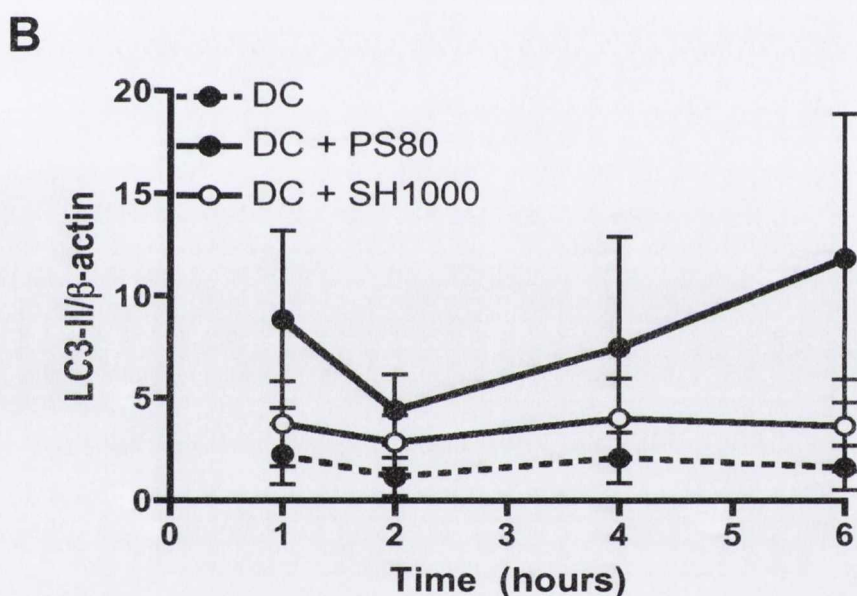
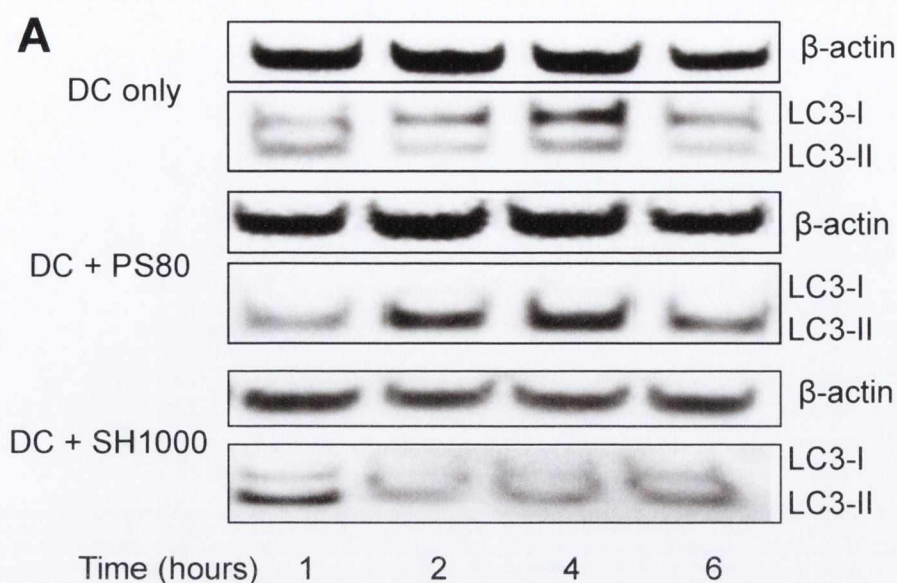


Figure 3.10: *S. aureus* strain PS80 inhibits normal autophagic flux in BMDCs.

BMDCs were infected with *S. aureus* strains PS80 or SH1000 and gentamicin treated to kill extracellular bacteria, or were left uninfected. At indicated time points cells were lysed and processing of LC3 analysed by Western immunoblotting. Bands show conversion of LC3-I to LC3-II. β-actin was measured as a loading control. A representative blot is shown (A). Densitometry analysis of 3-5 independent experiments are shown as mean±SEM (B).

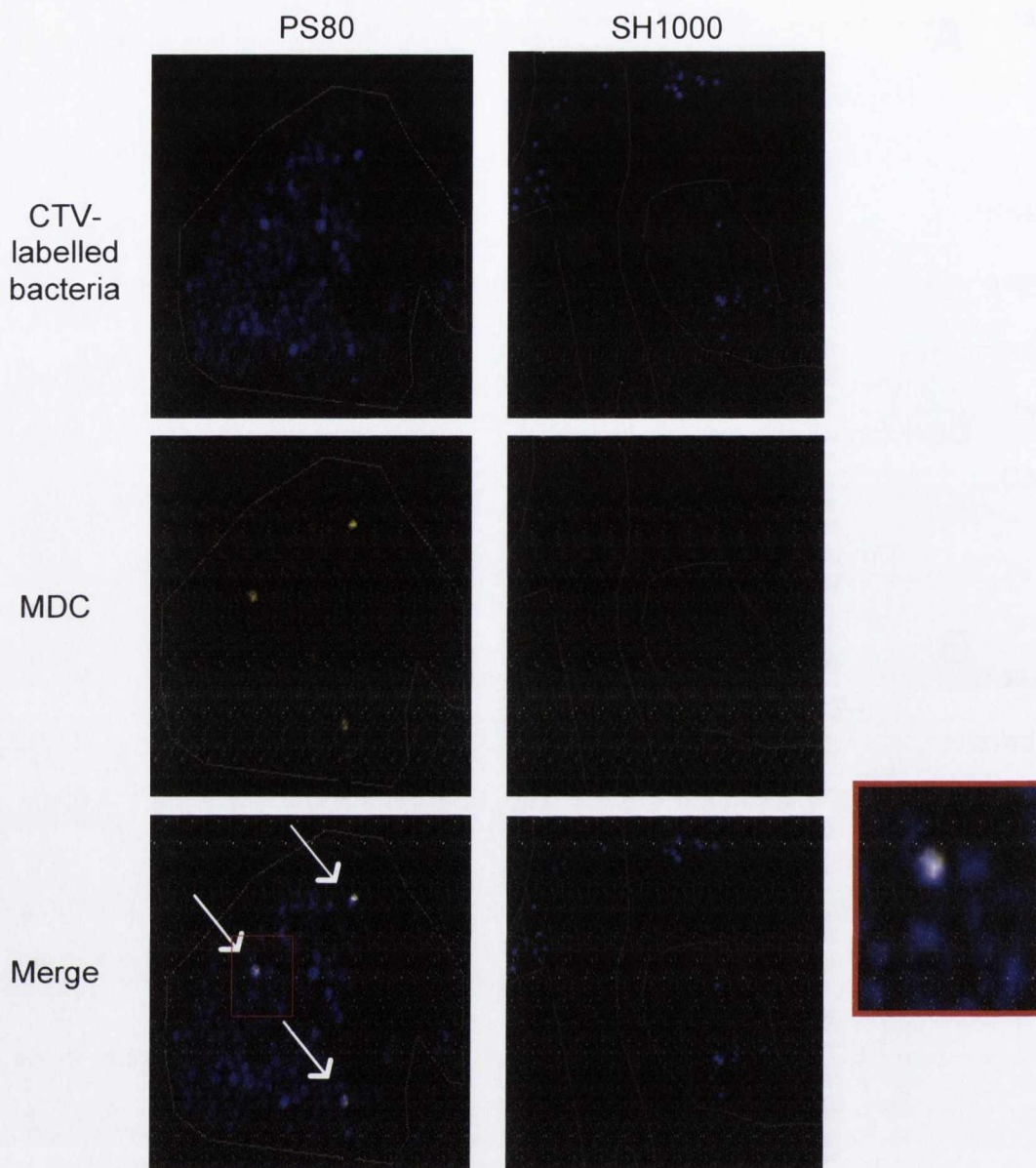


Figure 3.11: PS80 associates with LC3+ autophagosomes in BMDCs.

At 6 hours post infection with CTV-labelled bacteria, DCs were stained with MDC, fixed and viewed under a fluorescent microscope. Blue: bacteria, yellow: MDC. White arrows indicate colocalisation of bacteria and LC3-II. Magnification: 60X with 4X zoom. See also the enlarged image showing co-localisation.

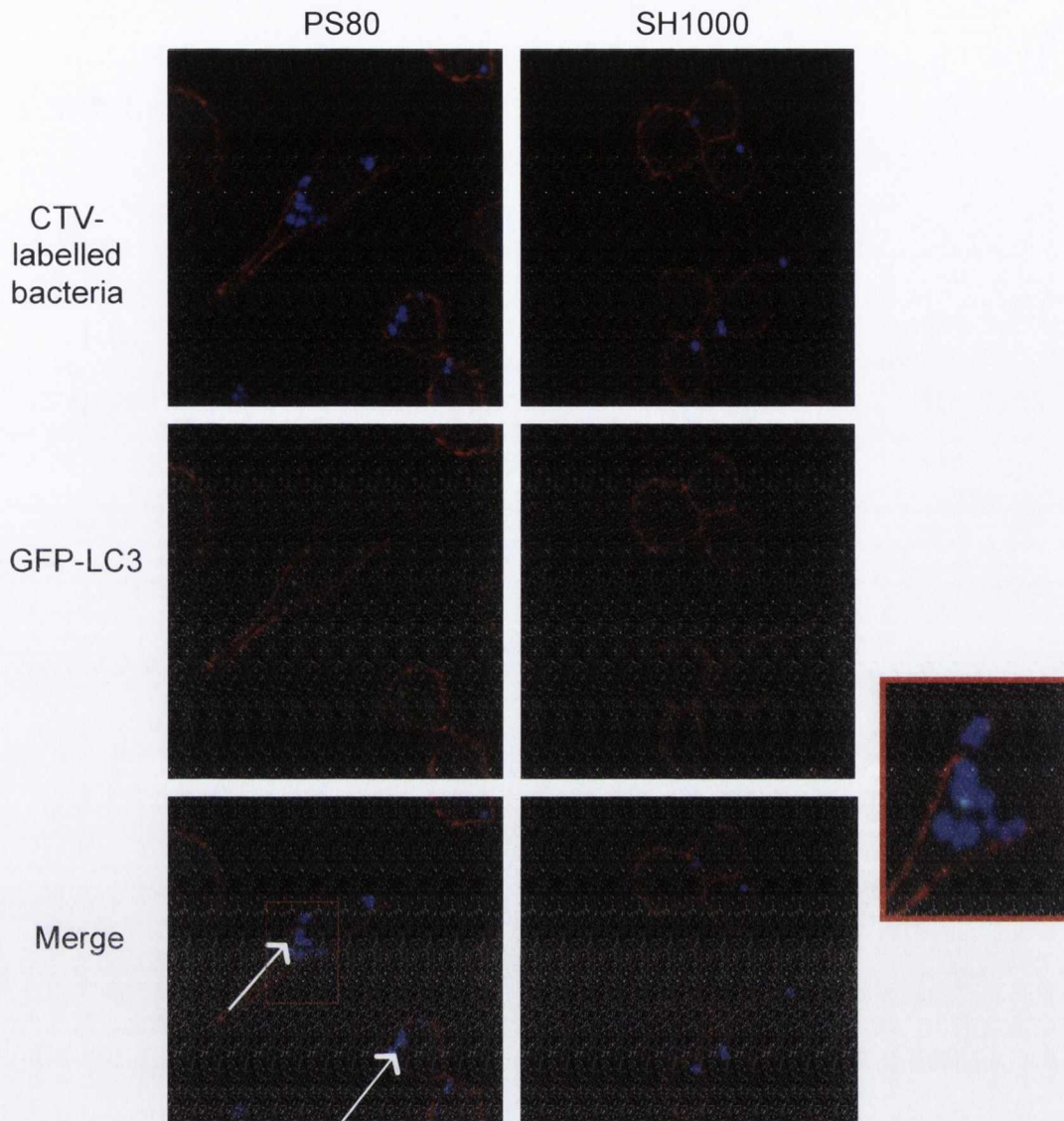


Figure 3.12: PS80 associates with LC3+ autophagosomes in iBMMs.

At 3 hours post infection with CTV-labelled bacteria, GFP-LC3 iBMM were fixed, permeabalised and stained for phalloidin to be viewed under a fluorescent microscope. Blue: bacteria, green: LC3, red: phalloidin. White arrows indicate colocalisation of bacteria and LC3-II. Magnification: 60X. See also the enlarged image showing co-localisation.

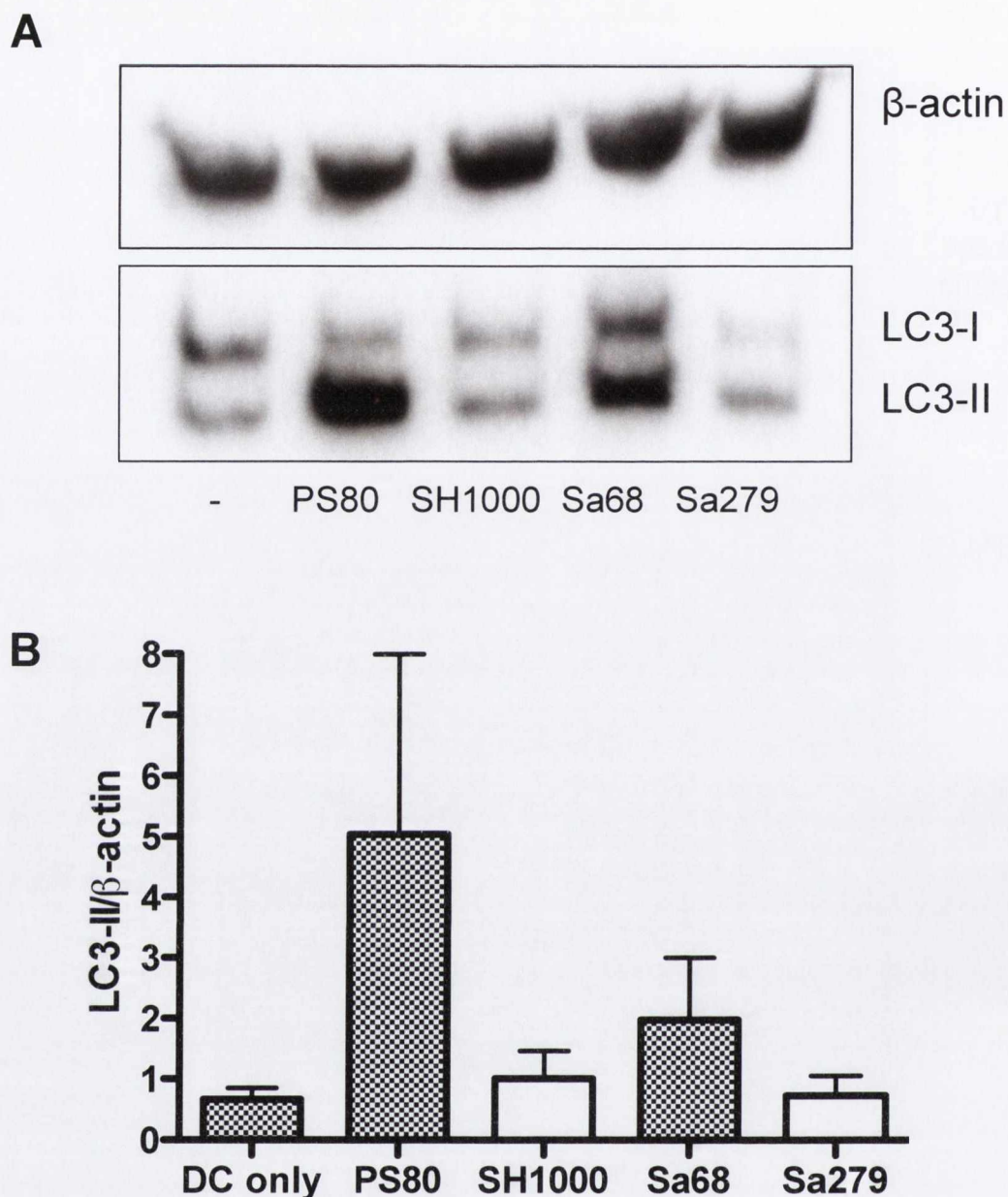


Figure 3.13: Sa68 inhibits autophagic flux while Sa279 does not.

BMDCs were infected with *S. aureus* strains PS80, SH1000, Sa68 or Sa279 and gentamicin treated to kill extracellular bacteria. At 6 hours cells were lysed and processing of LC3 analysed by Western immunoblotting. Bands show conversion of LC3-I to LC3-II. β -actin was measured as a loading control. A representative blot is shown (A). Densitometry analysis of 4 independent experiments were pooled and are shown as mean $\pm$ SEM (B).

3.2.8 Treatment with an autophagy inhibitor restores the bactericidal capabilities of BMDCs and prevents PS80 or Sa68-induced cell death.

Having shown that *S. aureus* strains that were capable of evading killing by BMDCs were inhibiting autophagic flux, the importance of normal autophagic turnover in killing these bacteria was assessed by pre-treating BMDCs with 3-methyladenine (3-MA), a well-established PI3K inhibitor that inhibits the induction of autophagy (206), prior to infection with these two strains (PS80 and Sa68).

Initially, it was important to determine whether 3-MA was capable of affecting *S. aureus* bacterial viability directly. Thus, bacteria were incubated for 16 hours in either media only or in media containing 10mM 3-MA. Importantly, 3-MA had no direct effect on bacterial viability (Figure 3.14).

The escape of *S. aureus* into the supernatant was then assessed at 6 and 12 hours. In the presence of 3-MA, PS80 and Sa68 escape into the cell culture supernatant was completely inhibited (Figure 3.15A). Associated with this, 3-MA treatment restored the viability of the infected BMDCs, with the level of LDH activity in the culture supernatant being significantly reduced following infection with both PS80 and Sa68 (Figure 3.15B). This clearly illustrates that the accumulation of autophagosomes is necessary for the survival of PS80 and Sa68 within the BMDC and their cytotoxic effects on BMDCs.

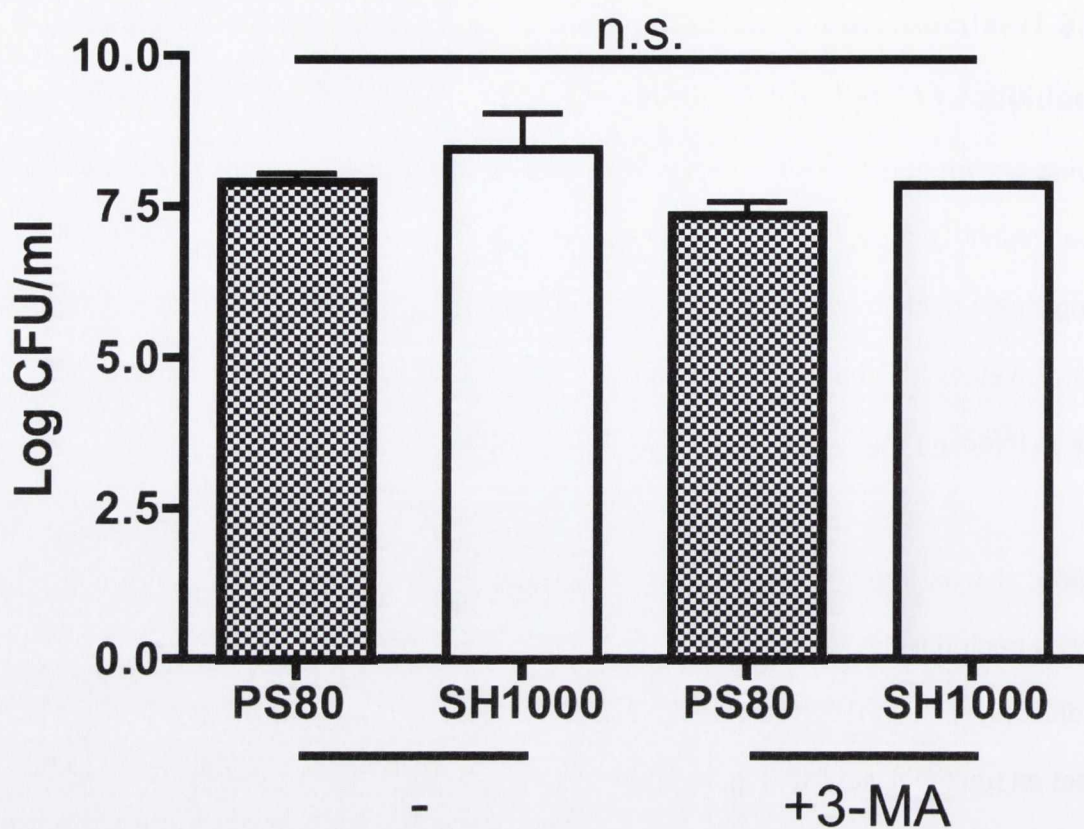


Figure 3.14: Treatment with 3-MA does not have a direct effect on *S. aureus* viability.

S. aureus strains were incubated in cell free media only or media containing 10 μ M 3-MA for 16 hours then plated on TSA to quantify bacterial survival. Results expressed as mean $\pm$ SEM, n=3, n.s. = results are not significant.

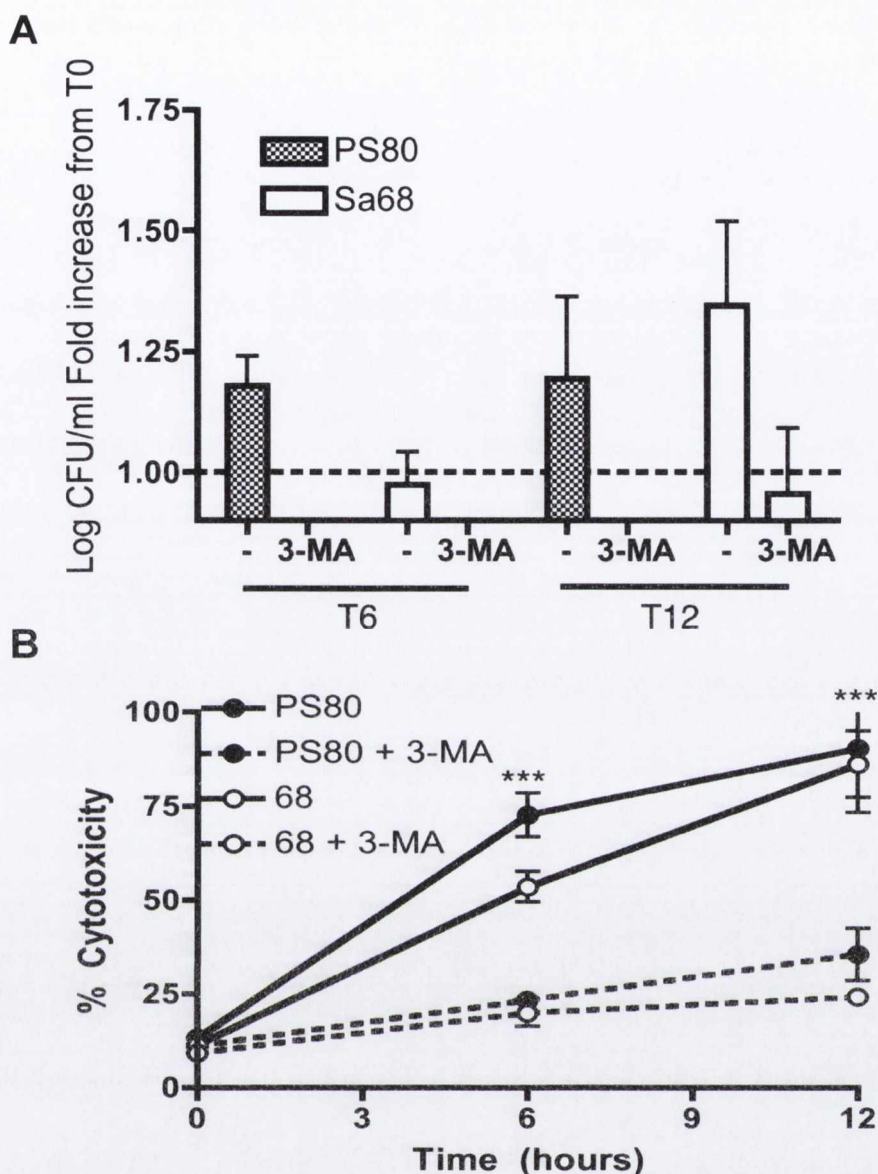


Figure 3.15: Inhibition of autophagic flux facilitates escape of *S. aureus* from phagocytes and prevents cell death.

BMDCs were pretreated with 3-MA for 30min and infected with either PS80 or Sa68 (MOI 100). Escape of each strain into the cell culture media was assessed at 6 hours and 12 hours post gentamicin treatment and expressed as fold increase from Log T0 (Dotted Line) (A). LDH levels were assessed in the supernatant of 3-MA pretreated and untreated BMDCs that were infected with either PS80 or Sa68 and gentamicin treated (B). Results expressed as mean±SEM (A) or mean±SD (B), n=4/6 (A), representative of 3 independent experiments (B), ***p<0.001.

3.2.9 Distinct intracellular survival phenotypes were observed between *S. aureus* strains *in vivo*

Having established that both laboratory and clinical strains of *S. aureus* can manipulate autophagy to evade phagocytic killing it was important to determine whether this phenomenon affected infection outcome *in vivo*. Groups of wild-type mice were infected with *S. aureus* strains PS80, SH1000, Sa68 or Sa279 by i.p. injection. At 3 hours post challenge blood was collected and total bacterial burden in the blood was quantified (Table 1). Interestingly, bacterial strains that did not induce accumulation of autophagosomes (SH1000 and Sa279) had significantly reduced survival in the blood in comparison to strains (PS80 and Sa68) that engaged with the autophagic machinery of cells.

	PS80	SH1000	Sa68	Sa279
Log CFU/ml (Mean±SEM)	3.63±0.12	2.93±0.26	3.92±0.39	2.37±0.49
Significance of reduction compared to PS80	N/A	p<0.01 **	n.s.	p<0.001 ***

Table 3.1: Bacterial burden in the blood

To prove that differences in bacterial burden in the blood were due to the differential abilities of individual strains to survive intracellularly, mice were infected with *S. aureus* strains PS80, SH1000, Sa68 or Sa279. At 3 hours post infection the total blood leukocytes were separated from the RBC and extracellular bacteria by centrifugation through Histopaque 1083. Leukocytes were washed thoroughly and then lysed to quantify viable intracellular *S. aureus*. The number of intracellular bacteria recovered was significantly higher in PS80 infected animals compared to SH1000 infected animals (Figure 3.16A). The same trend was seen in the clinical strains, with significantly higher levels of Sa68 recovered from the isolated leukocytes in comparison to Sa279 (Figure 3.16B). This suggests that PS80 and Sa68 are capable of surviving within circulating leukocytes *in vivo*, potentially facilitating systemic dissemination and persistence.

Consistent with this, animals infected with PS80 demonstrated an increased bacterial burden in the spleen at 12 hours post challenge as compared to the animals infected with SH1000 (Figure 3.17A). Although not significant, the same trend is observed in the livers of PS80 and SH1000 infected mice (Figure 3.17B).

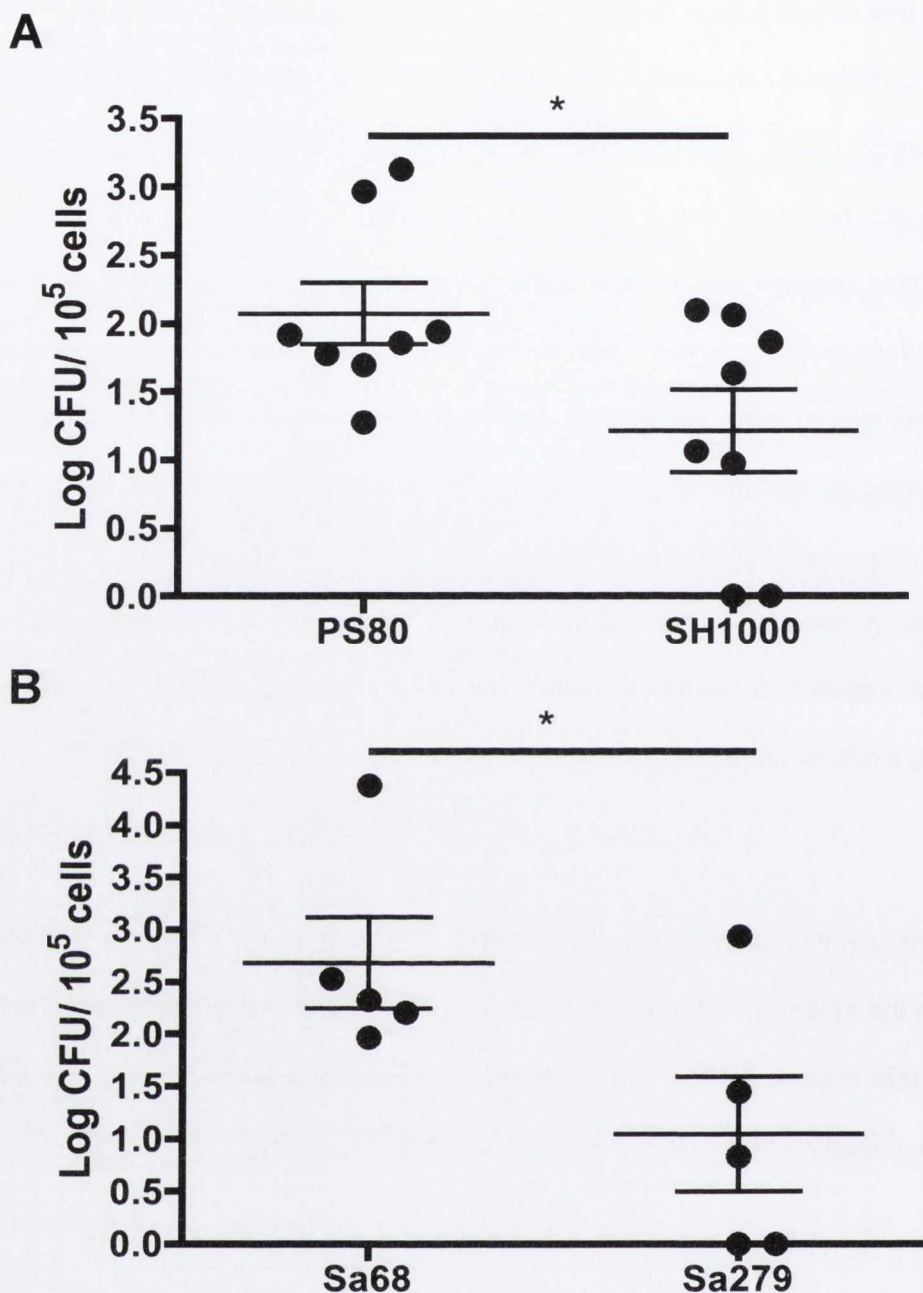


Figure 3.16: PS80 and Sa68 survive at higher levels intracellularly in blood leukocytes compared to SH1000 or Sa279.

Groups of mice were exposed to *S. aureus* strains PS80 and SH1000 (A) or Sa68 and Sa279 (B) (5×10^8 CFU) via i.p. injection. At 3 hours post infection, blood leukocytes were isolated, washed thoroughly and lysed to quantify intracellular bacteria. Line represents mean $\pm$ SEM, $n=5/8$, $*p<0.05$

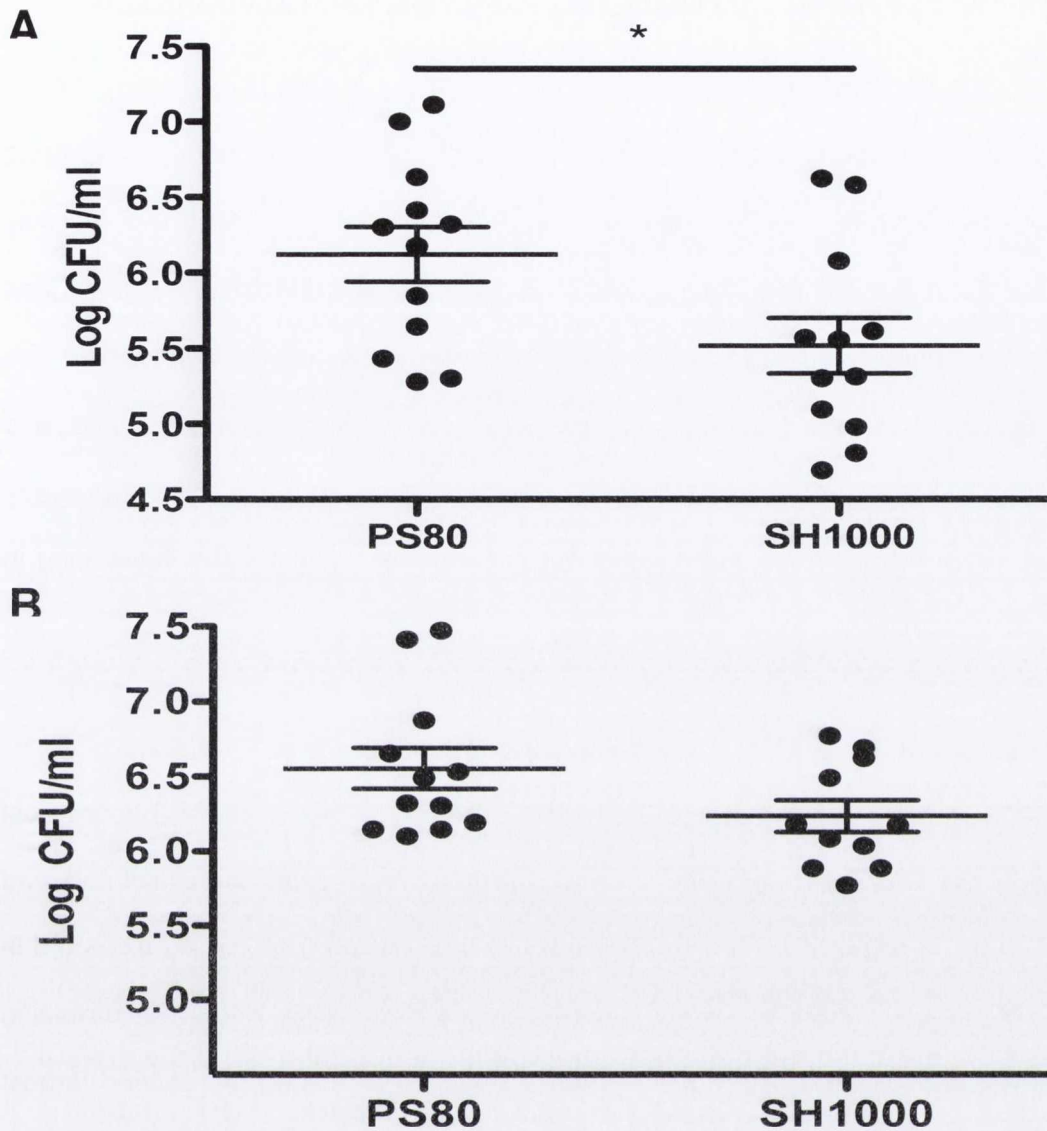


Figure 3.17: Reduced intracellular survival results in reduced persistence in the periphery.

Groups of mice were infected with PS80 or SH1000 (5×10^8 CFU) via i.p. injection. At 12 hours post infection, spleens (A) and livers (B) were harvested, homogenised and total bacterial burden quantified. Line represents mean $\pm$ SEM, n=12, *p<0.05.

3.2.10 PS80 primarily survives inside neutrophils and dendritic cells during *in vivo* infection

To establish which specific leukocyte populations *in vivo* were harbouring intracellular bacteria a mechanism to detect cell-associated bacteria *ex vivo* was needed. To this end, a GFP-expressing strain of PS80 was generated by colleagues in the Department of Microbiology, TCD. To confirm that the GFP bacteria were constitutively expressing GFP, bacteria were grown to exponential phase and analysed either by fluorescent microscopy (Figure 3.18A) or by flow cytometry (Figure 3.18B). In both instances, all of the bacteria were found to be fluorescing in the FITC channel.

GFP-PS80 was then injected into the peritoneal cavity of WT mice. At 3 hours post infection the PECs and the blood were collected. Leukocytes were isolated and stained with a panel of antibodies to identify phagocyte populations. As expected in terms of sheer numbers, the predominant cell type associated with GFP-expressing PS80 was found to be neutrophils. Surprisingly, DCs contained the second largest population of PS80-GFP+ cells. In contrast, only a low number of monocytes were associated with PS80-GFP+ (Figure 3.19A&B). However, when the proportions of cell type were analysed, CD11c+ DCs were found to have the largest percentage of cells contain GFP-bacteria, significantly higher than both neutrophils and monocytes (Figure 3.19 C). The same trend was observed in leukocytes isolated from the blood. This supports the contention of this study that DCs play an important direct role in phagocytosis and clearance of *S. aureus* and in fact may suggest that DCs are a preferential cell type for intracellular survival of the bacteria.

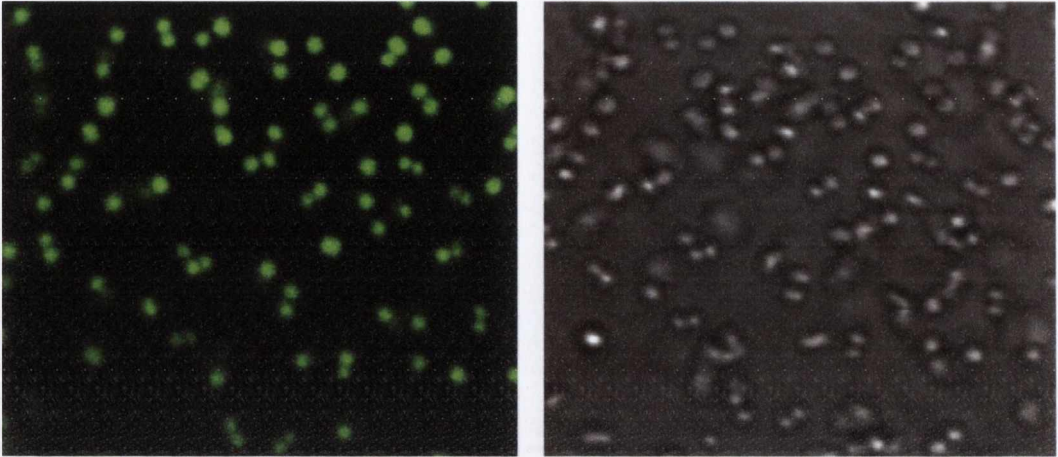
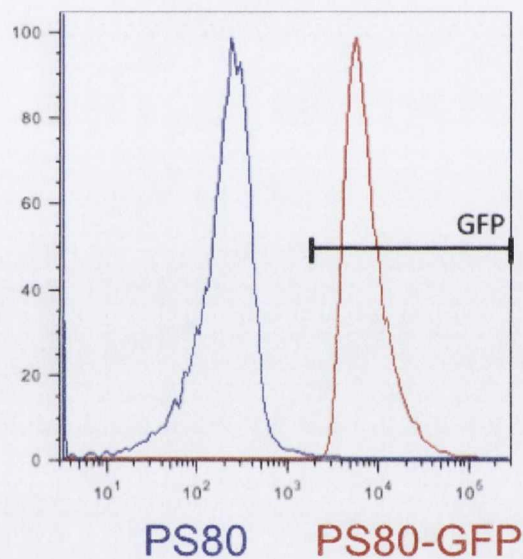
A**B**

Figure 3.18: PS80-GFP can be detected by fluorescent microscopy or flow cytometry.

GFP-PS80 or PS80 were grown to exponential phase growth and fixed on a coverslip to be viewed under a fluorescent microscope (A). Alternatively, bacteria were fixed and analysed by flow cytometry (B). *PS80-GFP generated by Dr. Ian Monk, School of Microbiology, TCD.*

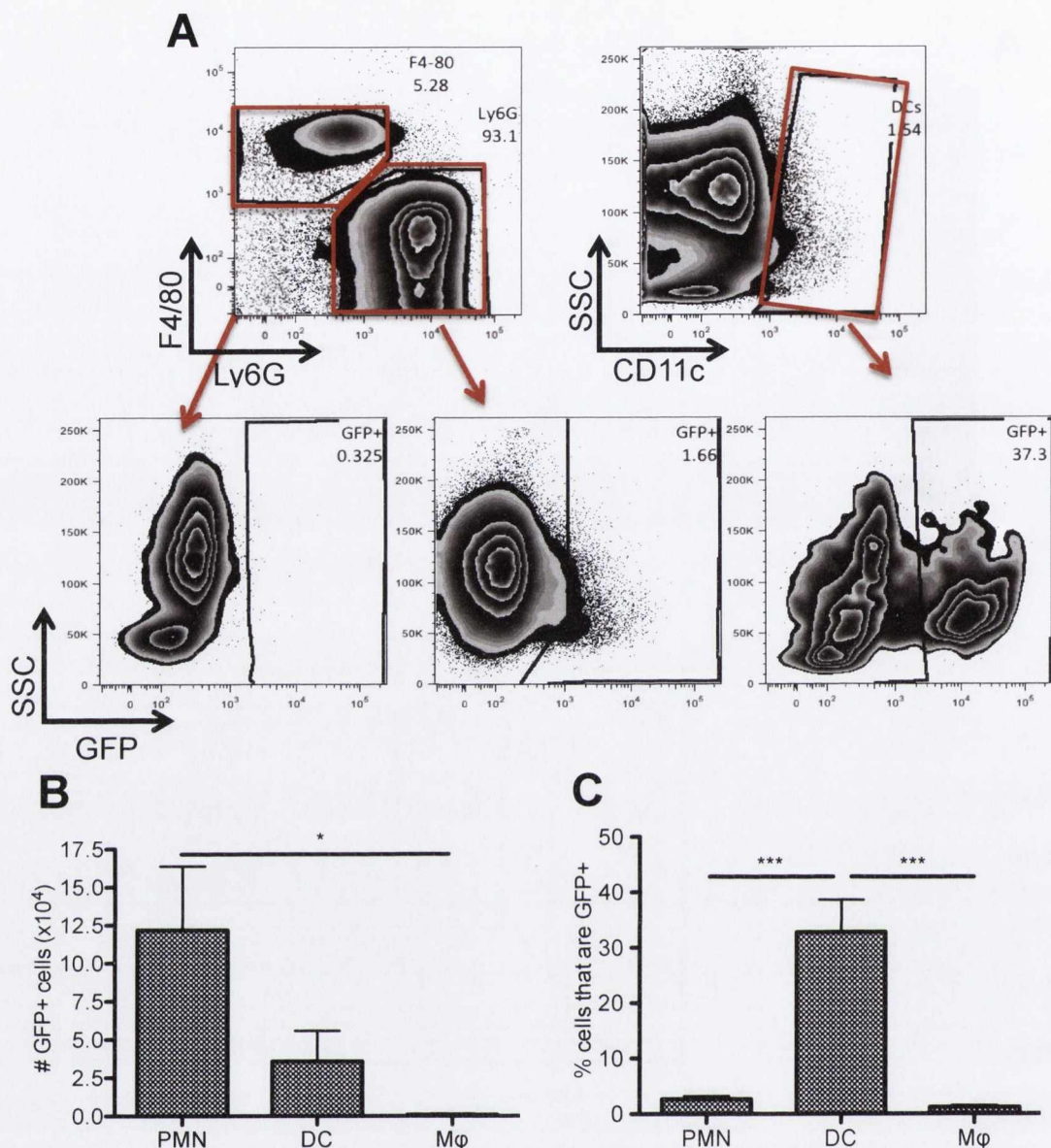


Figure 3.19: PS80-GFP is found within different phagocyte populations *in vivo*.

Mice were exposed to 5×10^8 CFU of GFP-PS80 via i.p. injection. Leukocytes were isolated 3 hours post challenge and analysed by flow cytometry. CD11b+F480-Ly6G+ (neutrophil (PMN)), CD11c+ (dendritic cells (DC)) and CD11b+F480+Ly6G- (monocyte (Mφ)) populations that were GFP+ were determined in the PEC as absolute numbers (B) and the % of each cell type that were GFP+ (C). Representative FACS plots are shown (A). Results expressed as mean $\pm$ SEM (B&C), $n=5$, * $p<0.05$, ** $p<0.01$

3.2.11 Capsular polysaccharide expression does not affect bacteremia levels

It has previously been documented that Capsular Polysaccharide (CP) + *S. aureus* strains seed the blood stream from the peritoneal cavity in greater numbers than CP- strains (109, 207). PS80 is known to express CP 8 (181), whereas SH1000 is a-capsular (182) and the CP-expression of the clinical strains are unknown. In order to prove that the different abilities of these strains to seed the blood and survive intracellularly were not simply due to differences in CP expression levels, mice were infected with PS80 or an isogenic mutant of PS80, RMS-1 that is a-capsular (207). 3 hours post infection, the blood and spleens were isolated and total bacterial burden quantified. There was no significant difference in the levels of bacteria recoverable from the blood (Figure 3.20A) or spleens (Figure 3.20B) of the two groups, confirming that the observed differences in bacteremia levels were not as a result of differential CP expression.

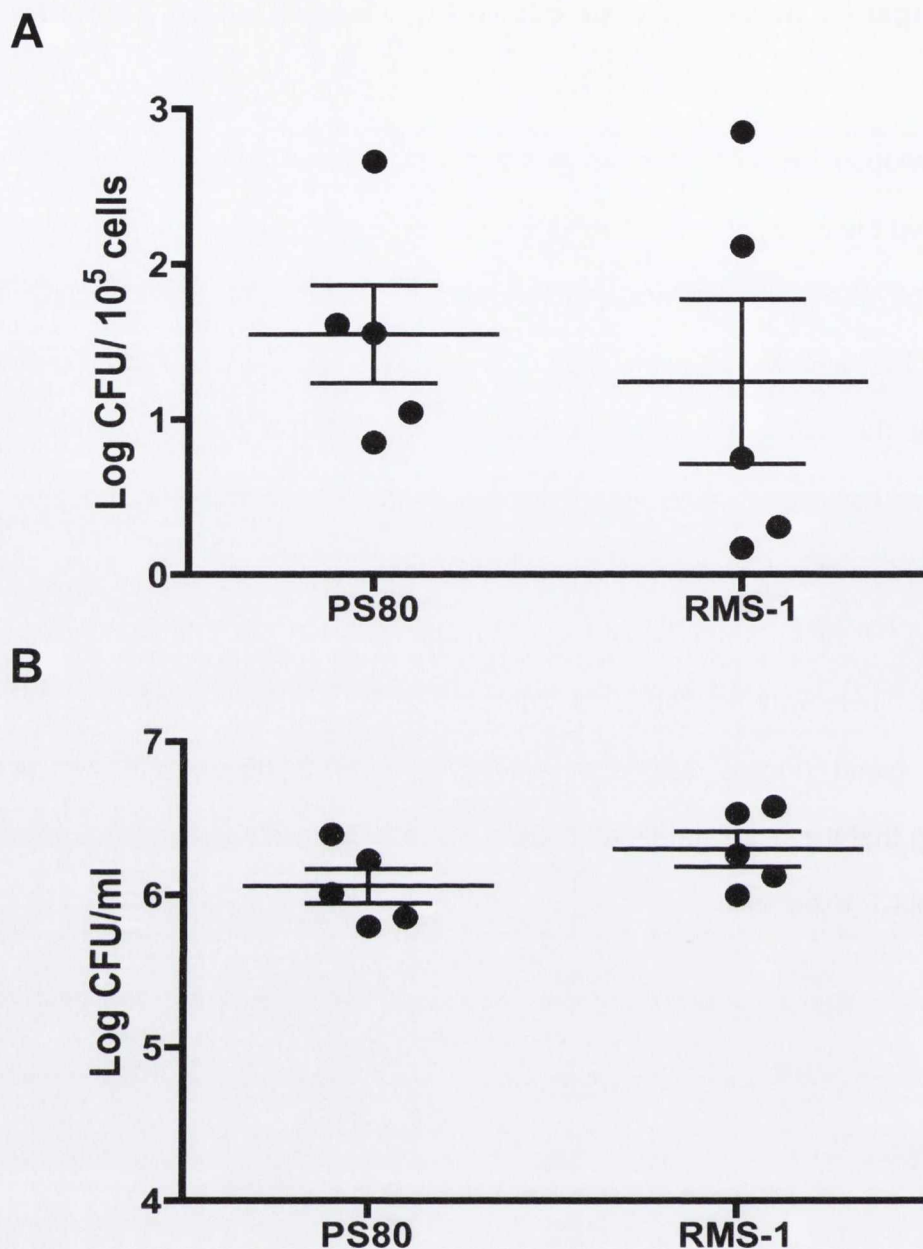


Figure 3.20: Differences in bacterial survival are not due to presence or absence of capsule.

Groups of mice were exposed to PS80 or RMS-1 (5×10^8 CFU) via i.p. injection. At 3 hours post infection, blood leukocytes were isolated, washed thoroughly and lysed to quantify intracellular bacteria (A). Spleens were harvested 3 hours post infection, homogenised and bacterial burden quantified (B). Line represents mean $\pm$ SEM, $n=5$.

3.2.12 Analysis of *agr* activity levels of strains reveals two distinct phenotypes

S. aureus' ability to divert from the endosomal pathway to autophagosomes is driven by factors that are under the control of the Agr regulatory system (175). It was hypothesised that the different abilities of strains to delay autophagic flux in this study may be associated with the level of expression of Agr. Consequently, collaborators in the University of Bath measured the Agr activity of the four strains used in this study using a vesicle lysis test (VLT). This assay measures the interaction of PSM toxins with lipid vesicles (208). PSM α peptide δ -toxin is translated from a short open reading frame located within the regulatory RNAIII molecule while transcription of the other *psm* genes is activated directly by the AgrA response regulator of the Agr two component signal transduction system. Expression of these toxins correlates to the level of expression of Agr in the stationary phase of growth (209). PS80 and Sa68 induced significantly more vesicle lysis than SH1000 and Sa279 (Figure 3.21A) suggesting a greater level of Agr activity in these strains.

To validate the results of the VLT, RNA III expression was also measured. RNA III regulates the transcription of a large number of virulence factors of *S. aureus* and is also an mRNA that codes for δ -toxin (210). Consistent with the VLT, RNAIII was expressed at higher levels by *S. aureus* strains PS80 and Sa68 as compared to SH1000 and Sa279 (Figure 3.21B). Taken together it can be concluded that the *S. aureus* strains PS80 and Sa68 that induce autophagosome accumulation, exhibit a greater level of *agr* activity than SH1000 and Sa279, which have no interactions with autophagosomes.

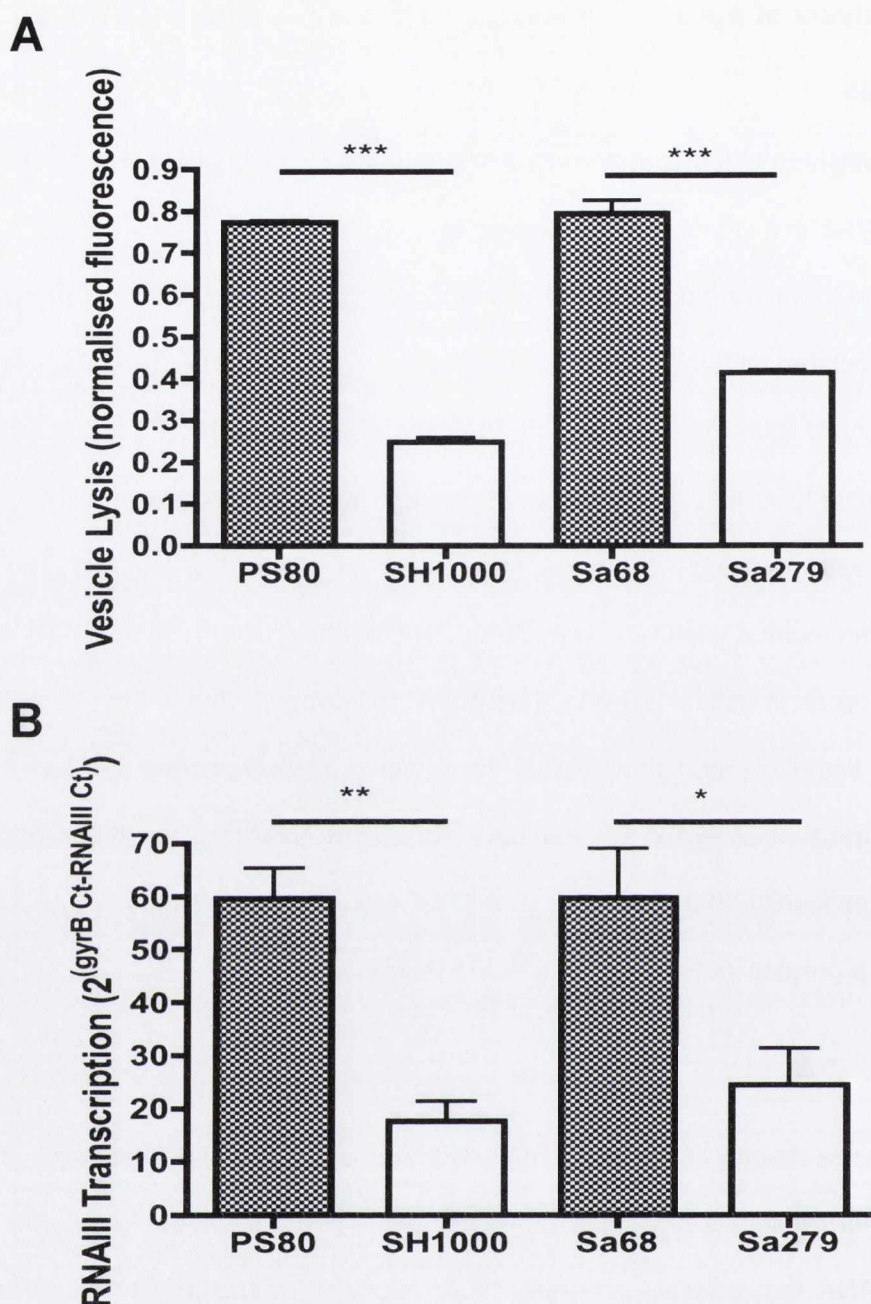


Figure 3.21: *S. aureus* strains exhibit distinct levels of Agr expression.

Bacterial supernatant was incubated at 1:1 ratio with lipid vesicles and fluorescence intensity recorded as a measure of vesicle lysis (A). RNAIII activity was measured using quantitative RT-PCR, as a ratio of RNA III and *gyrB* transcript number (B). Results expressed as mean $\pm$ SEM. n=3/4, *p<0.05, **p<0.01 ***p<0.001. Experiments performed by Dr. Maisem Laabei, University of Bath, UK.

3.2.13 Agr expression is critical for evasion of BMDC killing

Having observed that the strains used in this study expressed different levels of Agr, the role of Agr in controlling bacterial killing and the delay of normal autophagic flux was assessed. This was achieved through the generation of an *agr* mutant strain of PS80 by collaborators in the Department of Microbiology, Moyne Institute of Preventative Medicine, TCD. To confirm that the mutant was a true *agr* knockout, the presence of toxins that are under the control of the Agr system was tested by streaking WT PS80 and PS80 Δ *agr* alongside RN4220 to test for hemolysis on sheep's blood agar. α -haemolysin produced by PS80 causes the blood cells to lyse, resulting in a clear zone around the streak of bacteria. At the intersection of the PS80 and RN4220 the β -haemolysin (from RN4220) and δ -haemolysin (from PS80) synergise to produce a zone of clear haemolysis. As the clear zones of hemolysis are only seen surrounding the WT PS80 and not PS80 Δ *agr*, it is evident that PS80 Δ *agr* is a knockout and is not producing either δ -haemolysin or α -haemolysin (Figure 3.22).

To confirm that *agr*-expression influences bacterial killing by BMDCs, cells were infected with PS80 or PS80 Δ *agr* and bacterial killing monitored over time. By 6 h post infection almost 100% of PS80 Δ *agr* were killed (Figure 3.23A) as compared to the parental strain, which failed to be killed. In addition, the ability of PS80 Δ *agr* to escape from the BMDCs was then assessed. After 12 hours there was no increase in the amount of PS80 Δ *agr* in the culture supernatant, while WT PS80 increased significantly (Figure 3.23B).

To establish whether the absence of Agr activity prevented PS80 killing the BMDCs, cells were infected with WT PS80, PS80 Δ *agr* or left uninfected. Following gentamicin killing of extracellular non-phagocytosed bacteria, the LDH release was monitored at

6 h and 12 h. As previously shown, PS80 induced significant release of LDH from the cells, however the level of LDH found in the supernatant of cells infected with PS80 Δ *agr* was at a similar level to the uninfected control cells (Figure 3.23C).

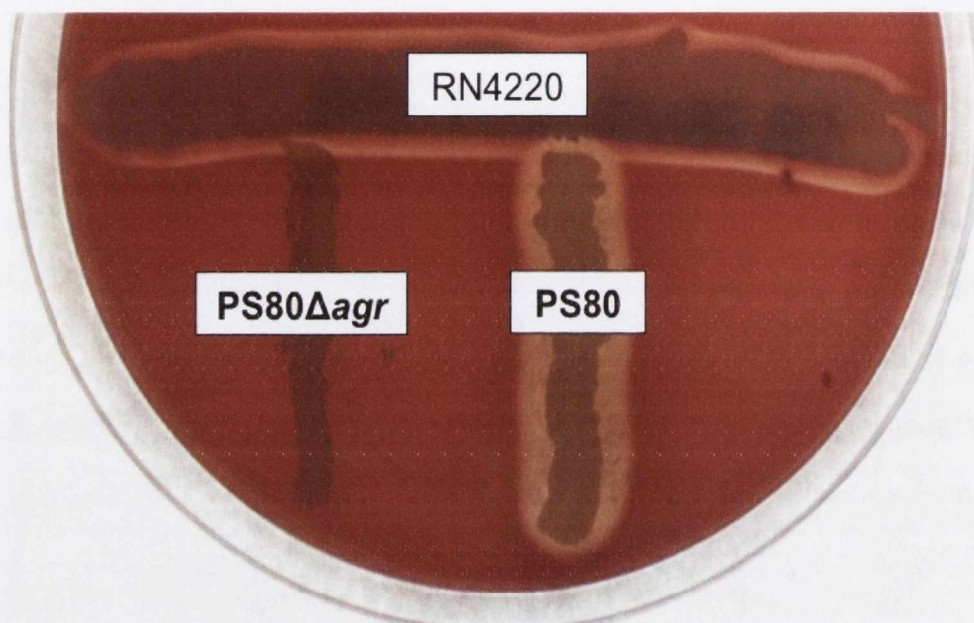


Figure 3.22: PS80 Δ *agr* produces no α -haemolysin or δ -haemolysin.

PS80 or PS80 Δ *agr* were streaked on a sheep's blood agar plate, perpendicular to *S. aureus* strain RN4220.

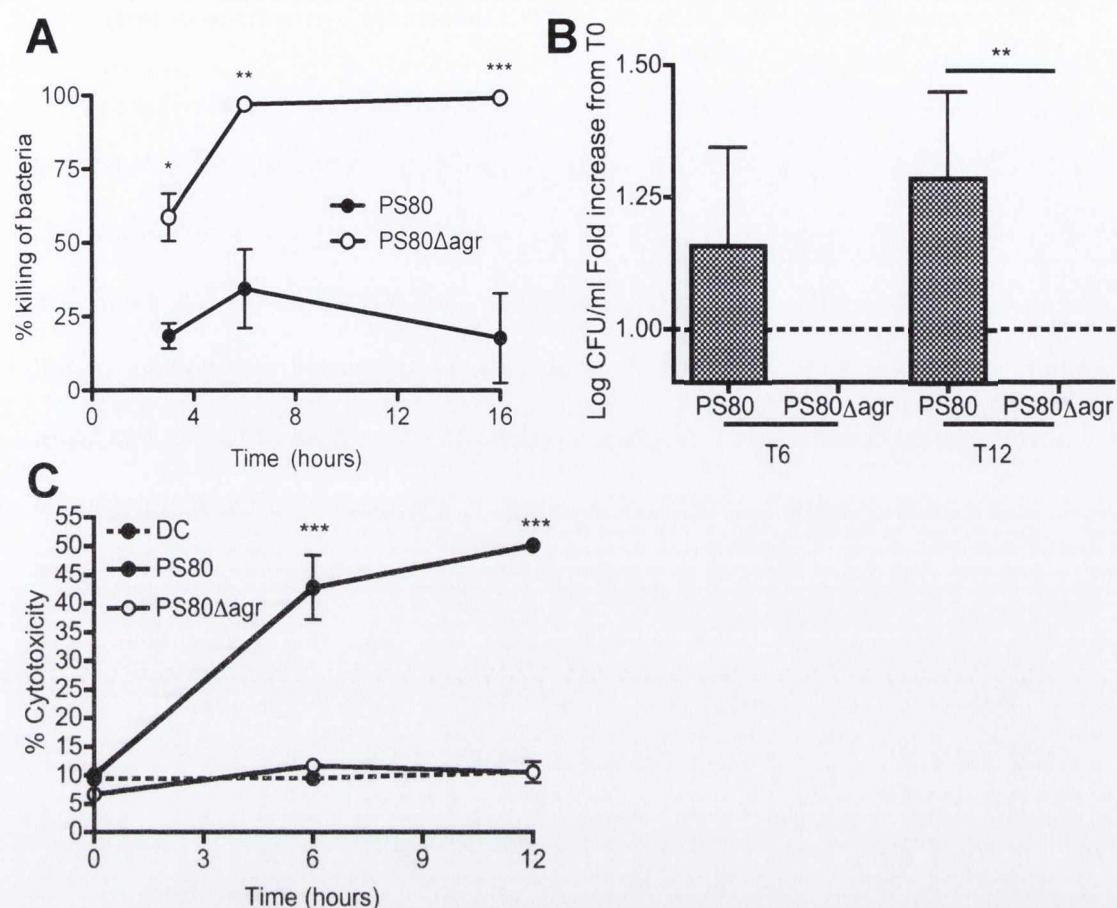


Figure 3.23: PS80Δagr is killed by BMDCs.

BMDCs were infected with *S. aureus* strains PS80 or PS80Δagr at MOI 100. % killing of bacteria was determined by comparing total CFU in the presence of BMDCs to bacteria in media only (A). Escape of each strain into the cell culture media was assessed at 6 hours and 12 hours post gentamicin treatment (B). LDH levels were assessed in the supernatant of both infected and uninfected BMDCs (C). Results expressed as mean±SEM (A&B) or mean±SD (C). n=3 (A&B), representative of 3 independent experiments (C), *p<0.05, **p<0.01 ***p<0.001.

3.2.14 Agr activity contributes to *S. aureus* induced autophagosome accumulation in BMDCs

Having established that Agr activity is necessary for the evasion of BMDC killing by PS80, the effect that *agr* expression by *S. aureus* has on the manipulation of autophagy was assessed. After 6 h of infection with WT PS80 or PS80 Δ *agr*, the accumulation of LC3-II in infected BMDCs was measured by immunoblotting. LC3-II expression was reduced in cells infected with PS80 Δ *agr* compared to the wild type, further proving that the *agr* locus plays a role in PS80's ability to block autophagic flux. However, the LC3-II processing was not reduced to baseline levels (Figure 3.24A&B), suggesting that PS80 may also be expressing alternative, non-Agr regulated genes, which have some capacity to delay autophagic flux.

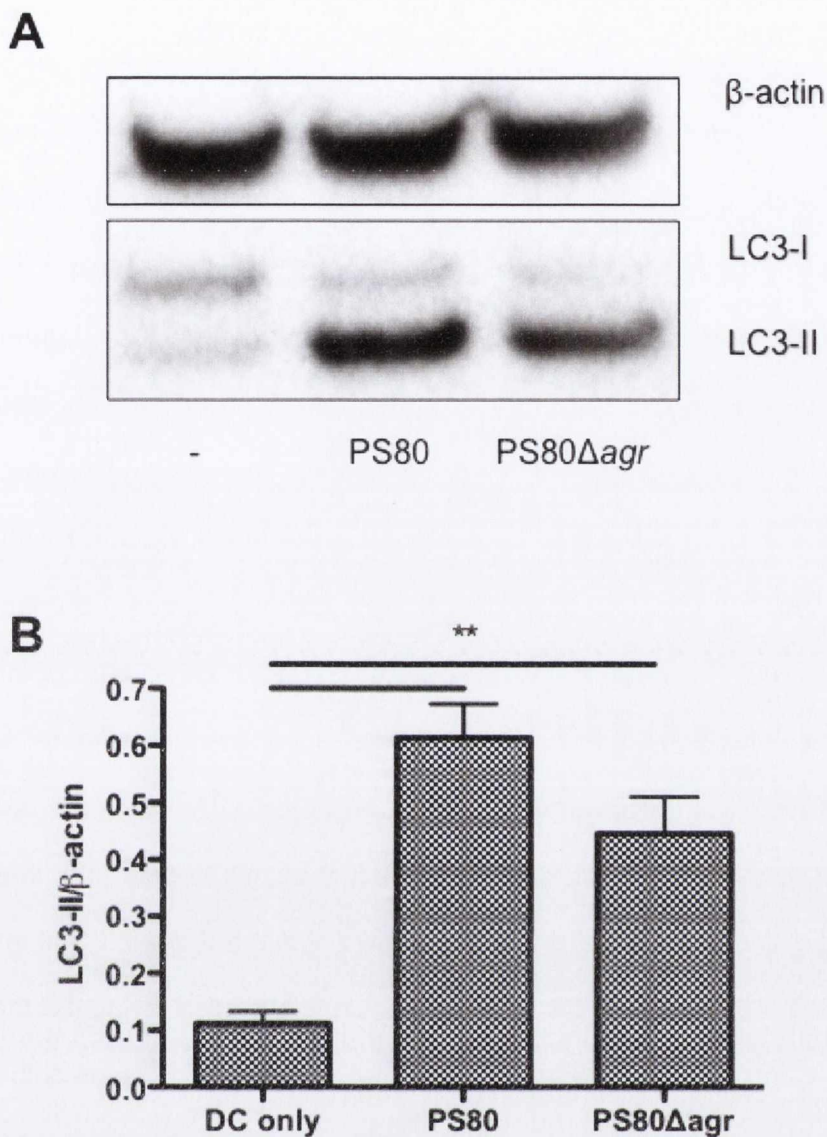


Figure 3.24: PS80Δagr reduces accumulation of LC3-II.

At 6 hours post infection and gentamicin treatment, BMDCs were lysed and processing of LC3 analysed by Western immunoblotting. β-actin was measured as a loading control. Representative blot is shown (A). Densitometry analysis of 3 independent experiments are shown as mean±SEM (B), **p<0.01.

3.2.15 Agr expression affects intracellular survival *in vivo*

As Agr activity has been shown above to play a vital role in the evasion of phagocytic killing of *S. aureus in vitro*, it was important to determine whether different levels of Agr activity accounted for the different phenotypes observed between strains *in vivo*. Thus, groups of WT mice were injected i.p. with either PS80 or PS80 Δ agr (5×10^8 CFU). After 3 hours of infection, blood was collected and total bacterial burden was assessed. Mice infected with PS80 Δ agr had a significantly lower bacterial load than those infected with PS80 (Figure 3.25A). To confirm that these bacteria were intracellular, leukocytes were isolated by centrifuging the blood through Histopaque 1083, washed well and lysed to determine the intracellular bacterial burden. The number of intracellular bacteria in the leukocytes isolated from PS80 Δ agr-infected mice was significantly lower than that of the PS80-infected mice (Figure 3.25B). Finally, the persistence of these bacteria was assessed by determining the bacterial burden in the spleens and livers of PS80 Δ agr-infected mice 12 hours after infection. The bacterial load was found to be significantly lower compared to the PS80 infected mice (Figure 3.25C). Taken together, these data provide further evidence that Agr activity plays an important role in the intracellular survival of *S. aureus* in blood leukocytes *in vivo*, potentially allowing more efficient dissemination to the periphery.

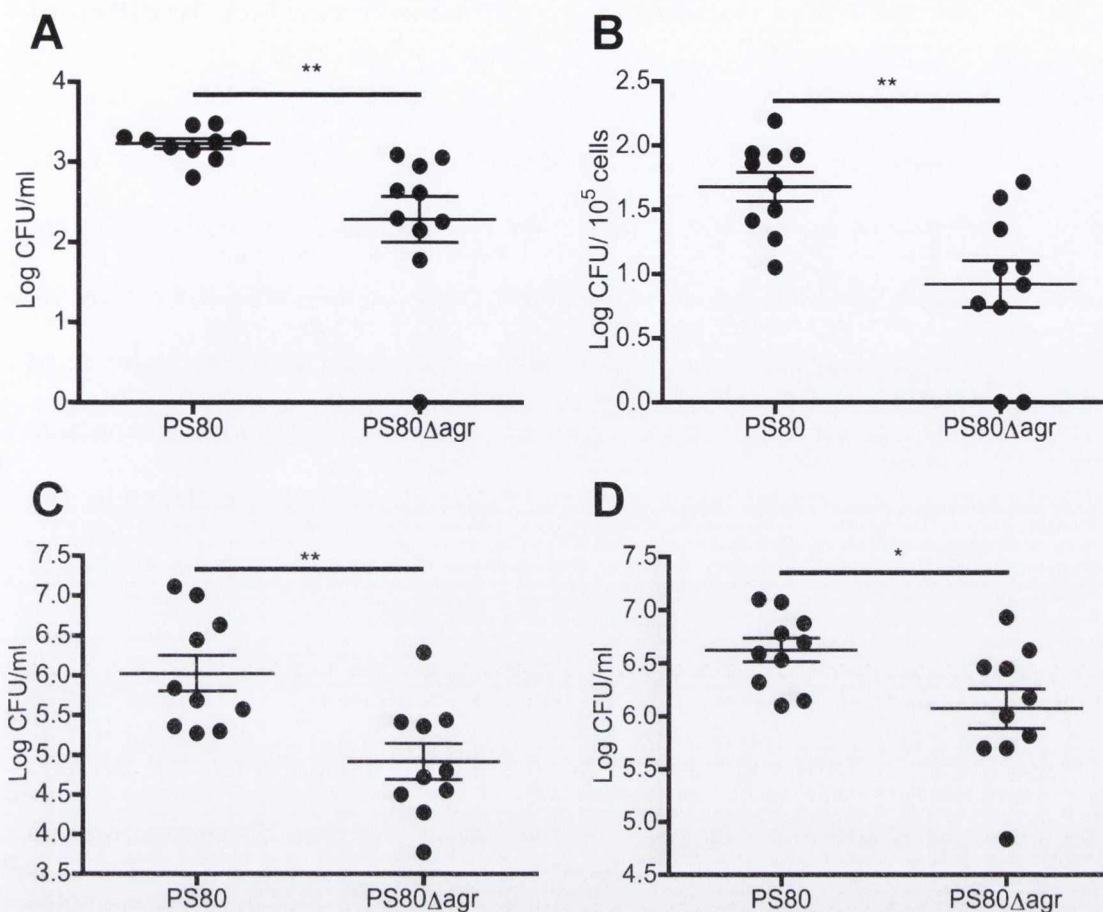


Figure 3.25: PS80 persists both in blood leukocytes and in the periphery at a higher level than an Agr deficient mutant.

Mice were exposed to PS80 or PS80 Δ agr (5×10^8 CFU) via i.p. injection. At 3 hours post infection, the bacterial burden in the blood was assessed (A). Total leukocytes in the blood were isolated, washed well and lysed to quantify intracellular bacteria (B). At 12 hours post infection, spleens (C) and livers (D) were harvested and total bacterial load assessed. Line represented mean $\pm$ SEM, $n=10$, ** $p<0.01$.

3.2.16 Differential Hla expression is not sufficient to account for different phenotypes observed between strains

To date, Hla has been identified as the only known *S. aureus* virulence factor implicated in the induction of autophagy (174). The expression of *hla* is under the control of the Agr system, thus it was important to characterise Hla expression by the strains used in this study to determine whether their patterns of Hla expression could account for the divergent phenotypes observed. To this end, culture supernatants from each strain of *S. aureus* underwent SDS-PAGE and were immunoblotted for Hla expression. *S. aureus* strains PS80 and Sa68 were comparable in their abilities to manipulate autophagy in order to evade phagocytic killing, however PS80 was a high Hla producer whereas Sa68 was Hla negative. Furthermore, SH1000 and Sa279 were both killed by DCs and failed to accumulate autophagosomes but SH1000 expressed appreciable if low levels of Hla whereas there was no Hla expression detectable in Sa279 (Figure 3.26). Consequently it appears that the discrepancies among strains in terms of their ability to engage autophagy to facilitate intracellular survival in phagocytes cannot be explained by expression of Hla and raises the likelihood that other Agr-regulated factors might also be engaged in *S. aureus*' manipulation of autophagy.

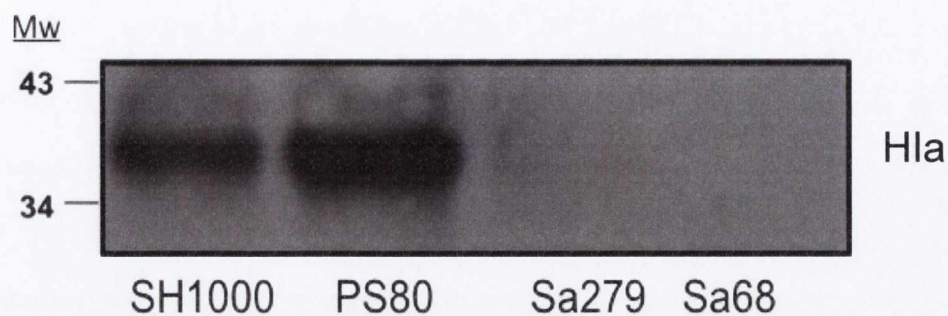


Figure 3.26: Hla expression of *S. aureus* strains.

Supernatants from stationary phase cultures were filtered, concentrated and expression of Hla assessed by Western blotting. A representative blot from 3 independent experiments is shown. *Experiments performed by Dr. Joan Geoghegan, School of Microbiology, TCD.*

3.2.17 Whole genome sequencing of *S. aureus* strains

In order to look for possible genetic factors that would account for the different phenotypes between strains, all 4 strains were sequenced by Dr. Michéal MacAogain, Department of Clinical Microbiology, School of Medicine, TCD. This sequencing was conducted concurrently with a large batch of strains in another study, providing the opportunity to take a very preliminary overview of mutations between the strains in this study. As there are only four strains in this study, it is impossible to draw any significant conclusions from these data as determination of any association between genotype and phenotype requires a much larger number of samples. However, whole genome sequencing can give a preliminary overview of similarities and differences in gene expression between strains and may expose interesting discrepancies that warrant further research.

In annotating the strains, it was decided that analysis would look for genes responsible for “phagocyte susceptibility” or “phagocyte resistance” (the two observed phenotypes associated with different *agr* expression) and would focus specifically on genes under the control of the *agr* regulon as this has previously been associated with the subversion of autophagy. This biased approach highlighted several interesting non-synonymous mutations (Table 2). A nonsense mutation in the PSM3 gene of SH1000 was found to truncate the protein, this could account for increased “phagocyte susceptibility”, however there was no similar mutation in Sa279, despite these strains having the same phenotype. Furthermore, through analysis by both VLT and RT-PCR, it has been shown that not only are PSMs reduced in SH1000 and Sa279 (as demonstrated by decreased vesicle lysis), RNA III is also inhibited in these strains (See Figure 3.21). As RNA III is upstream of PSM3, it is unlikely that the truncation of PSM3 in SH1000 plays any role in the phenotype

observed. Additionally, PS80 was found to have a SNP in PSM3, further implying that this gene is not related to the different phenotypes between PS80 and SH1000.

Interestingly, both SH1000 and 279 were found to have SNPs in the AgrC region. AgrC is a component of the Agr pathway that is a receptor that binds the autoinducing peptide (AIP) to activate the Agr system. If these strains have loss-of-function mutations in AgrC, preventing the recognition or signalling of AIP, it could potentially explain why they had reduced Agr activity and thus were more susceptible to phagocytic killing. These preliminary data, while not sufficient to allow conclusions to be drawn, highlight differences between AgrC as a potential candidate for mechanistic studies on exactly how some strains of *S. aureus* can inhibit autophagic flux and survive intracellularly.

Strain	PS80	SH1000	Sa68	Sa279
Phenotype	<u>Phagocyte resistant</u>	<u>Phagocyte susceptible</u>	<u>Phagocyte resistant</u>	<u>Phagocyte susceptible</u>
Mutation to PSM3	Yes (Asn22Tyr)	Yes -Truncated (Ala5Pro)	No	No
Mutation to AgrC	No	Yes (Gly25Val)	No	Yes (Gly330Asn)

Table 3.2: Pertinent SNPs between *S. aureus* strains.

3.3 Discussion

Undoubtedly, the success of *S. aureus* as a pathogen can be attributed to its inherent ability to disarm the host's protective immune responses. In particular, *S. aureus* possesses a unique arsenal of virulence factors that can circumvent the bactericidal effects of phagocytes and can manipulate these cells, even parasitising them to facilitate an intracellular lifestyle. Analysis of several *S. aureus* strains revealed that, despite being phagocytosed to similar extents, some strains could elude phagocytic killing, subsequently lysing phagocytes and escaping. The strains identified as resistant to killing included a laboratory strain (PS80) and a clinically isolated strain (Sa68) that behaved similarly, illustrating the clinical relevance of these results. The ability to evade killing was directly associated with the capacity of these strains to inhibit normal autophagic flux within the cells. Infection with strains that were capable of inhibiting autophagosomal turnover resulted in an increased persistence intracellularly *in vivo*, suggesting that these strains exploit the autophagosome as an intracellular survival niche to facilitate persistence during infection. The ability of *S. aureus* to subvert autophagic pathways and survive within phagocytes was found to be associated with Agr activity as both strains with lower levels of Agr and mutants with no Agr activity exhibited normal, homeostatic turnover of autophagosomes and were killed by the phagocytes. Furthermore, both Agr-low SH1000 and the mutant of PS80 lacking Agr activity were less infectious and invasive *in vivo*, suggesting that the ability of *S. aureus* to survive intracellularly within phagocytes *in vivo* is also Agr dependent.

Previous studies have documented a protective role for dendritic cells during *S. aureus* infection. Depletion of DCs was associated with increased mortality during *S. aureus* blood stream infection (76), and impaired bacterial clearance in a *S. aureus* pneumonia model (201). In both cases the beneficial effects afforded by DCs were

dependent upon their ability to control inflammation through their influence on neutrophils and cells of the adaptive immune response. Analysis of phagocyte recruitment to the site of *S. aureus* peritoneal infection *in vivo* led to the observation that DCs were significantly increased in number during *S. aureus* infection as compared to the numbers of macrophages, which did not increase post-infection, raising the question whether DCs may play a more important direct role in killing of bacteria than previously suggested. When a GFP-expressing strain of *S. aureus* was used to determine which of the phagocytes were interacting directly with the bacteria, there were a higher number of GFP+ DCs than GFP+ MΦ in both the PEC and in the blood of infected mice and DCs were proportionally the cell type with the largest GFP+ associated population. *In vitro*, the direct bactericidal qualities of BMDCs were compared to BMMs and peritoneal macrophages and they were found to be equally as efficient at killing *S. aureus* strain SH1000 as either type of macrophage. This highlights the fact that DCs are without a doubt important cells in the immune response to *S. aureus*. Of note, this finding that BMDCs can kill *S. aureus* strain SH1000 contrasted with a previously published study, which concluded that BMDCs do not play a role in direct killing of this strain (76). In that study, dendritic cells were infected with *S. aureus* at a very low ratio (MOI 0.1). However, as it has previously been shown that bacterial killing by macrophages is directly linked to MOI (211), the threshold for DC killing was tested. While no killing of SH1000 was observed at an MOI 0.1, the ability of BMDCs to kill SH1000 by 16 hours became apparent at as low an MOI as 2 (97.7±1.7% killing). This suggests that the MOI must reach a critical threshold *in vitro* in order to activate the DCs to kill the bacteria.

Although the phagocytes were shown to kill SH1000 efficiently, it was found that BMDCs, BMMs and peritoneal macrophages were all unable to kill *S. aureus* strain PS80. Further analysis revealed that both PS80 and SH1000 were phagocytosed to

the same extent by the BMDCs, suggesting that the survival of PS80 in phagocytes is due to a superior capacity for evasion of their killing mechanisms. An identical trend was observed when the clinical strains were examined, with Sa68 surviving in the presence of phagocytes (like PS80) while Sa279 was killed (similar to SH1000), although both strains were phagocytosed to the same extent.

Upon invasion of non-professional phagocytes *S. aureus* has been shown to subvert autophagy enabling replication within the autophagosome, and subsequent lysis of the host cell (175), thus the subversion of autophagy by *S. aureus* within the DCs as a potential mechanism of immune evasion was examined. This study proves that *S. aureus* strains PS80 and Sa68 are capable of engaging with autophagy in professional phagocytes as a means of evading phagocytic killing. Using both immunoblotting for LC3 processing and confocal microscopy assessing the colocalisation of GFP-bacteria with the LC3+ autophagosome, it was shown that these strains, but not SH1000 or Sa279, led to the accumulation of and association with autophagosomes. Furthermore, blocking autophagy using 3-MA prevented both PS80 and Sa68 escaping DC killing and inhibited their associated cytotoxic activities, providing definitive evidence that these strains of *S. aureus* are subverting autophagy. The correlation between a strain's ability to evade phagocytic killing and to subvert autophagy confirms that the bacteria is using the autophagosome as a niche in which it can survive.

The mechanism by which certain strains of *S. aureus* delay autophagic flux in phagocytes has not yet been elucidated. It has been shown that ability of *S. aureus* to subvert autophagy in non-phagocytic cells is controlled by the Agr system. *In vitro*, *agr* mutants of *S. aureus* fail to trigger autophagy and are delivered efficiently to the

lysosome for degradation. Thus, the role of Agr in manipulating autophagic flux in phagocytes was assessed. It was found that high levels of Agr expression (as found in PS80 and Sa68 but not SH1000 or Sa279) were associated with evasion of phagocytic killing. A mutant of PS80 lacking the *agr* locus was killed by BMDCs while *in vivo*, PS80 Δ *agr* had a reduced ability to survive in the blood and periphery. These data confirm that in phagocytes, the Agr system plays an important role in the subversion of autophagy for intracellular survival.

The Agr system controls a large number of *S. aureus* virulence factors. However, until this study, Hla (under the control of Agr) was the only known *S. aureus* virulence factor implicated in the induction of autophagy (174). A recently published *in vivo* study has shown that while autophagy plays an important role in conferring protection against *S. aureus* lethality by mediating tolerance towards Hla, infection with a Hla mutant strain causes increased bacterial burden in wild-type mice in comparison to Atg16L1^{HM} mice (that have reduced autophagy). This indicates that Hla may be dispensable in the exploitation of autophagy in the context of intracellular bacterial survival (212). Interestingly, when Hla expression was profiled among strains used in this study, it did not seem to correlate with the abilities of these strains to inhibit autophagic flux in phagocytes. This raises the question whether other Agr-regulated factors might also be capable of manipulating autophagy. Intriguingly, the VLT used to assess Agr activity measures PSM activity in culture supernatants of *S. aureus* and the pattern of vesicle lysis corresponds exactly with the observed phenotypes (208). Thus it is tempting to speculate that these toxins may also have an as yet undocumented role in the induction of autophagy in phagocytic cells. Interestingly, melittin, a component of bee sting venom that is an α -helical, amphipathic antimicrobial peptide, similar in structure to δ -toxin (213) has previously been shown to induce autophagic cell death in trypanosomes (214). In addition,

α PSMs trigger phagosomal escape by *S. aureus* in the monocytic cell line THP-1s (196), allowing the bacteria to replicate in the cytoplasm (197), where they may cause the induction of autophagy (171).

Whole genome sequencing of the four strains used in this study identified a SNP in the AgrC region of SH1000 and Sa279. AgrC is a membrane protein that recognises the AutoInducing Peptide (AIP), which activates AgrA to begin transcription of virulence proteins. Therefore, a SNP in AgrC could reduce the activity of Agr and thus downregulate the transcription of PSMs, resulting in an inability to evade phagocyte killing. Undoubtedly, extensive further research is required to test this hypothesis. In order to determine definitively whether the PSMs are driving the subversion of autophagy, additional mutants of PS80 would need to be constructed to determine whether a PS80 Δ *psm* mutant has reduced ability to evade phagocyte killing. It would then theoretically be possible to knockout each individual PSM sequentially in order to determine the precise gene responsible for the manipulation of autophagy.

This ability to survive and replicate within the autophagosome of phagocytes has several obvious advantages. Firstly, phagocytes are the cells designed to kill the bacterium, thus by surviving within these cells bacteria can evade the immune system's main killing mechanism. Furthermore, phagocytic cells are more motile than other cell types and can migrate within the blood making them an attractive host cell for dissemination, a fact that is exploited by other bacterial species such as *L. monocytogenes* (215). Strains of *S. aureus* that were capable of subverting autophagy in DCs *in vitro* were found at higher levels in leukocytes isolated from the blood of infected mice and at higher levels in the spleen and liver 12 hours post

infection, indicating increased persistence in the periphery of the host. This provides substantial evidence that *S. aureus* is capable of hijacking phagocytic cells to aid in its dissemination. Unfortunately due to limitations in cell numbers, autophagic flux in individual blood leukocyte populations *ex vivo* could not be analysed to definitively prove that this was the mechanism by which the bacteria survived intracellularly *in vivo*. Ideally, in order to prove that the bacteria are surviving and replicating within autophagosomes *in vivo* (and to show dissemination), intravital microscopy using GFP-bacteria and mice that have fluorescently tagged LC3 would be required, which is significantly beyond the scope of this current study.

These results contribute to the growing literature that links subversion of autophagosomes by *S. aureus* with intracellular survival (174, 175). These data demonstrate that *S. aureus* strain PS80 and a comparable clinical isolate that express high levels of Agr prevent constitutive degradation of LC3-II+ autophagosomes, to create a temporary intracellular survival niche where they can survive and potentially replicate, and thus escape killing by professional phagocytes (Figure 3.3.1). This study implicates autophagy as a mechanism to facilitate temporary intracellular survival of certain *S. aureus* strains within different phagocytes, maximising their potential for dissemination and persistence *in vivo*. The notion that *S. aureus* could parasitise neutrophils to facilitate dissemination has already been proposed (133) and our studies support the hypothesis that other phagocytes, in particular dendritic cells, may also act as “Trojan horses” for the metastasis of *S. aureus* provided that the infecting organism possesses the appropriate tools to subvert autophagosomes. Given that our findings were replicated in clinically relevant strains, it is possible that identifying *S. aureus* isolates that can inhibit autophagic flux may predict invasive disease potential.

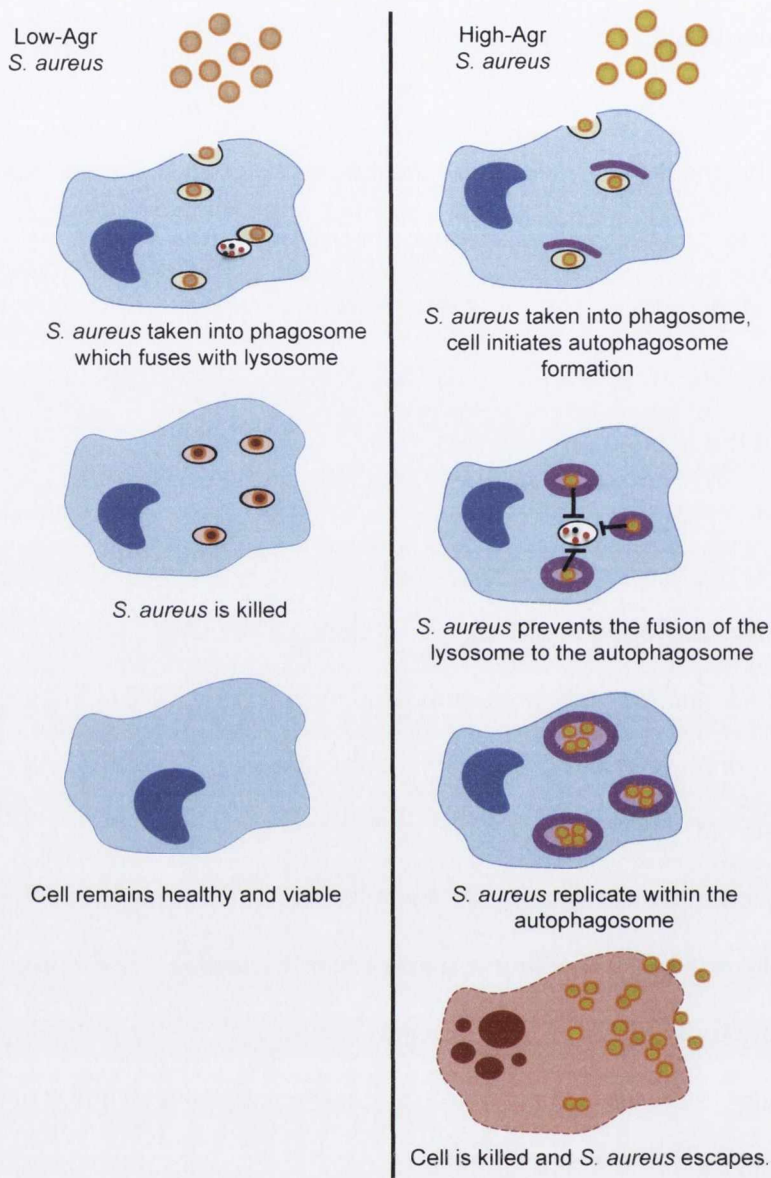


Figure 3.27 Summary of evasion of phagocytic killing by *S. aureus*.

Strains of *S. aureus* with low Agr activity are taken up into the phagosome, which fuses to the lysosome, killing the bacteria. The cell remains viable. *S. aureus* strains with high Agr activity are taken into the phagosome and cause initiation of autophagy, leading to autophagosome formation. Once in the autophagosome they can replicate and then escape from within the cell, resulting in cell death.

Chapter 4.

***S. aureus* manipulation of
neutrophil cell death facilitates
intracellular survival.**

4.1 Introduction

Polymorphonuclear Neutrophils (Neutrophils or PMN) are considered the primary host defence mechanism against *S. aureus* infection (216). Patients with defective neutrophil killing mechanisms have enhanced susceptibility to *S. aureus* infections and mice that have been depleted of neutrophils have been shown to suffer increased mortality in response to *S. aureus* exposure (45, 46). Despite the potent antibacterial capacity of neutrophils, *S. aureus* has evolved countless evasion mechanisms that allow it to prevent the recruitment and extravasation of neutrophils, evade phagocytosis by the cells, and even upon successful phagocytosis, *S. aureus* possesses numerous mechanisms by which it can elude intracellular killing (25) (discussed in detail in section 1.3). This ability to manipulate every facet of the neutrophil response means that *S. aureus* can actually survive within the cell (130). Thwaites et al. have referenced both laboratory and clinical data to develop their “Trojan horse” theory, which postulates that *S. aureus* is capable of surviving within neutrophils to facilitate dissemination around the host (133).

It is clear that an “appropriate” neutrophil response is key to the clearance of *S. aureus*. While neutrophils are indispensable in the resolution of *S. aureus* infection, it has been shown that excessive neutrophil recruitment to the site of infection is detrimental and results in increased bacterial burden (83). This is presumably due to the exploitation of these neutrophils by the bacteria as an intracellular survival niche. In contrast, inhibition of neutrophil recruitment (without the complete abrogation of the neutrophil response) actually afforded protection against bacterial growth, by reducing the availability of an environment in which the bacteria could replicate (83). Thus, it seems that there is an optimum number of neutrophils that facilitates clearance of the infection without providing an intracellular survival niche. In order to

achieve this, a balance must be struck between neutrophil recruitment and apoptosis and clearance.

It is well established that neutrophils are quickly recruited to the site of infection following *S. aureus* exposure. In order to balance this efficient recruitment, once the neutrophils have phagocytosed bacteria they need to undergo apoptosis and be cleared away by infiltrating macrophages. Under normal circumstances, neutrophils are very short-lived cells that constitutively expresses pro-apoptotic genes and as such, undergo apoptosis within a few days of leaving the bone marrow, or upon phagocytosis and killing of extracellular bacteria. The relatively short half life of neutrophils calls into question the value to the bacterium of surviving in such a fleeting niche and suggests that the short lifespan of the neutrophil likely requires modulation for it to be of value as an intracellular reservoir. Indeed it has previously been reported that certain strains of bacteria that are capable of survival within neutrophils (*Anaplasma phagocytophilum* (217) and *Chlamydia pneumoniae* (218)) inhibit the spontaneous apoptosis of the cells as a means of preserving their intracellular survival niche. These findings give rise to the supposition that *S. aureus* may also modulate neutrophil apoptosis to prolong their survival within these cells.

While there is a possibility that *S. aureus* may directly manipulate neutrophil apoptosis, there are many other facets of the immune system that play an appreciable role in directing neutrophil responses throughout the course of an infection. IFN γ and IL-17 are cytokines produced by cells of the immune system in response to *S. aureus* and are well established as key factors controlling neutrophil recruitment to the site of infection (84, 89). IFN γ has been shown to have an involvement in the intracellular survival of *S. aureus* through the induction of CXC

chemokines which recruit neutrophils that provide a survival niche (89). IL-17 has been shown to be important in enhancing neutrophil survival through its influence on G-CSF production, thus also facilitating bacterial persistence.

Although both cytokines have been shown to modulate recruitment, and perhaps to prolong the life of the cells, the role played by IFN γ and IL-17 in manipulating neutrophil apoptosis as a means of controlling the number of cells present at the site of infection has yet to be thoroughly studied in the context of infection. IFN γ has been shown to delay neutrophil apoptosis through IL-6 signalling in a murine model of acute peritoneal inflammation (91), suggesting that manipulation of apoptosis may be another mechanism by which IFN γ can control neutrophil numbers, and thus *S. aureus* intracellular survival at the site of infection.

The data presented in this chapter aim to prove the hypothesis that *S. aureus* is capable of directly manipulating neutrophil apoptosis in order to prolong the availability of an intracellular niche in which it can survive both *in vitro* and *in vivo*. Furthermore, it addresses the proposition that T cell cytokines produced in response to *S. aureus* may also modulate neutrophil apoptosis and thus indirectly facilitate bacterial survival.

4.2 Results

4.2.1 Neutrophil killing of *S. aureus* is strain dependent

Having shown in Chapter 3 that certain strains of *S. aureus* (such as SH1000) were killed by DCs and macrophages while others (such as PS80) were not killed and instead exerted cytotoxic effects on the cells, the response of neutrophils to these two strains of *S. aureus* was assessed. Intriguingly, it was found that there were also strain dependent differences in the ability of neutrophils to kill this bacteria. Neutrophils were capable of killing both strains of *S. aureus* to a comparable degree within one hour of infection. However, by 6 hours post infection there was no further increase in killing of PS80. In contrast, there was a substantial increase in the killing of SH1000 between 1 hour and 6 hours post infection, bringing the level of killing of SH1000 to almost 100% by 6 hours (Figure 4.1 A). This implies that while neutrophils can effectively kill SH1000, they can only kill a finite amount of PS80, as 50% of the bacteria are still viable after 6 hours of incubation. This suggests that this strain of *S. aureus* is capable of evading killing by the neutrophils.

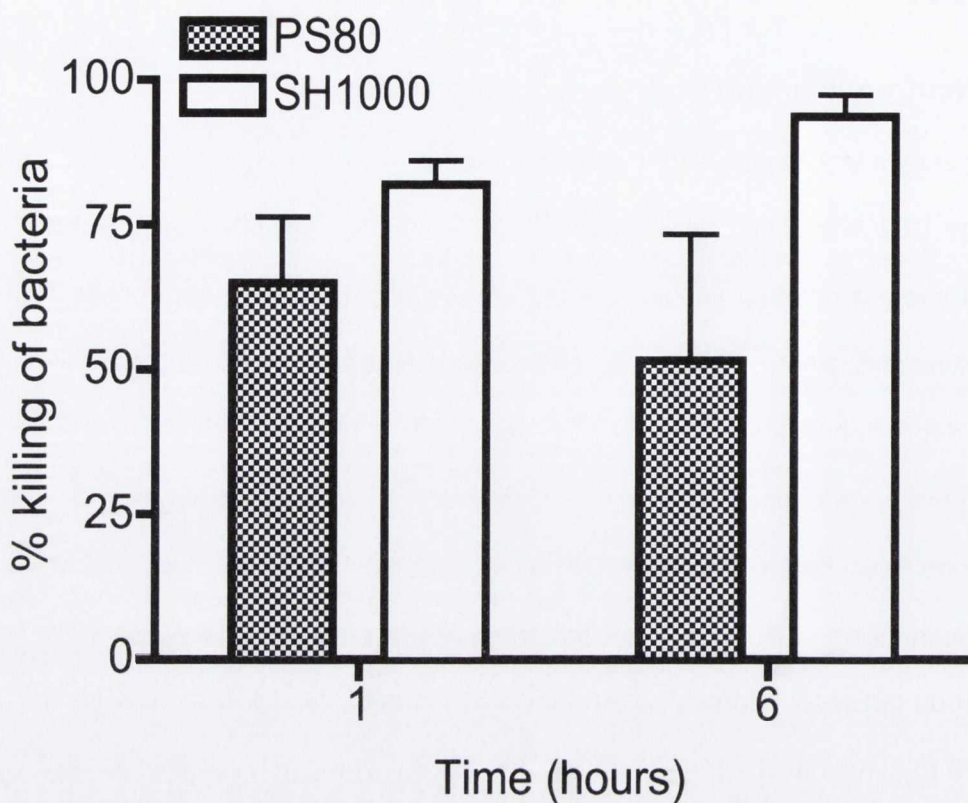


Figure 4.1: Human neutrophils can kill *S. aureus* in a strain specific manner.

Human PMN were infected with *S. aureus* strain PS80 or SH1000 at MOI 10 in the presence of opsonins. % killing of bacteria was determined by comparing the total CFU in the presence of phagocytes to bacteria in media only at 1 and 6 hours post-infection. Results expressed as mean $\pm$ SEM at each time point, n=5.

4.2.2 PS80 does not affect neutrophil viability at early timepoints

It was important to ascertain if *S. aureus* strain PS80 was exerting cytotoxic effects on neutrophils, which could explain the sub-optimal bacterial killing of PS80 in comparison to SH1000 (Figure 4.1 A). Neutrophils were infected at MOI 10 and viability was assessed at various timepoints by EB/AO staining. There was no significant difference in viability of infected or uninfected neutrophils within the first 14 hours of infection, however by 24 hours post infection the neutrophils were significantly killed in the presence of PS80 in comparison to the uninfected cells (Figure 4.2 A). To confirm that there was no difference in the viability of infected or uninfected neutrophils at early timepoints post-infection, neutrophils were cultured with/without PS80 and LDH release into the supernatant assessed at various timepoints post-infection. There was no significant difference in LDH release within 6 hours of infection with *S. aureus* strain PS80 compared to the control non infected cells (Figure 4.2 B). Thus, the reduced ability of neutrophils to kill PS80 observed after 6 hours can not be explained by reduced neutrophil viability and is presumably due to this strains' ability to somehow evade neutrophil killing.

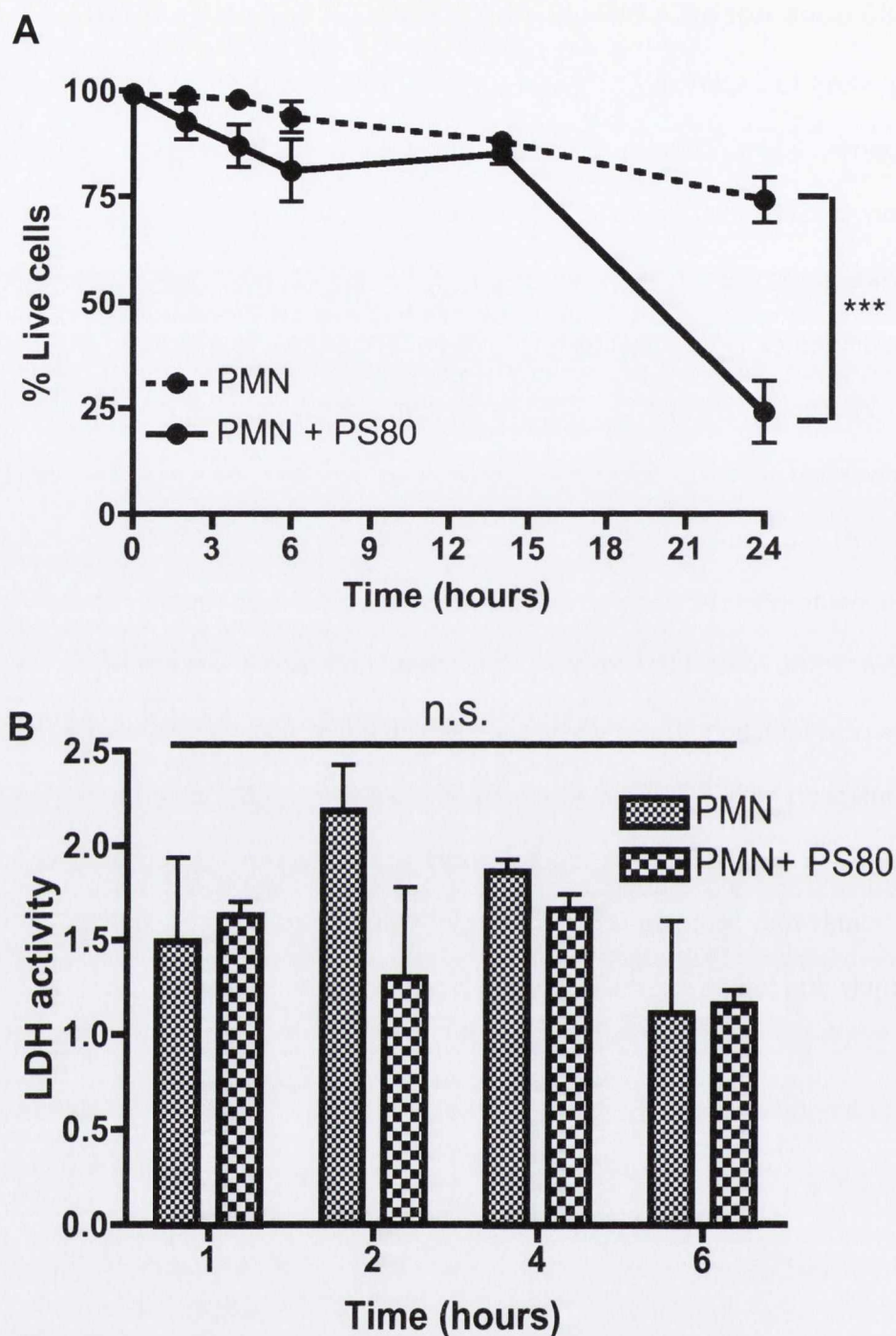


Figure 4.2: *S. aureus* strain PS80 exerts cytotoxic effects on neutrophils after 24 hours.

Human PMN were cultured with/without *S. aureus* strain PS80. At indicated time points, cells were counted in EBAO to determine the % live cells (A) or cell free supernatants were collected and the LDH levels determined (B). Results expressed as mean $\pm$ SEM, n=2-5, ***p<0.001.

4.2.3 Neutrophils constitutively undergo apoptosis

As demonstrated in Section 4.2.2, within 24 hours of incubation with *S. aureus* strain PS80 neutrophils are no longer viable, likely due to a build up of toxins in the artificial *in vitro* system which can lyse the cells (219). Therefore to, assess whether *S. aureus* could manipulate neutrophil apoptosis, experiments were restricted to the first 6 hours of infection, to exclude the lytic effects caused by toxin accumulation. Neutrophils were isolated from peripheral blood of healthy donors and incubated in cDMEM at 37°C, 5% CO₂ under constant rotation, allowing spontaneous apoptosis to occur. Neutrophils were then fixed and the levels of staining of neutrophils with TUNEL was measured. TUNEL is a fluorescent dye that is a widely used indicator of late-stage apoptosis as it detects strand breaks in DNA. TUNEL staining was assessed at 2 and 6 hours post infection and maximal TUNEL expression was observed at 6 hours (Figure 4.3 A&B).

In order to further investigate the mechanism of spontaneous apoptosis occurring in the human neutrophil population, cleavage of Caspase 3 was assessed. Cleavage of Caspase 3 from the pro-form is an important step in the initiation of apoptosis, thus it is detected at earlier time points than the late-stage DNA fragmentation detected by TUNEL. Human neutrophils were cultured in cDMEM for up to 2 hours at 37°C, 5% CO₂ under constant rotation and at various intervals, cells were lysed. The neutrophil lysates were run on SDS-PAGE, transferred to a PVDF membrane and probed for Caspase 3. Substantial levels of Caspase 3 cleavage were detected after 2 hours incubation (Figure 4.4 A&B), thus cleavage of Caspase 3 at this timepoint was used as an early indicator of apoptosis in future experiments.

Phagocytosis of *S. aureus* by neutrophils was facilitated by the addition of opsonins (IVIG and complement) to the culture throughout the study, so it was important to ensure that incubation with opsonins alone had no effect on neutrophil apoptosis. There was no difference observed in Caspase 3 cleavage between neutrophils cultured in media alone and those cultured in media supplemented with opsonins (Figure 4.5) after 2 hours of incubation.

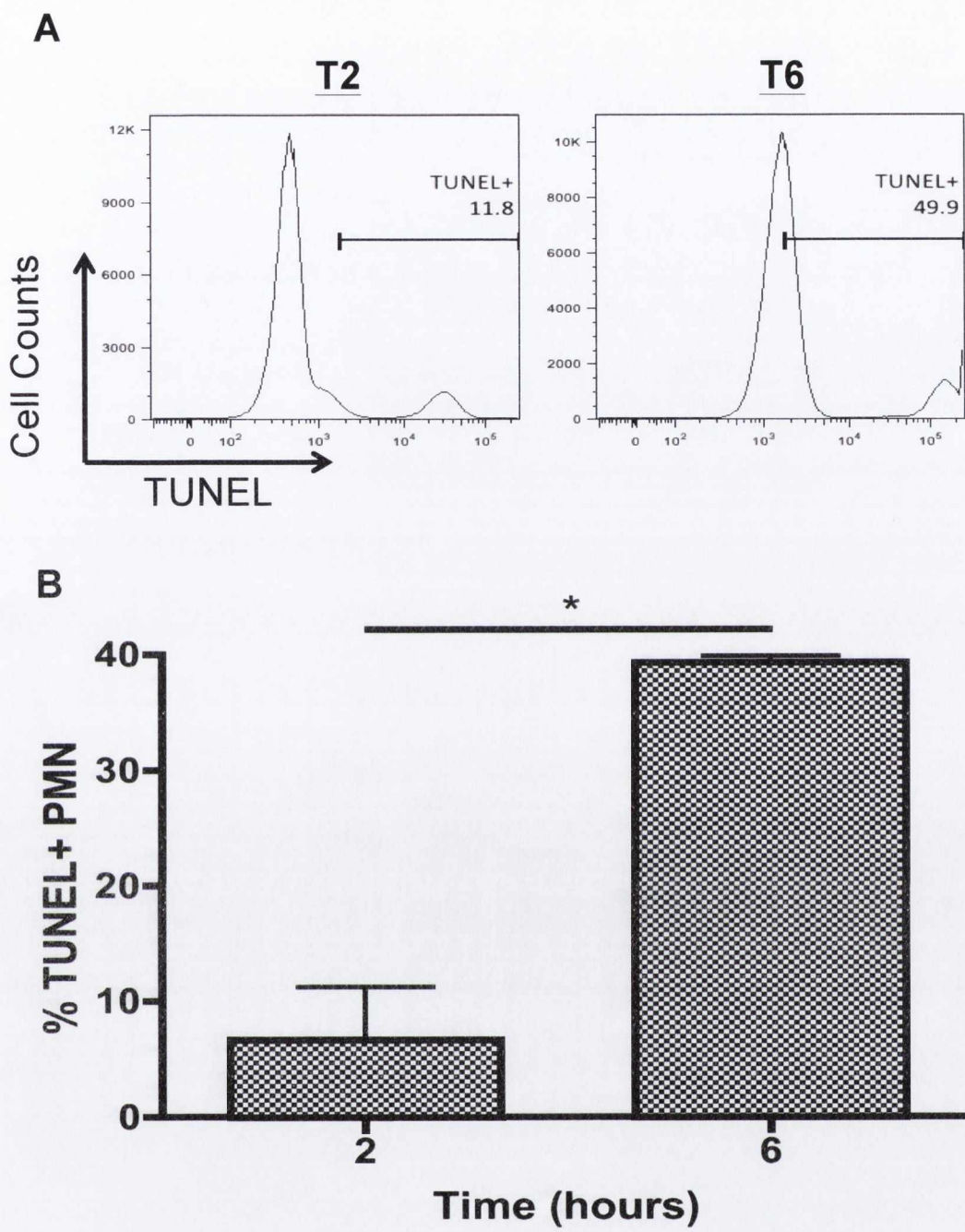


Figure 4.3: Human neutrophils spontaneously apoptose within 6 hours of *in vitro* culture, as measured by TUNEL staining

Human PMN were incubated at 5% CO₂, 37°C in media for specified times. Cells were then fixed, permeabilised and stained with fluorescein-conjugated TUNEL to be analysed by flow cytometry. Results expressed as representative FACS plots (A) and % TUNEL + cells, mean ± SEM (B). n=2, *p<0.05.

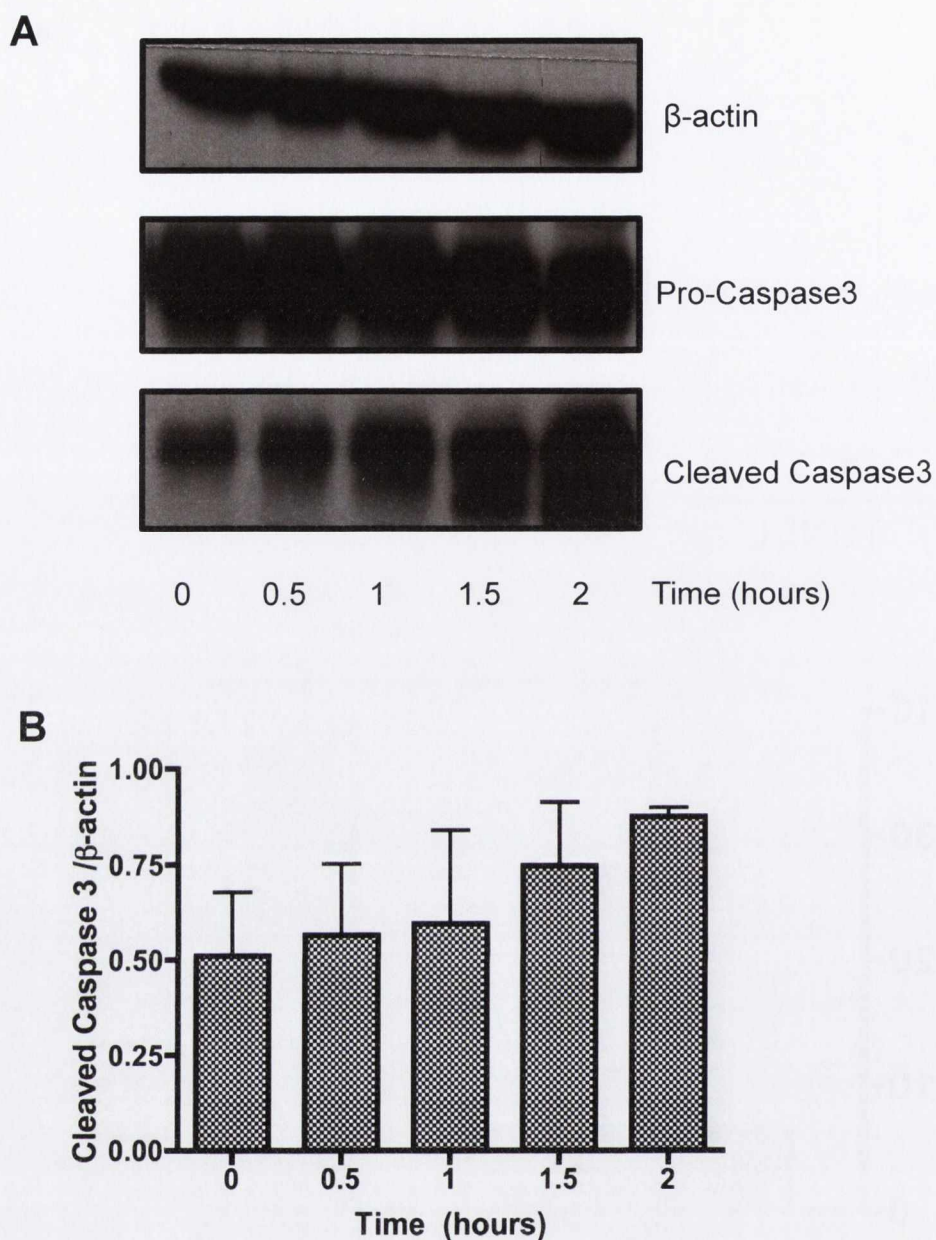
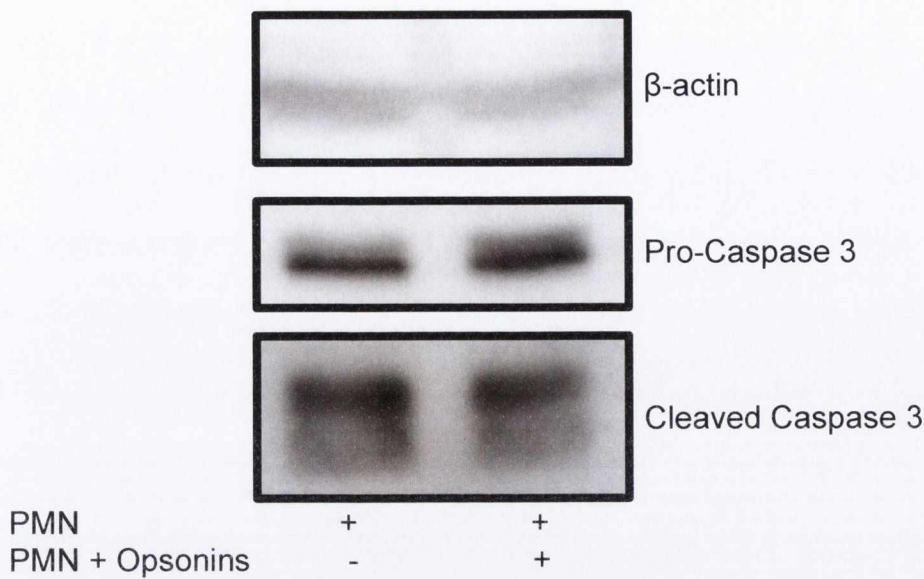


Figure 4.4: Human neutrophils spontaneously apoptose *in vitro* as measured by Caspase 3 cleavage

Human PMN were incubated at 5% CO₂, 37°C in media for specified times. Cells were lysed and run on SDS-PAGE and transferred to PVDF membrane. This was then probed with anti-Caspase 3. β -actin was probed for as a loading control. Results expressed as representative Western Blot (A) and plotted normalised to β -actin, mean $\pm$ SEM (B) n=2

A



B

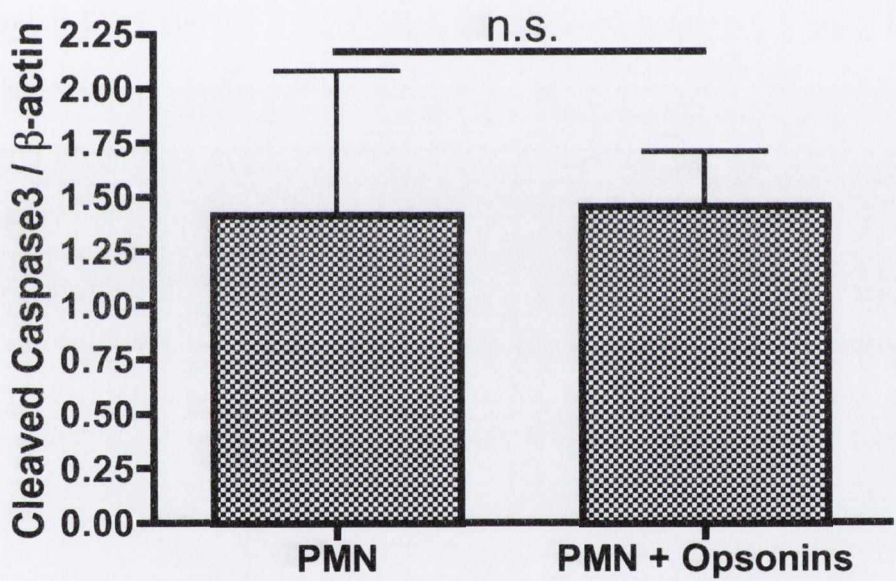


Figure 4.5: Opsonins have no impact on Caspase 3 cleavage.

Human PMN were incubated for 2 hours with/without opsonins. They were then lysed and run on SDS-PAGE and transferred to PVDF membrane. This was probed with anti-Caspase 3. β -actin was probed for as a loading control. Results expressed as representative Western Blot (A) and plotted normalised to β -actin, mean $\pm$ SEM (B), n.s. = results are not significant, n=3.

4.2.4 *S. aureus* inhibits spontaneous neutrophil apoptosis

Having optimised methods for detecting neutrophil apoptosis *in vitro*, the direct effects of *S. aureus* infection on neutrophil apoptosis were assessed. Human neutrophils were infected with *S. aureus* strain PS80 (hereafter referred to as *S. aureus*) and 6 hours after infection, staining by TUNEL was analysed by flow cytometry. Neutrophils that had been infected with *S. aureus* were found to have significantly reduced levels of TUNEL staining in comparison to uninfected neutrophils (Figure 4.6), and thus were less apoptotic.

To confirm that this reduction in neutrophil apoptosis was also associated with inhibition of Caspase 3 cleavage, cells were infected with *S. aureus* for 2 hours, lysed and cleavage of Caspase 3 assessed. *S. aureus*-infected neutrophils demonstrated significantly reduced levels of Caspase 3 cleavage at 2 hours post infection when compared to uninfected, control neutrophils (Figure 4.7). These results clearly illustrate that *S. aureus* strain PS80 is capable of directly delaying the normal process of apoptosis in human neutrophils.

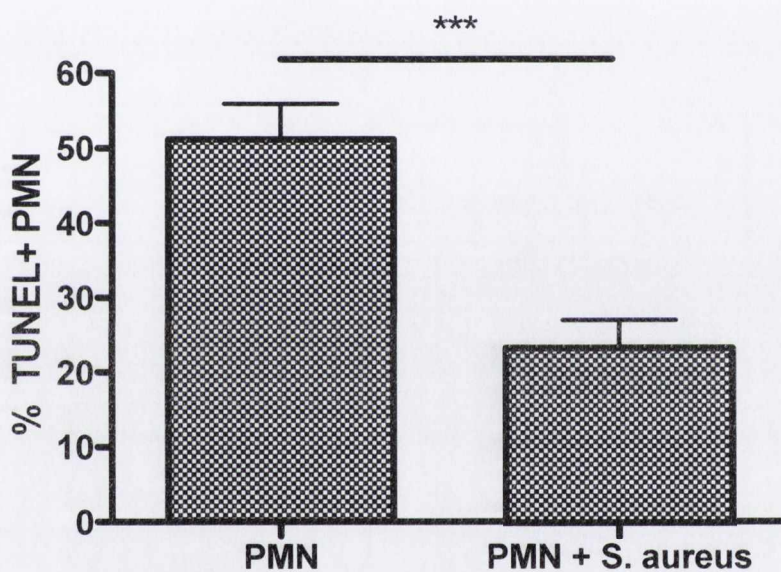
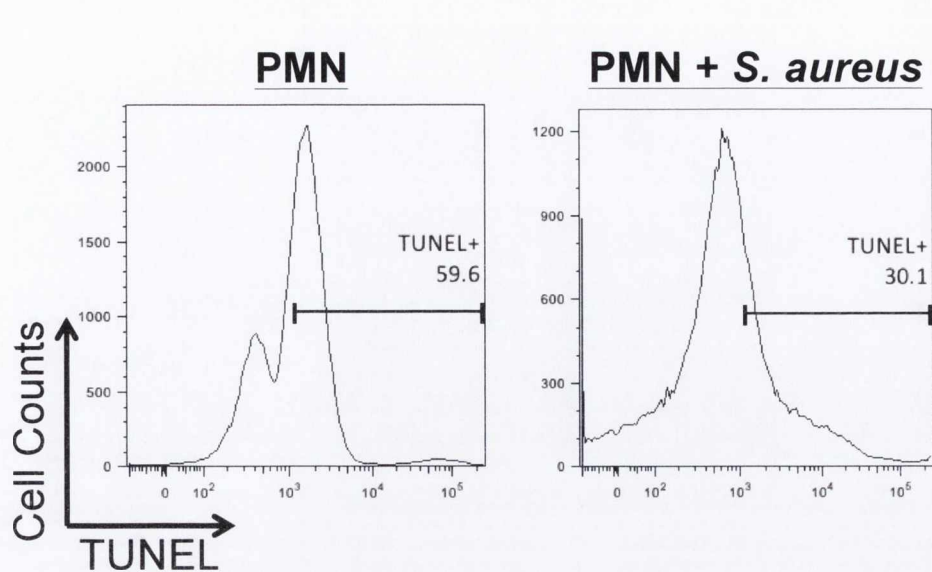


Figure 4.6: Infection with *S. aureus* inhibits spontaneous apoptosis of neutrophils as measured by TUNEL staining.

Human PMN were cultured with/without *S. aureus* (strain PS80) in the presence of opsonins. After 6 hours cells were fixed, permeabilised and stained with fluorescein-conjugated TUNEL and analysed by flow cytometry. Results expressed as representative flow cytometry plots (A) and % TUNEL+ cells, mean $\pm$ SEM (B). $n = 7$, *** $p < 0.001$ versus uninfected controls.

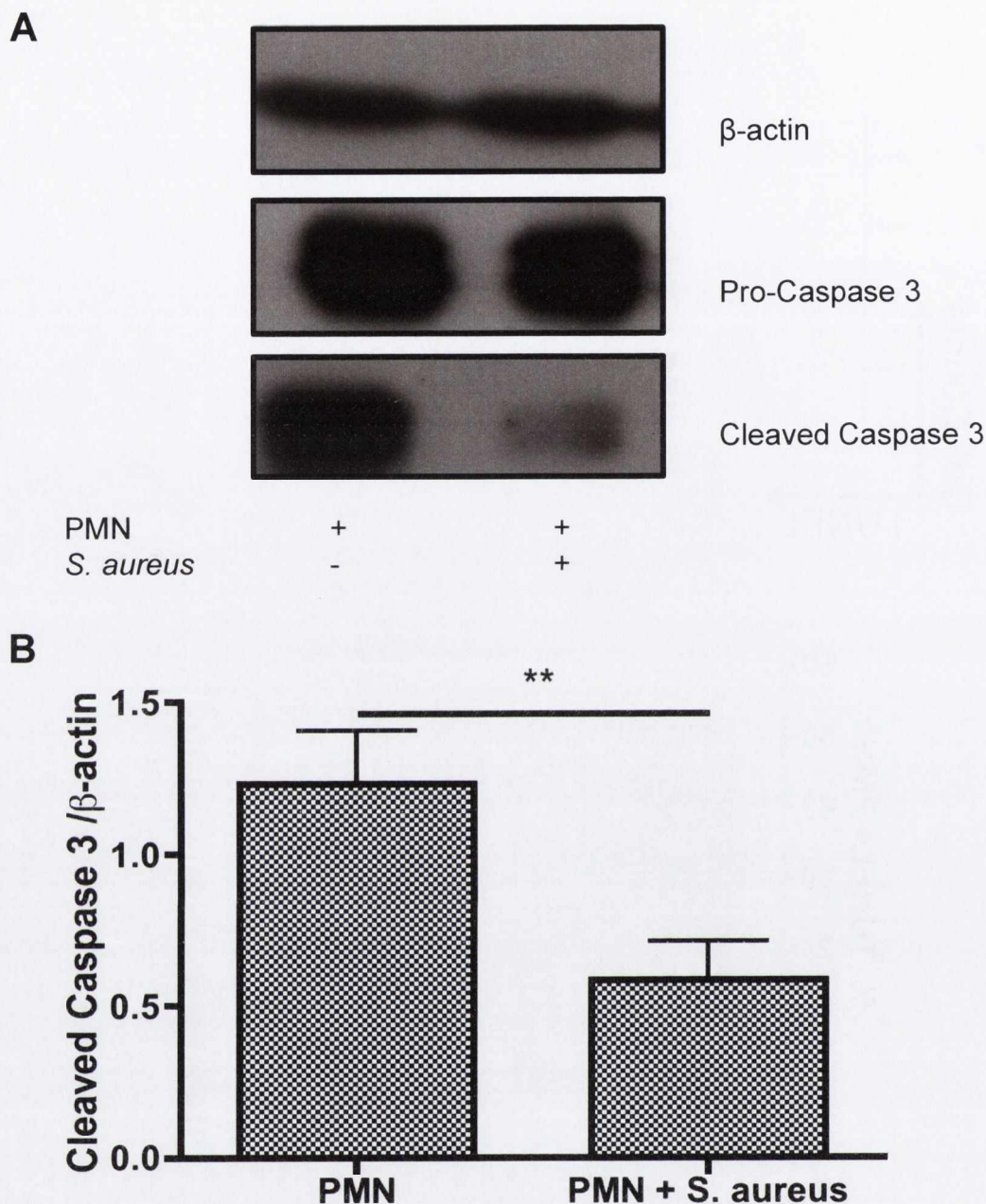


Figure 4.7: Infection with *S. aureus* inhibits cleavage of Caspase 3 in human neutrophils.

Human PMN were incubated for 2 hours with/without *S. aureus* (strain PS80) in the presence of opsonins. They were then lysed and run on SDS-PAGE and transferred to PVDF membrane. This was probed with anti-Caspase 3. β -actin was probed for as a loading control. Results expressed as representative Western Blot (A) and plotted normalised to β -actin, mean $\pm$ SEM (B), ** $p < 0.01$, $n = 13$.

4.2.5 Effects of T-cell derived cytokines on spontaneous human neutrophil apoptosis *in vitro*.

Having determined that infection with *S. aureus* was sufficient to inhibit the spontaneous apoptosis of human neutrophils, the ability of T cell cytokines that are known to be produced in response to a *S. aureus* infection to also modulate apoptosis was assessed. Neutrophils were incubated with recombinant IFN γ (100ng/ml) or IL-17 (100ng/ml) for 6 hours, and apoptosis assessed by TUNEL staining. rIFN γ inhibits the spontaneous apoptosis of human neutrophils, with the levels of TUNEL expression decreasing from $47.5\pm6.1\%$ to $29.2\pm7.5\%$ in the presence of IFN γ . IL-17 on the other hand did not have an appreciable effect on spontaneous neutrophil apoptosis. TUNEL expression in neutrophils treated with rIL-17 were similar to that of untreated cells at $44.3\pm9.8\%$ (Figure 4.8 A&B)

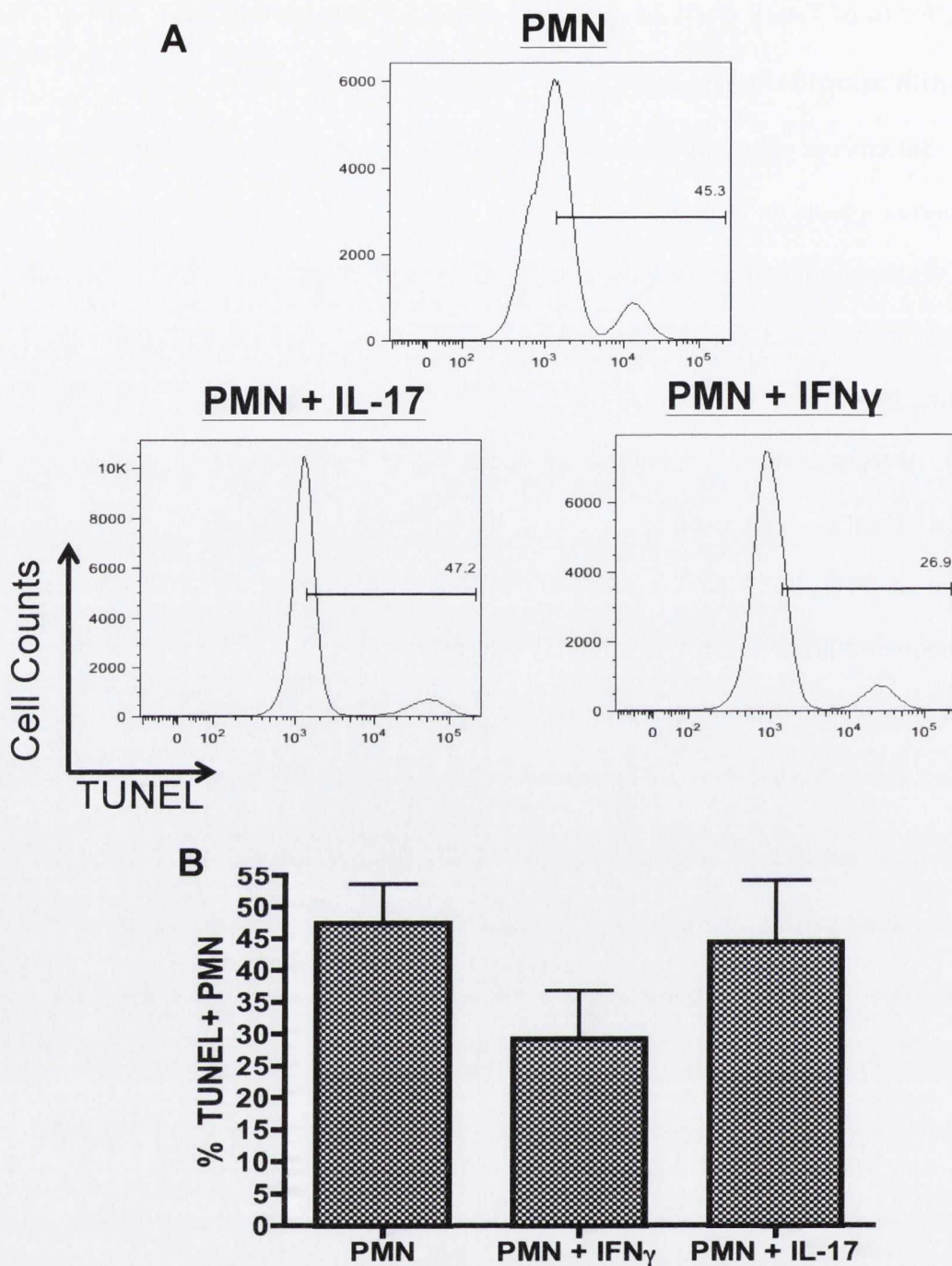


Figure 4.8: IFN γ but not IL-17 inhibits spontaneous neutrophil apoptosis.

Human PMN were cultured with 100ng/ml recombinant IFN γ or IL-17 at 5% CO₂, 37°C in media. After 6 hours cells were fixed, permeabilised and stained with fluorescein-conjugated TUNEL to be analysed by flow cytometry. Results expressed as representative flow cytometry plots (A) and % TUNEL + cells, mean $\pm$ SEM (B). n=4/7.

4.2.6 IFN γ does not significantly enhance *S. aureus* inhibition of neutrophil apoptosis.

As both IFN γ and *S. aureus* were capable of independently inhibiting neutrophil apoptosis *in vitro*, it was important to determine whether there was an additive or synergistic effect when *S. aureus*-infected cells were also exposed to this cytokine. Neutrophils were infected with *S. aureus* in the presence or absence of rIFN γ (100ng). At 6 hours post infection, neutrophil apoptosis was assessed by TUNEL staining. There was no enhancement of anti-apoptotic effects when neutrophils were exposed to both cytokine and bacteria. The level of neutrophil apoptosis in both rIFN γ treated ($21.4 \pm 5.6\%$) and untreated *S. aureus* infected cells ($22.5 \pm 4.3\%$) was similar. Under both conditions neutrophil apoptosis was significantly inhibited compared to that seen in the control untreated, uninfected neutrophils ($49.33 \pm 8.7\%$) (Figure 4.9 A&B).

To establish whether exposure to IFN γ had any effect on the cleavage of Caspase 3 in *S. aureus* infected cells, neutrophils were incubated for 2 hours with or without *S. aureus* in the presence or absence of IFN γ and Caspase 3 cleavage assessed. As before, Caspase 3 cleavage was significantly reduced in *S. aureus* infected cells as compared to uninfected neutrophils (Figure 4.10 A&B). In the presence of IFN γ , this reduction was enhanced, suggesting that IFN γ may further inhibit apoptosis in these infected cells.

Cumulatively, results thus far suggest that *S. aureus* strain PS80 is capable of directly inhibiting neutrophil apoptosis *in vitro*, presumably as a means to preserve its intracellular survival niche within the cell. These data also demonstrate that during

infection with *S. aureus*, IFN γ (but not IL-17), may also be capable of manipulating the rate of neutrophil apoptosis.

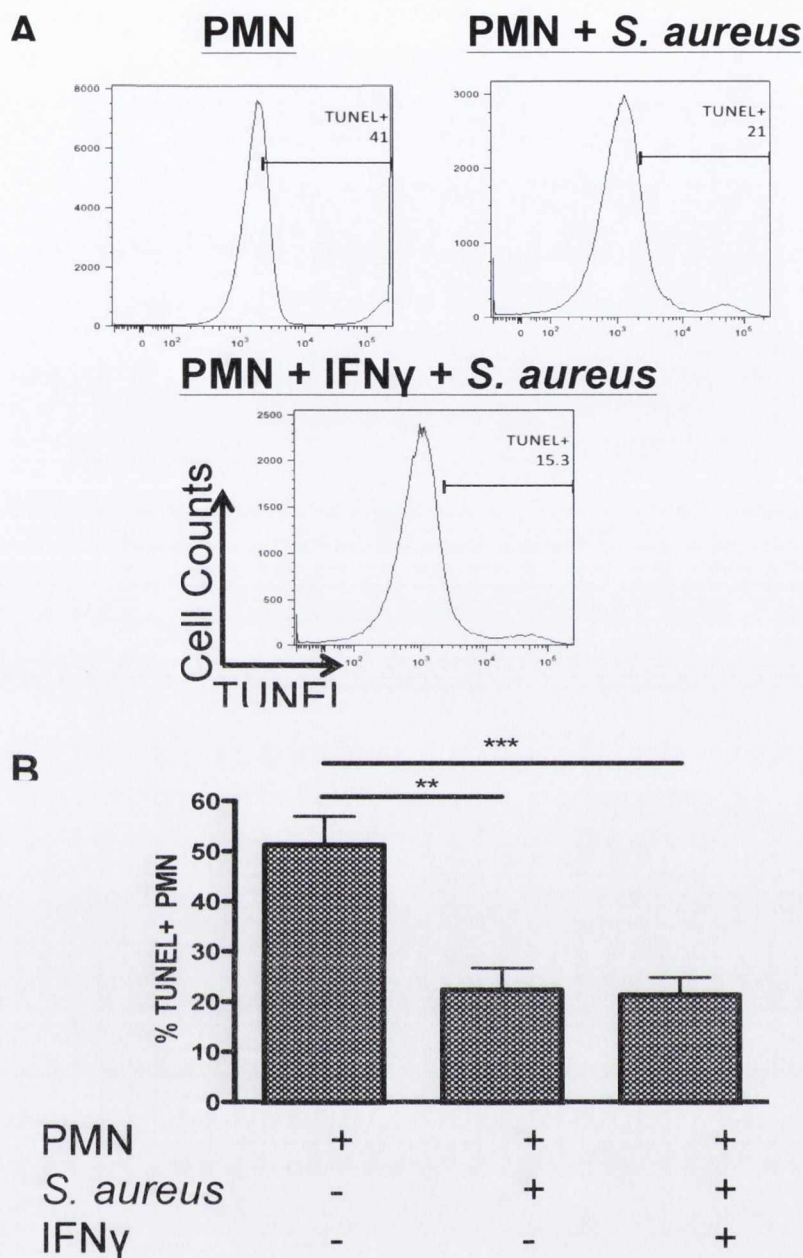


Figure 4.9: Addition of IFN γ to *S. aureus*-infected cells has no cumulative effect on inhibition of apoptosis as measured by TUNEL staining.

Human PMN were cultured with/without *S. aureus* (strain PS80) in the presence or absence of IFN γ (100ng/ml). After 6 hours cells were fixed, permeabilised and stained with fluorescein-conjugated TUNEL to be analysed by flow cytometry. Results expressed as representative flow cytometry plots (A) and % TUNEL+ cells, mean $\pm$ SEM (B). n=6, **p<0.01, ***p<0.001.

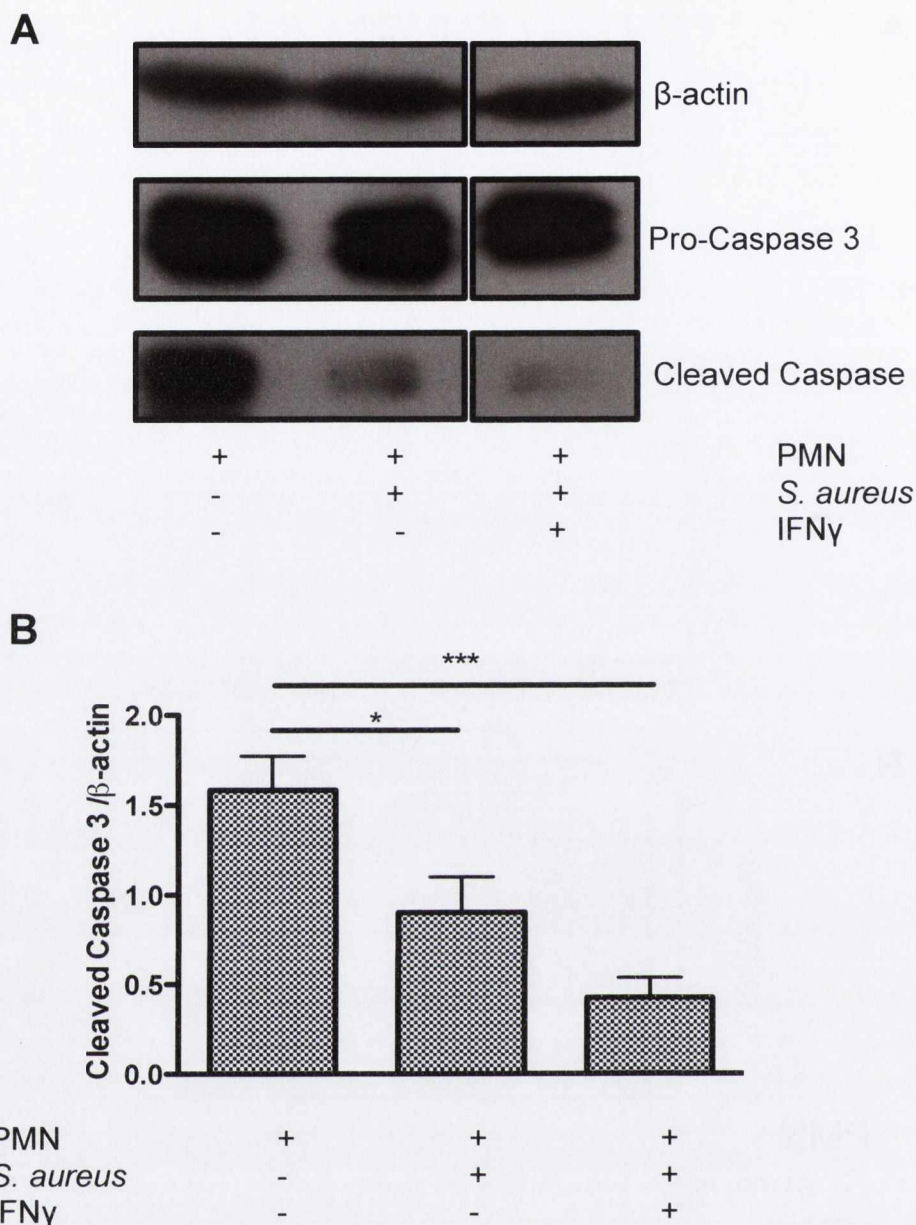


Figure 4.10: IFN γ may enhance inhibition of Caspase 3 cleavage by *S. aureus*

Human PMN were cultured with/without *S. aureus* in the presence or absence of IFN γ (100ng/ml). Cells were lysed, run on SDS-PAGE and transferred to PVDF membrane. This was probed with anti-Caspase 3. β -actin was probed for as a loading control. Results expressed as representative Western Blot (A) and plotted normalised to β -actin, mean $\pm$ SEM (B). n=6, *p<0.05, ***p<0.001. Full membrane shown in Appendix 1.

4.2.7 *S. aureus* inhibits neutrophil apoptosis *in vivo*.

Having identified that *S. aureus* is capable of inhibiting neutrophil apoptosis *in vitro*, a model of *S. aureus* peritonitis was used to determine whether *S. aureus* is capable of *in vivo* manipulation of neutrophil apoptosis. It has already been shown that there was a significant influx of neutrophils to the peritoneal cavity following i.p challenge with *S. aureus* (See Section 3.2.1). To establish whether *S. aureus* can influence the rate of neutrophil apoptosis, mice were challenged with *S. aureus* (strain PS80) or PBS and the proportions of CD11b+Ly6G+ neutrophils that were TUNEL+ assessed by flow cytometry over 48 hours. Although there were much higher numbers of neutrophils present in the peritoneal cavity after *S. aureus* challenge than PBS, the neutrophils isolated from infected mice were significantly less apoptotic than cells isolated from control mice (Figure 4.11).

As an alternative mechanism to assess neutrophil apoptosis, Annexin V staining was used. Annexin V binds to phosphatidylserine (PS) that flips to the outer leaflet of the membrane during early stages of apoptosis (220). The levels of Annexin V staining on recruited neutrophils was reduced in infected mice at both 12 and 24 hours post-infection with *S. aureus* in comparison to PBS treated mice (Figure 4.12). These data further support the hypothesis that *S. aureus* is capable of directly manipulating the apoptosis of neutrophils *in vivo*.

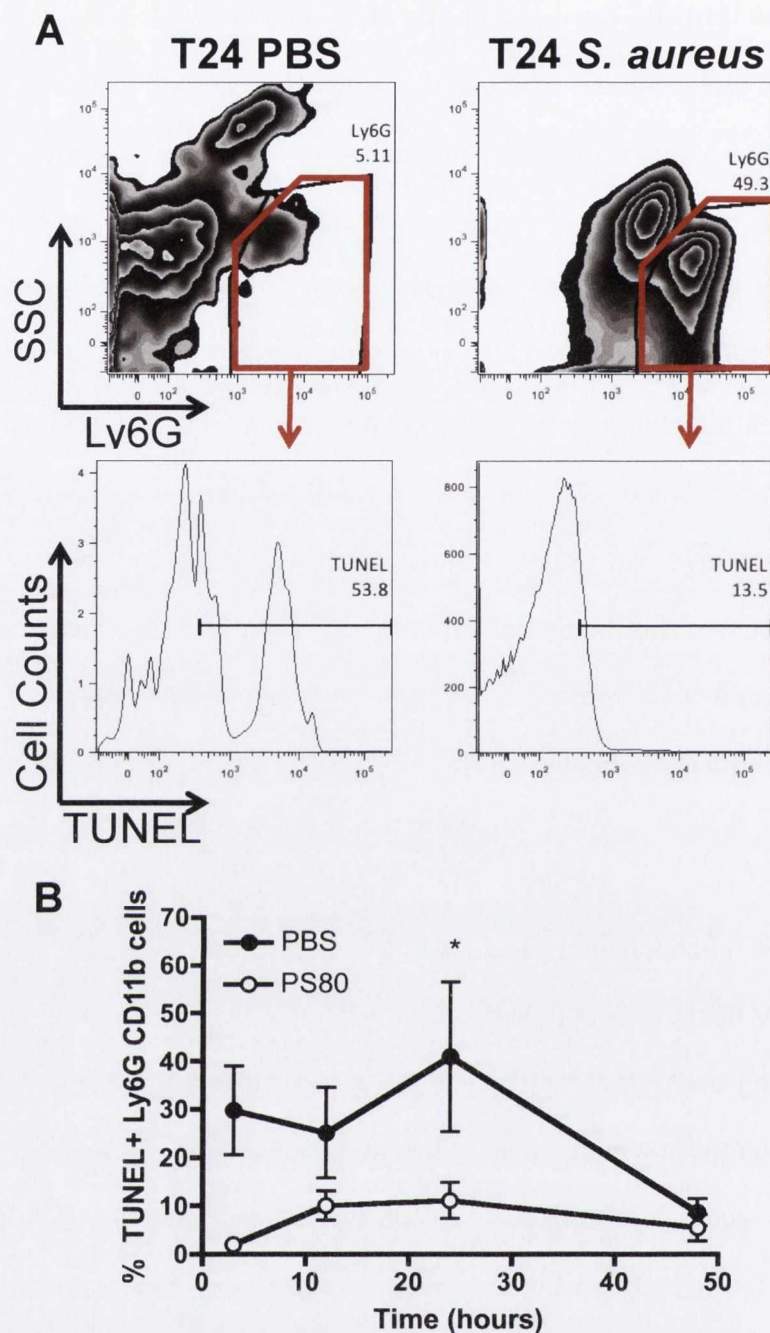


Figure 4.11: During *in vivo* infection, *S. aureus* inhibits neutrophil apoptosis.

Groups of mice were exposed to *S. aureus* strain PS80 (5×10^8 CFU) or PBS via i.p. injection. At indicated time points post challenge, gates were set on neutrophils (CD11b+Ly6G+) and the proportions of TUNEL+ PMN were assessed in the peritoneal cavity by flow cytometry (B). Representative Ly6G and TUNEL FACS plots are shown in (A). Results expressed as mean $\pm$ SEM, n=6, *p<0.05.

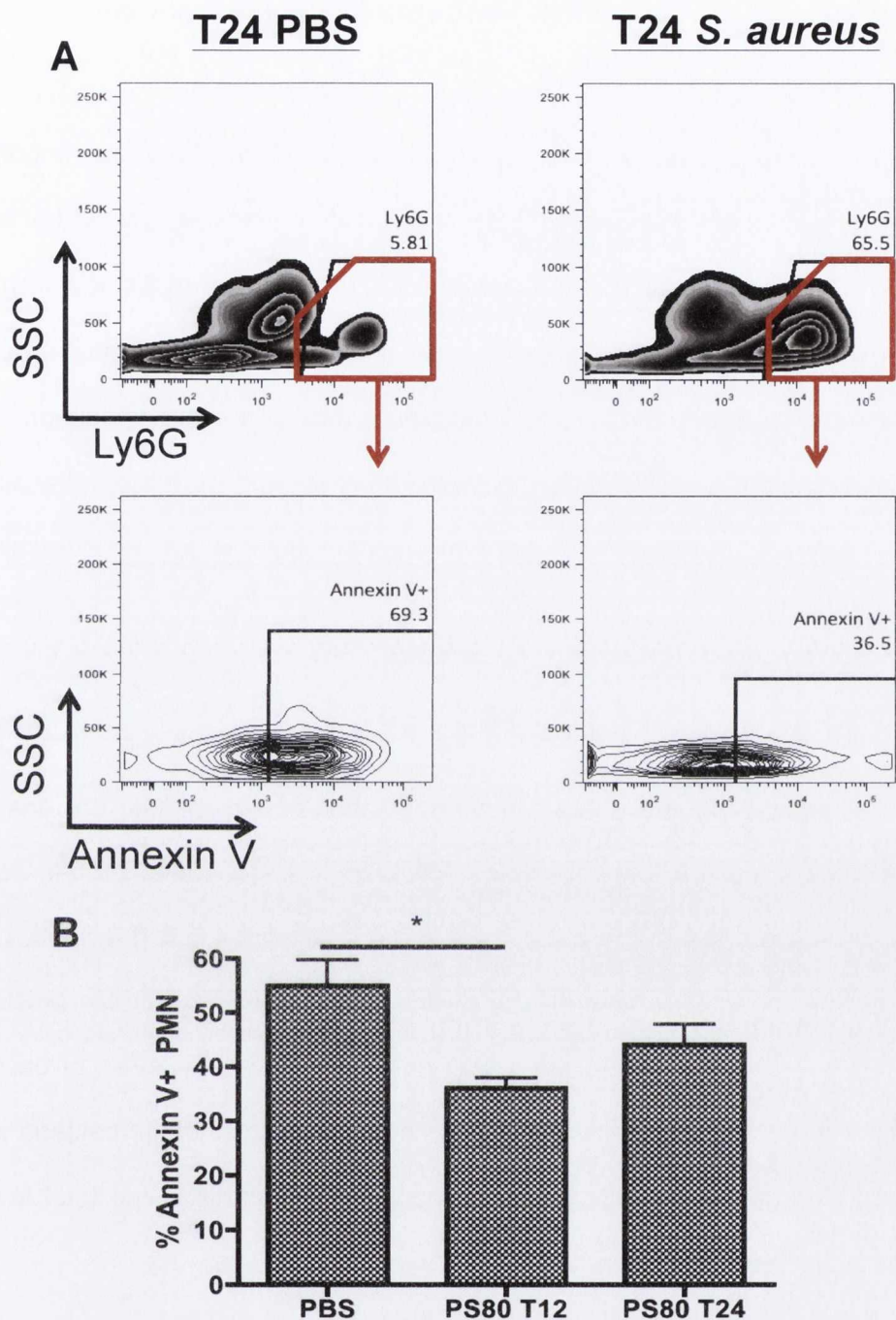


Figure 4.12: During *in vivo* infection, *S. aureus* inhibits apoptosis, as measured by Annexin V binding to exposed phosphatidylserine

Groups of mice were exposed to *S. aureus* strain PS80 (5×10^8 CFU) or PBS via i.p. injection. At indicated time points post challenge, proportions of Annexin V+ PMN were assessed in the peritoneal cavity by flow cytometry (B). Representative Ly6G and TUNEL FACS plots are shown in (A). Results expressed as mean $\pm$ SEM, n=5/11.

4.2.8 *S. aureus* can survive within neutrophils isolated from the peritoneal cavity.

Having shown that *S. aureus* can inhibit neutrophil apoptosis *in vivo*, it was important to establish whether this had any effect on survival of bacteria within the cells. Groups of mice were infected with *S. aureus* strain PS80 for up to 24 hours and at indicated timepoints, a peritoneal lavage was collected. The total bacterial burden in the peritoneal cavity was determined by plating an aliquot of the lavage fluid on TSA. The peritoneal exudate cells (PEC cells) were then isolated from the extracellular fluid of the lavage and treated with gentamicin for 1 hour to kill all extracellular bacteria. Isolated peritoneal cells were washed, lysed and plated on TSA to determine the intracellular population of bacteria. This technique determined whether the bacteria were intracellular or extracellular. A higher bacterial burden in the total lavage fluid illustrated that there was a large population of extracellular bacteria that were killed upon exposure to gentamicin. However, if the bacterial burden in the isolated peritoneal cell population was similar to the total lavage fluid this implies that the gentamicin cannot kill the bacteria and thus, they must be protected intracellularly. At 1 hour post-infection it was determined that the levels of bacteria were higher extracellularly, however by 3 hours post-infection all of the bacteria were internalised as there was no difference between bacterial loads in the total lavage fluid or the isolated cells (Figure 4.13 A).

In the method described above, there is no distinction made as to the cell type that contains that bacteria, it simply demonstrates that bacteria are protected from the antibiotic, and therefore are presumably inside a eukaryotic cell membrane. In order to prove that neutrophils specifically act as reservoirs for *S. aureus* intracellular survival, mice were infected with *S. aureus*. At 24 hours post infection (the point at which TUNEL expression was reduced in infected mice (See Figure 4.11)),

CD11b+Ly6G⁺ and CD11b+Ly6G⁻ cells were sorted by flow cytometry, washed, lysed and plated on TSA to determine intracellular bacterial burden. There was a significantly higher bacterial load in Ly6G⁺ cells in comparison to Ly6G⁻ cells, highlighting the importance of neutrophils in providing an intracellular survival niche for *S. aureus* (Figure 4.13 B).

To further prove that *S. aureus* is capable of predominantly surviving intracellularly in neutrophils following *S. aureus* i.p challenge, a flow cytometry based assay using GFP labelled *S. aureus* (strain PS80) was developed. Mice were injected with GFP-expressing *S. aureus* and PECs were isolated after 24 hours. These PEC cells were assessed by flow cytometry and cells expressing GFP (and thus containing bacteria) were assessed. Of the GFP⁺ cells, the proportion that were Ly6G⁺ or Ly6G⁻ was determined. A significantly higher proportion of GFP⁺ cells were Ly6G⁺, providing additional evidence that neutrophils are the key intracellular reservoir for *S. aureus* survival (Figure 4.14).

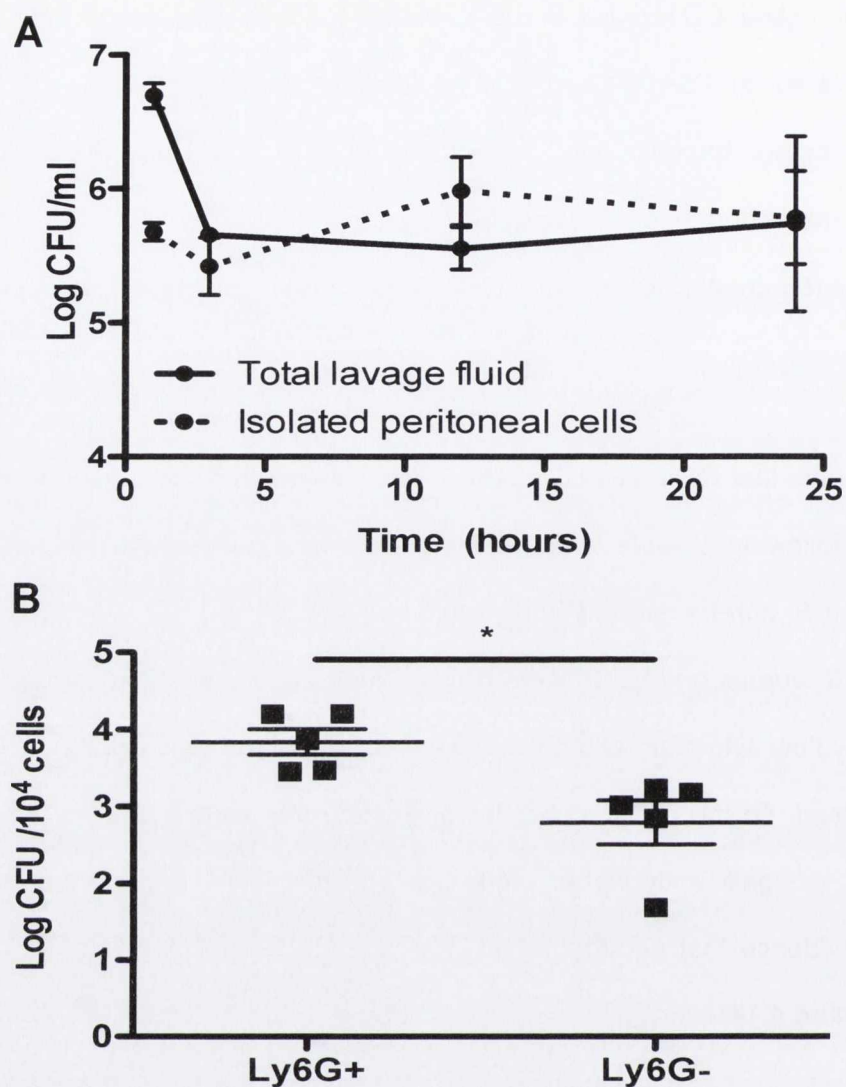


Figure 4.13: *S. aureus* is found intracellularly within neutrophils in the peritoneal cavity.

Groups of mice were exposed to *S. aureus* strain PS80 (5×10^8 CFU) via i.p. injection. At indicated time points post challenge, peritoneal lavages were collected and an aliquot plated on TSA. PEC cells were isolated from the lavage and incubated in gentamicin (200 μ g/ml) for 1 hour then washed, lysed and plated to determine intracellular bacteria. Alternatively, CD11b+Ly6G+ or CD11b+Ly6G- cells were sorted by flow cytometry, washed, lysed in sterile H₂O and plated to determine the intracellular CFUs (B). Results expressed as mean $\pm$ SEM, n=3/5.

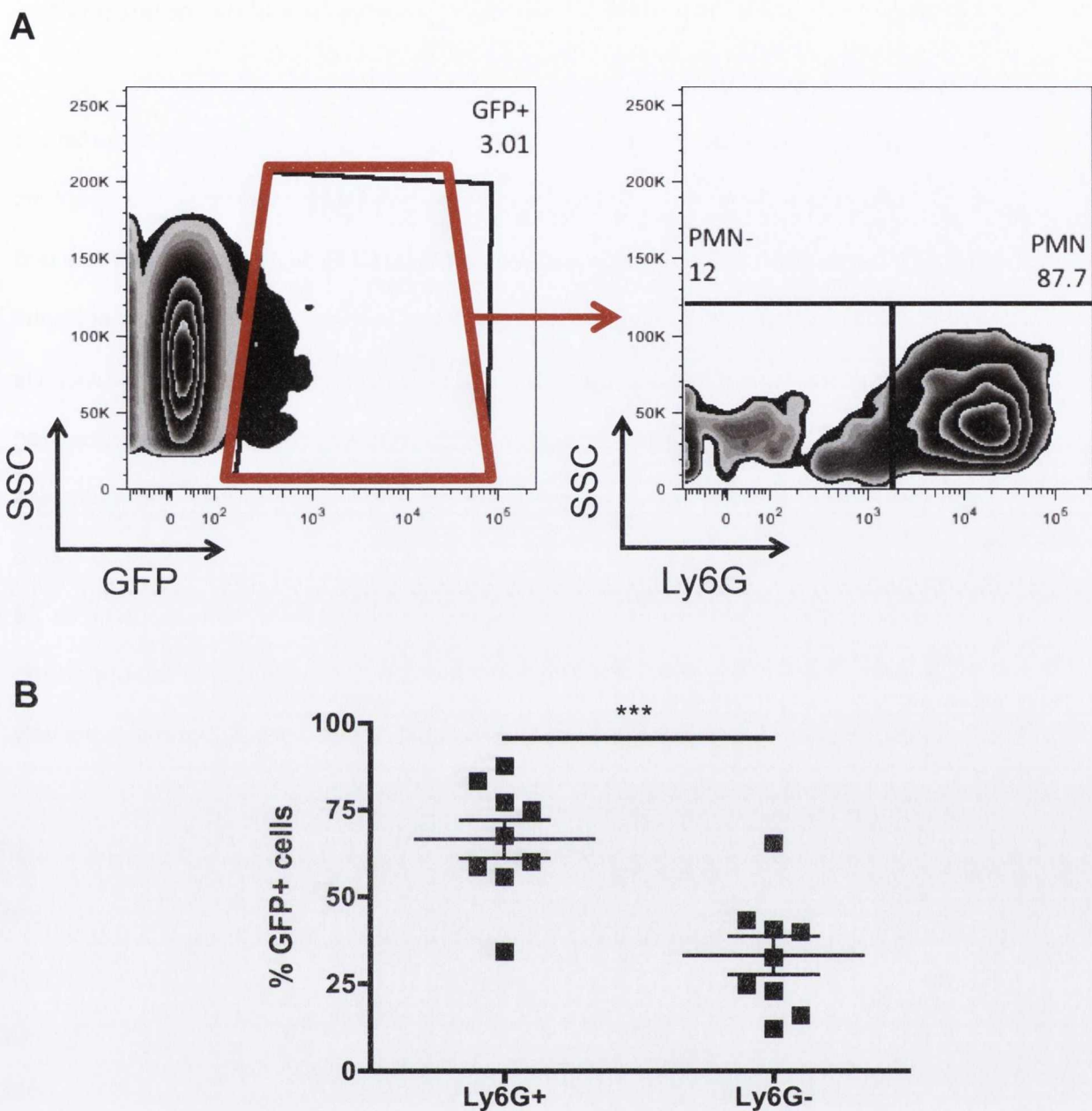


Figure 4.14: GFP-PS80 enables identification of intracellular bacteria within neutrophils.

Groups of mice were exposed to GFP-*S. aureus* strain PS80 (5×10^8 CFU) via i.p. injection. At 24 hours post injection the peritoneal cavity was lavaged and PEC cells isolated. Gates were set on GFP+ cells and then the proportions of these cells that were CD11b+Ly6G+ or CD11b+Ly6G- assessed by flow cytometry. Representative FACS plots are shown in (A) Lines represent mean $\pm$ SEM (B). n=9, ***p<0.001.

4.2.9 Intracellular survival of *S. aureus* in neutrophils is associated with low levels of apoptosis

Having established a method for identifying populations of neutrophils that contained viable intracellular *S. aureus in vivo*, it was then important to determine the apoptotic state of these neutrophils. Mice were infected with GFP-expressing *S. aureus* and PECs isolated at various timepoints post-infection. Cells were stained for neutrophil surface markers and gates were set on GFP+CD11b+Ly6G+ cells (i.e. neutrophils that contained bacteria). The level of TUNEL staining of these cells was then assessed to determine their apoptotic state. GFP+ neutrophils demonstrated significantly lower levels of TUNEL staining across all time points assessed. More than 75% of GFP+ cells were TUNEL negative and therefore non apoptotic at 24 hours post-infection (Figure 4.15). This provides substantial evidence to support the hypothesis that while surviving intracellularly within neutrophils, *S. aureus* is actively inhibiting neutrophil apoptosis to maintain its survival niche.

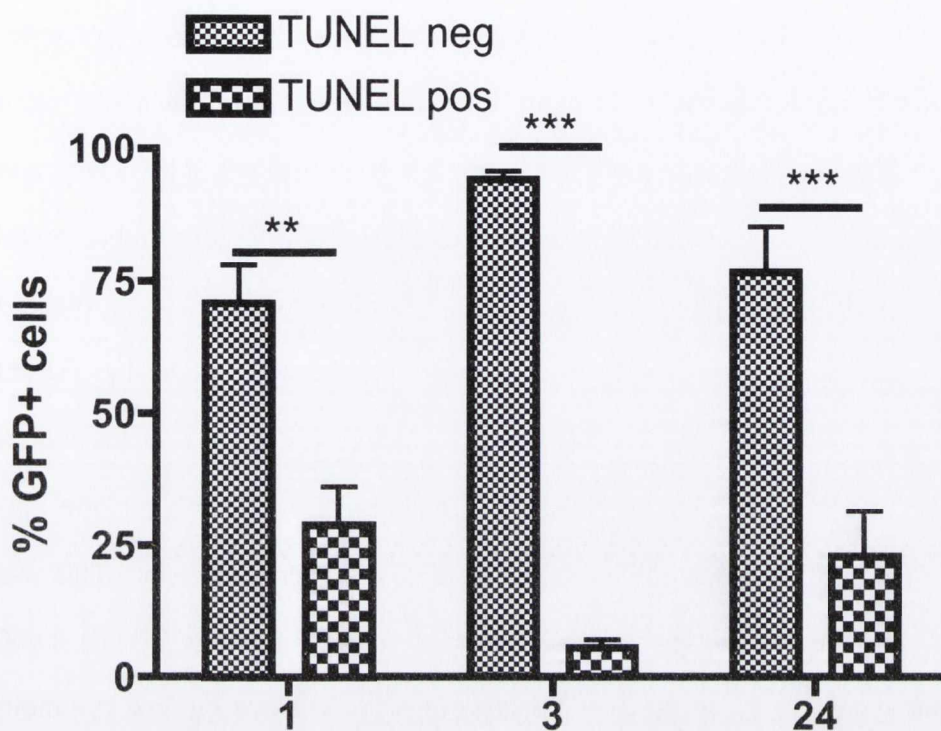


Figure 4.15: Neutrophils harbouring GFP-bacteria were non-apoptotic.

Mice were injected with GFP-*S. aureus* strain PS80 (5×10^8 CFU) via i.p. injection. At indicated time points post challenge, PEC cells were isolated, surface stained for CD11b and Ly6G expression, fixed and stained with TUNEL. Gates were set on GFP+ CD11b+Ly6G+ cells and the proportion of these cells that were TUNEL+ were assessed in the peritoneal cavity by flow cytometry. Results expressed as mean $\pm$ SEM, n=5, **p<0.01, ***p<0.001.

4.2.10 *S. aureus* infected neutrophils are less apoptotic in the wound

It has previously been shown using a *S. aureus* surgical wound infection model that *S. aureus* could survive intracellularly within neutrophils and that IFN γ enhanced neutrophil recruitment to the site of infection, thus propagating an intracellular niche for *S. aureus* survival (89). It was therefore important to investigate if IFN γ was also controlling neutrophil accumulation through the modulation of apoptosis in this model. Initially, a method to isolate infiltrating neutrophils from the surgical wound infection site was developed, to allow for further analysis of apoptotic state by TUNEL staining. *S. aureus* surgical wounds were established in WT mice and the wound tissue excised at day 7. The wound tissue was collagenase digested for one hour and then filtered through a 40 μ m filter to obtain a single cell suspension. This was then stained with antibodies against the neutrophil surface markers CD11b, Ly6G and apoptosis assessed by analysis of TUNEL staining. Analysis by flow cytometry revealed a population of CD11b⁺ Ly6G⁺ neutrophils (Figure 4.16 A) that stained positively with TUNEL (Figure 4.16 B), representing apoptotic neutrophils.

Having established that neutrophils could be isolated from the wound site and assessed for apoptosis, surgical wounds were established in wild type mice and either infected with *S. aureus* or treated with PBS as a control. At days 3, 7 and 10 post infection wounds were excised and neutrophil influx assessed. As expected mice that had been infected with *S. aureus* had increased levels of neutrophils present at the wound site in comparison to PBS-treated mice (Figure 4.17). To determine the levels of neutrophil apoptosis, gates were set on CD11b⁺Ly6G⁺ neutrophils and the percentage of these that stained positively for TUNEL was assessed. The *S. aureus*-infected mice demonstrated substantially decreased levels of neutrophil apoptosis when compared to PBS controls across all time points (Figure 4.18 A&B). This decrease was significant at day 7 (Figure 4.18 C).

These data corroborate the previous *in vitro* and *in vivo* findings of this study and suggest that *S. aureus* can inhibit neutrophil apoptosis during *S. aureus* surgical wound infection presumably to cultivate an intracellular survival niche.

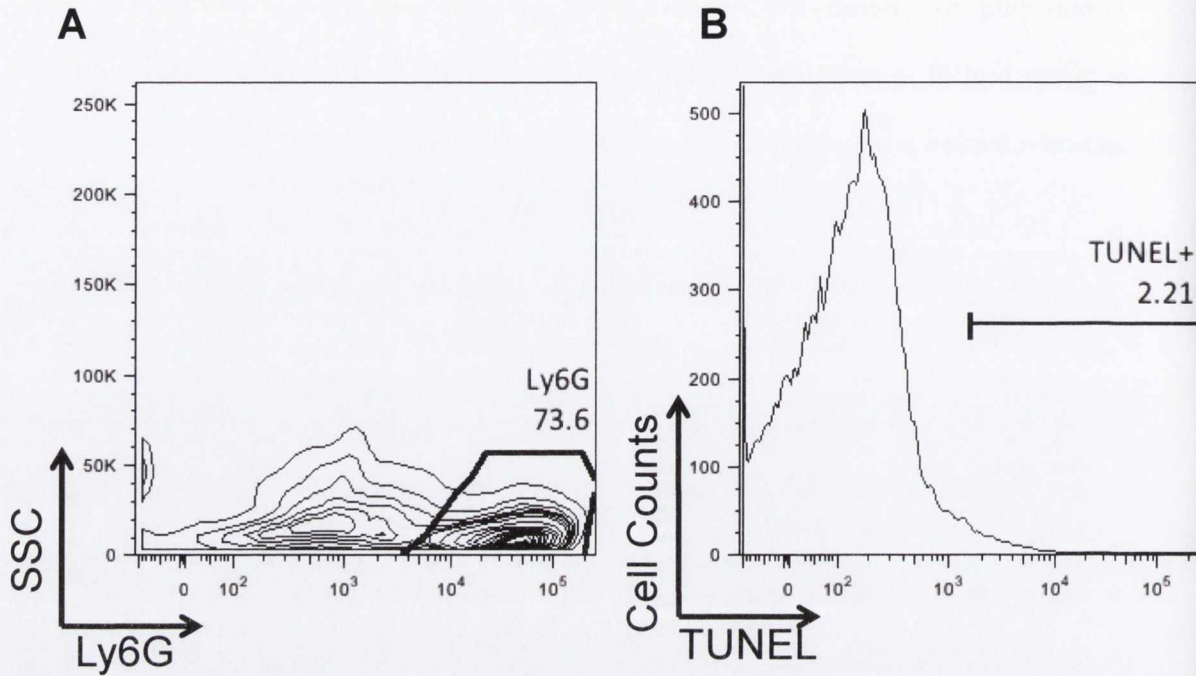


Figure 4.16: Isolation of CD11b+Ly6G+ cells from a *S. aureus* surgical wound infection site and quantification of TUNEL staining of these neutrophils.

Surgical wounds were established in wild type mice and infected with *S. aureus* strain PS80 (10^2 CFU). At day 7 post-infection, the wounds were excised and infiltrating CD11b+Ly6G+ PMNs were assessed for TUNEL staining. Gates were set on CD11b+ cells and representative flow cytometry plots show proportions of CD11b+Ly6G+ PMNs (A) and the proportion of these CD11b+Ly6G+ PMN that stain positively for TUNEL (B).

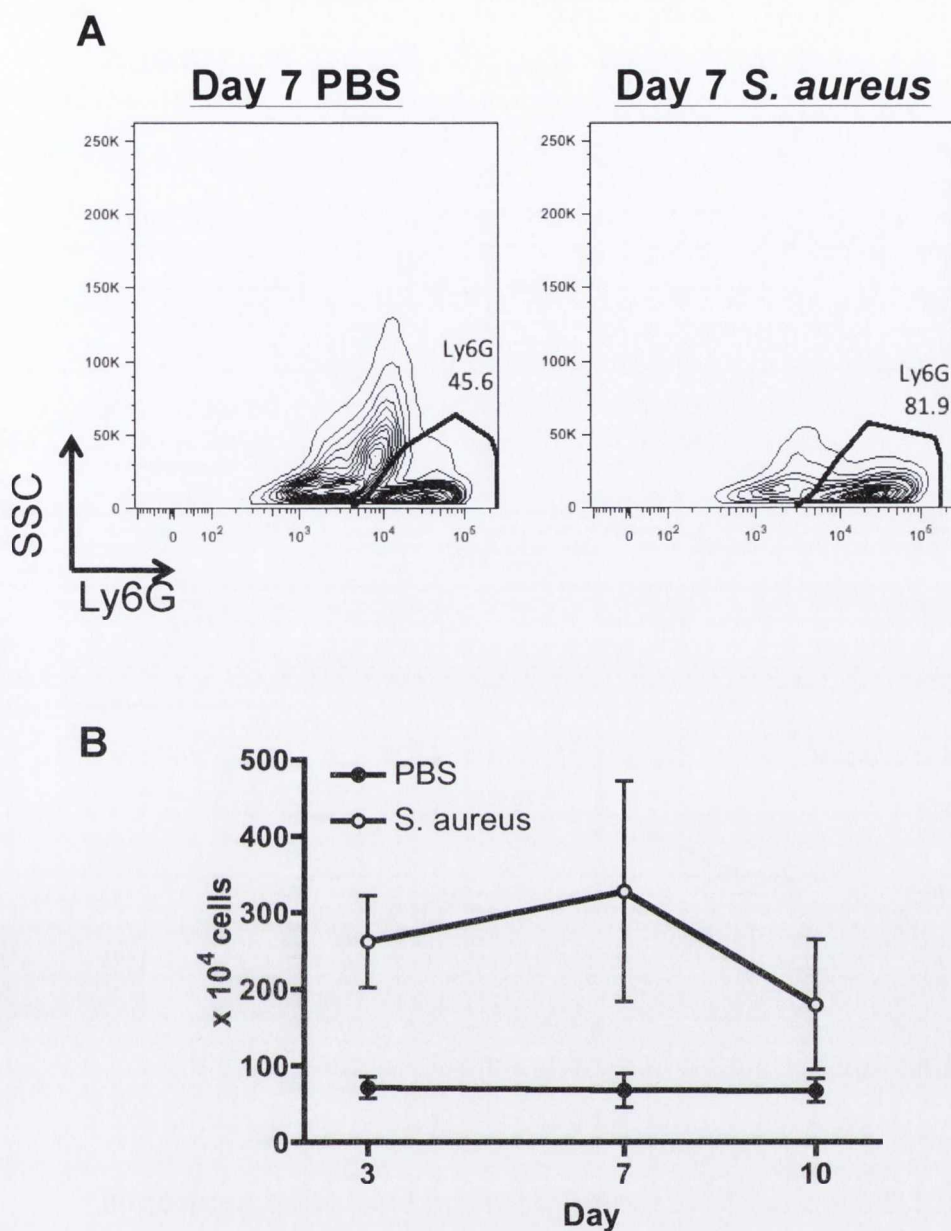


Figure 4.17: Neutrophils are recruited to the wound site following *S. aureus* infection.

Surgical wounds were established in WT mice and challenged with *S. aureus* strain PS80 (10^2 CFU) or PBS. At indicated time points post challenge, absolute numbers of PMN (CD11b+Ly6G+) were assessed in the wound by flow cytometry (B). Gates were set on CD11b+ cells and representative FACS plots show % of CD11b+Ly6G+ cells (A). Results expressed as mean $\pm$ SEM, n=6.

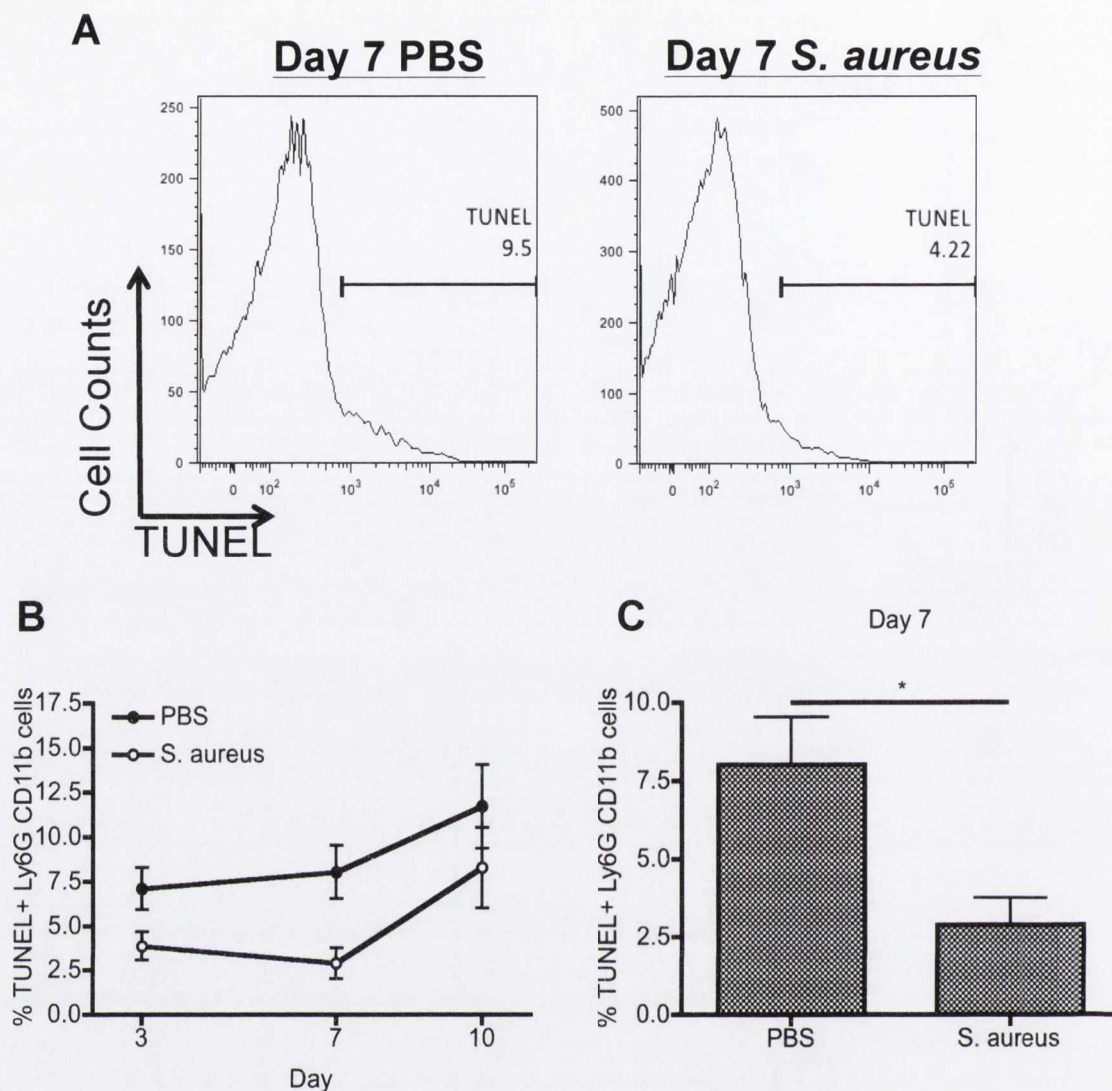


Figure 4.18: During *S. aureus* surgical wound infection neutrophil apoptosis is reduced.

Surgical wounds were established in WT mice and challenged with *S. aureus* strain PS80 (10^2 CFU) or PBS. At indicated time points post challenge, CD11b+Ly6G+ PMN were gated on and the proportions of PMN that stained positively with TUNEL were assessed in the wound by flow cytometry (B). Gates were set on CD11b+ cells and representative FACS plots show % of CD11b+Ly6G+ cells (A). % TUNEL+ PMN at Day 7 are shown in (C). Results expressed as mean $\pm$ SEM, n=6, *p<0.05.

4.2.11 IFN γ ^{-/-} mice have reduced neutrophil numbers, higher apoptosis levels and reduced bacterial burden at the wound site

To establish whether IFN γ played any role in modulating neutrophil apoptosis during *S. aureus* surgical wound infection, surgical wounds were established in groups of WT or IFN γ ^{-/-} mice and challenged with *S. aureus* (10² CFU). Neutrophil recruitment to the wound site was assessed at day 7. IFN γ ^{-/-} mice had significantly reduced numbers of CD11b+Ly6G+ neutrophils at the wound infection site 7 days post-infection in comparison to WT mice (Figure 4.19). Previous work had identified role for IFN γ in controlling neutrophil recruitment at early timepoints of infection i.e up to day 3 (89). It appears that this cytokine also affects neutrophil numbers at a later timepoint, suggesting that it may also be influencing neutrophil clearance.

To determine whether IFN γ was modulating neutrophil apoptosis, gates were set on CD11b+Ly6G+ neutrophils and the percentage of cells staining positively with TUNEL was assessed. At day 7 post-infection IFN γ ^{-/-} mice were found to have substantially higher levels of cells staining positively for TUNEL in comparison to their WT counterparts (Figure 4.20). Although these data did not reach statistical significance, there is clear biological significance as the proportion of TUNEL+ neutrophils was 1.87 fold greater in the IFN γ ^{-/-} mice as compared to the WT mice.

In order to ascertain whether manipulation of apoptosis played a role in the outcome of infection, bacterial burden was assessed. Previous research has demonstrated that IFN γ ^{-/-} mice have a reduced bacterial burden in the surgical wound after 3 days of infection, presumably due to influences of the cytokine on neutrophil recruitment. Here, it is confirmed that this effect is still observed at day 7, most likely due to the observed IFN γ -mediated effects on neutrophil clearance. *S. aureus* infected wounds

were established in groups of WT or IFN γ ^{-/-} mice. At day 7 post infection, the wounds were excised and bacterial burden assessed. IFN γ ^{-/-} mice were found to have a reduced bacterial burden in comparison to the WT mice (Figure 4.21). Consistent with what was observed in the previous study at day 3 (89), infection with 10² CFU of *S. aureus* resulted in a reduction in bacterial burden of approximately 1 LOG in the IFN γ ^{-/-} mice compared to the WT, while infection with 10¹ CFU of *S. aureus* resulted in a reduction of 1.5 Log.

Taken together, the data discussed thus far suggest that *S. aureus* is capable of inhibiting neutrophil apoptosis in order to propagate its survival both *in vitro* and *in vivo*. Furthermore, it illustrates that IFN γ plays a role in modulating neutrophil apoptosis at the site of *S. aureus* surgical wound infection, tipping the balance from a proportional, beneficial neutrophil response to an accumulation of neutrophils at the site of infection, resulting in intracellular survival and reduced clearance of infection.

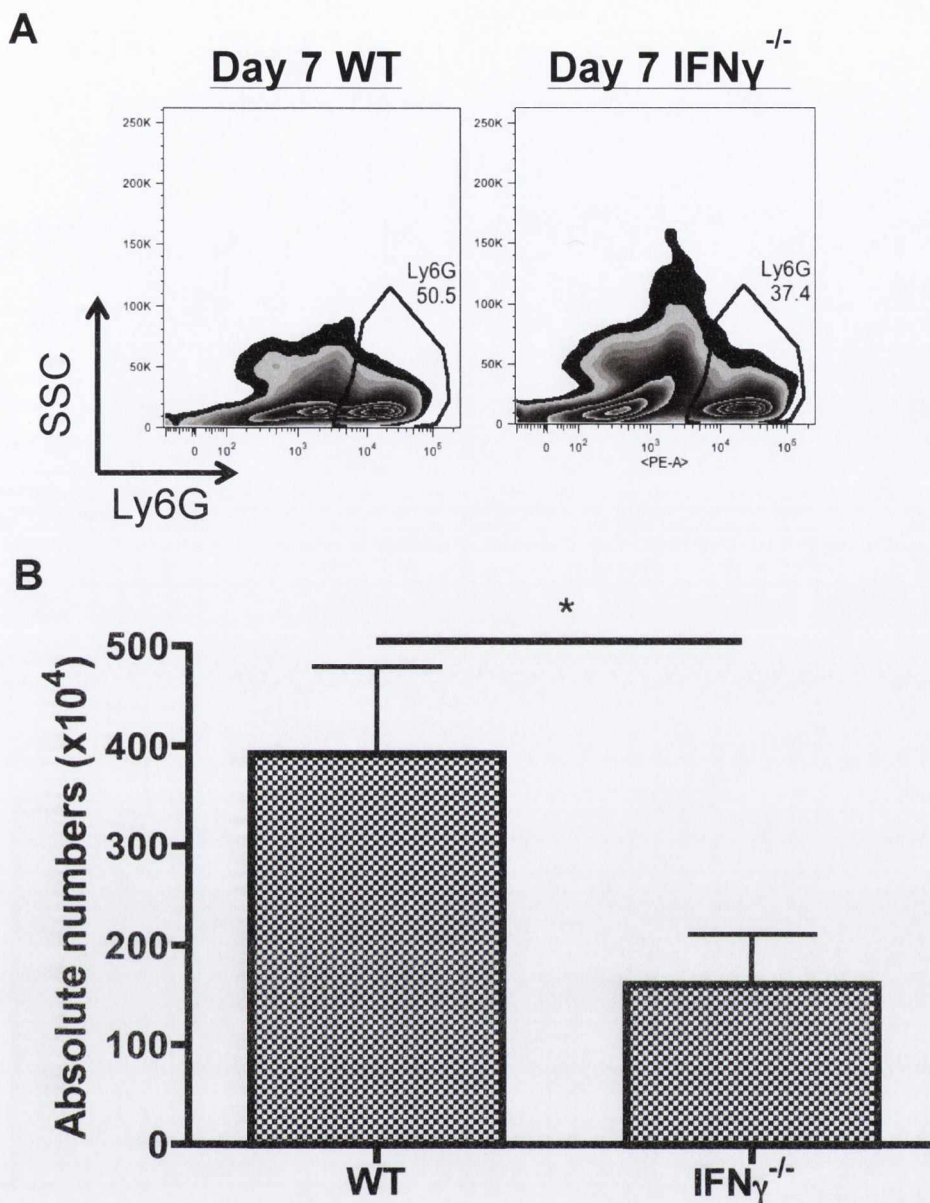


Figure 4.19: During *S. aureus* wound infection, neutrophil recruitment is reduced in IFN γ ^{-/-} mice.

Surgical wounds were established in WT and IFN γ ^{-/-} mice and challenged with *S. aureus* strain PS80 (10² CFU). At day 7 post-infection, the wounds were excised and infiltrating CD11b+Ly6G+ cells were assessed. Gates were set on CD11b+ cells and representative flow cytometry plots show % of CD11b+Ly6G+ cells (A) and mean $\pm$ SEM absolute numbers CD11b+Ly6G+ cells (B), n=13.

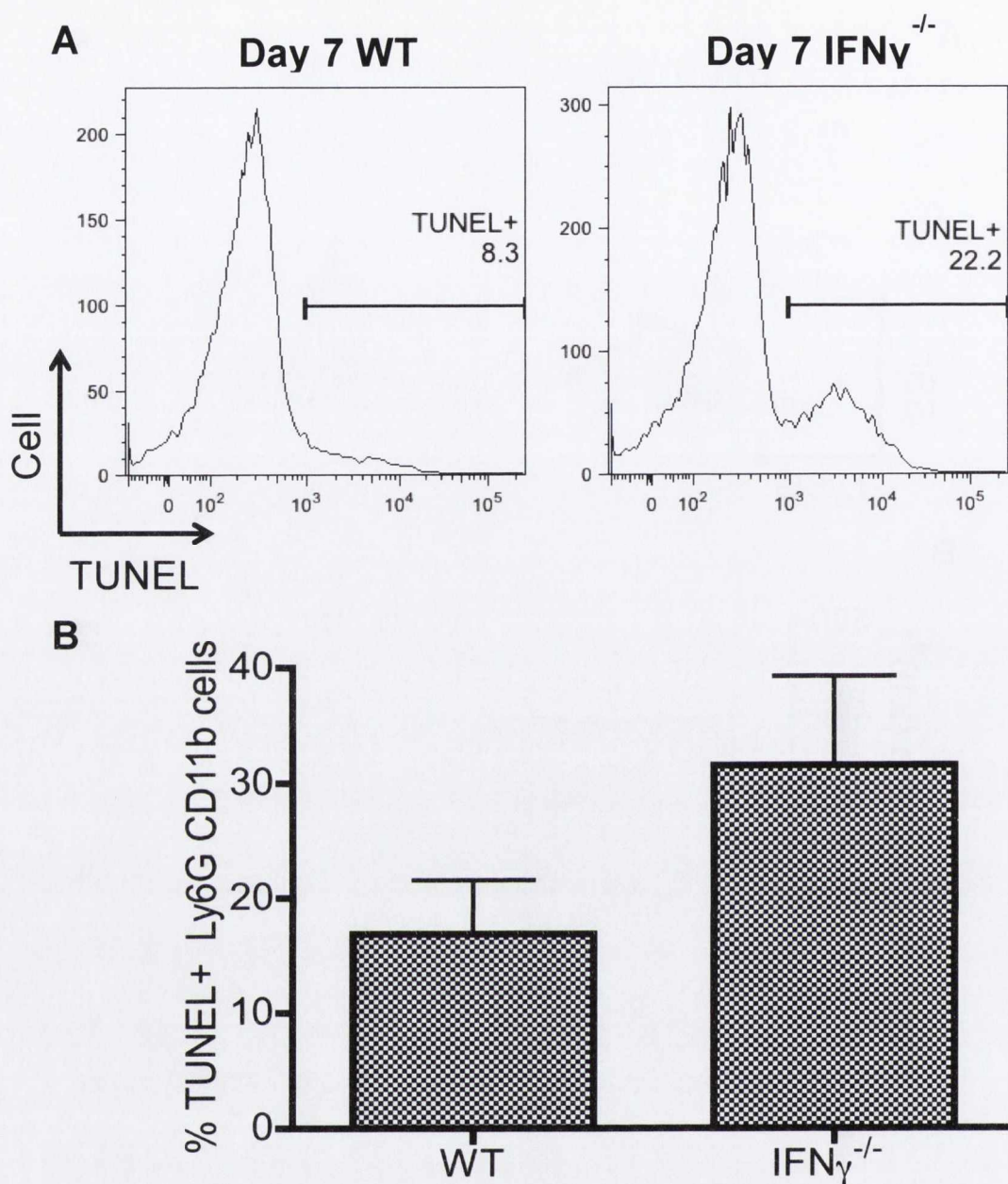


Figure 4.20: IFN γ ^{-/-} mice have increased TUNEL+ neutrophils at the wound site.

Surgical wounds were established in WT and IFN γ ^{-/-} mice and challenged with *S. aureus* strain PS80 (10^2 CFU). At 7 days post infection, wounds were excised, CD11b+Ly6G+ PMN gated on and the proportion of TUNEL+ cells assessed. Results are shown as representative flow cytometry plots (A) and mean $\pm$ SEM CD11b+Ly6G+ TUNEL+ cells (B), n=13.

4.2.12 Strain dependent differences in neutrophil response to *S. aureus*.

The results outlined in Chapter 3 clearly indicate that the ability of macrophages and dendritic cells to kill *S. aureus* is very much determined by the strain of *S. aureus* encountered. Thus far in Chapter 4 the experiments investigating *S. aureus* modulation of neutrophil apoptosis were conducted using *S. aureus* strain PS80. As shown in Figure 4.1 the degree of killing of *S. aureus* by neutrophils also depends upon the strain of bacteria encountered as neutrophils were much more effective at killing *S. aureus* strain SH1000 than PS80. This suggests that PS80 may be particularly adept at manipulating all phagocytes in a way that SH1000 cannot.

To determine whether the differences in neutrophil killing of the two strains of *S. aureus* are due to the differential abilities of these strains to manipulate neutrophil apoptosis, human neutrophils were isolated and infected with either *S. aureus* strain PS80 or SH1000. Six hours after infection, the proportion of cells that stained positively with TUNEL was analysed by flow cytometry. Infection with either *S. aureus* strain PS80 or SH1000 significantly reduced staining with TUNEL compared to uninfected neutrophils suggesting that *S. aureus* strain SH1000 was capable of inhibiting neutrophils apoptosis to a similar degree to PS80 (Figure 4.22). Furthermore, immunoblotting demonstrated that this inhibition of apoptosis was Caspase 3-dependent, suggesting that both strains of *S. aureus* employ similar mechanisms to inhibit apoptosis of neutrophils (Figure 4.23).

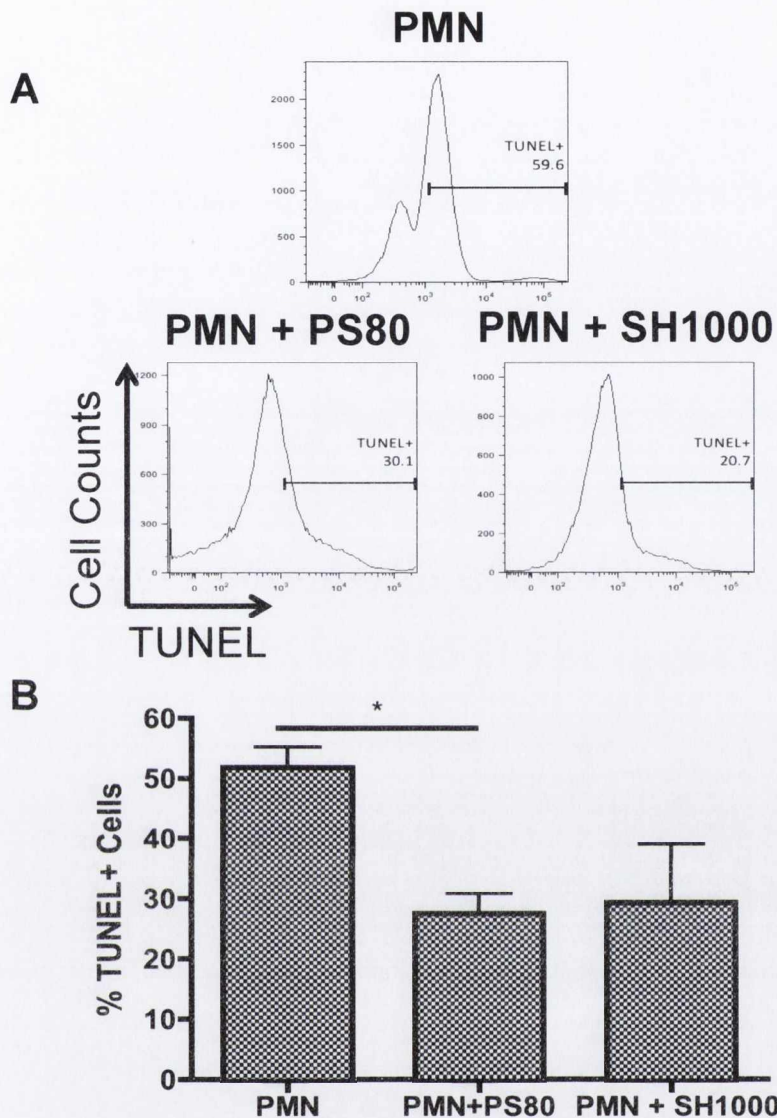


Figure 4.22: *S. aureus* strains PS80 and SH1000 both inhibit the spontaneous apoptosis of human neutrophils *in vitro*, as measured by staining with TUNEL.

Human PMN were cultured with *S. aureus* strain PS80 or SH1000, or left uninfected.

After 6 hours, cells were fixed, permeabilised and stained with fluorescein-conjugated TUNEL and analysed by flow cytometry. Results expressed as % TUNEL+ PMN, mean ± SEM (B). Representative FACS plots are shown (A).

* $p < 0.05$, $n = 5$.

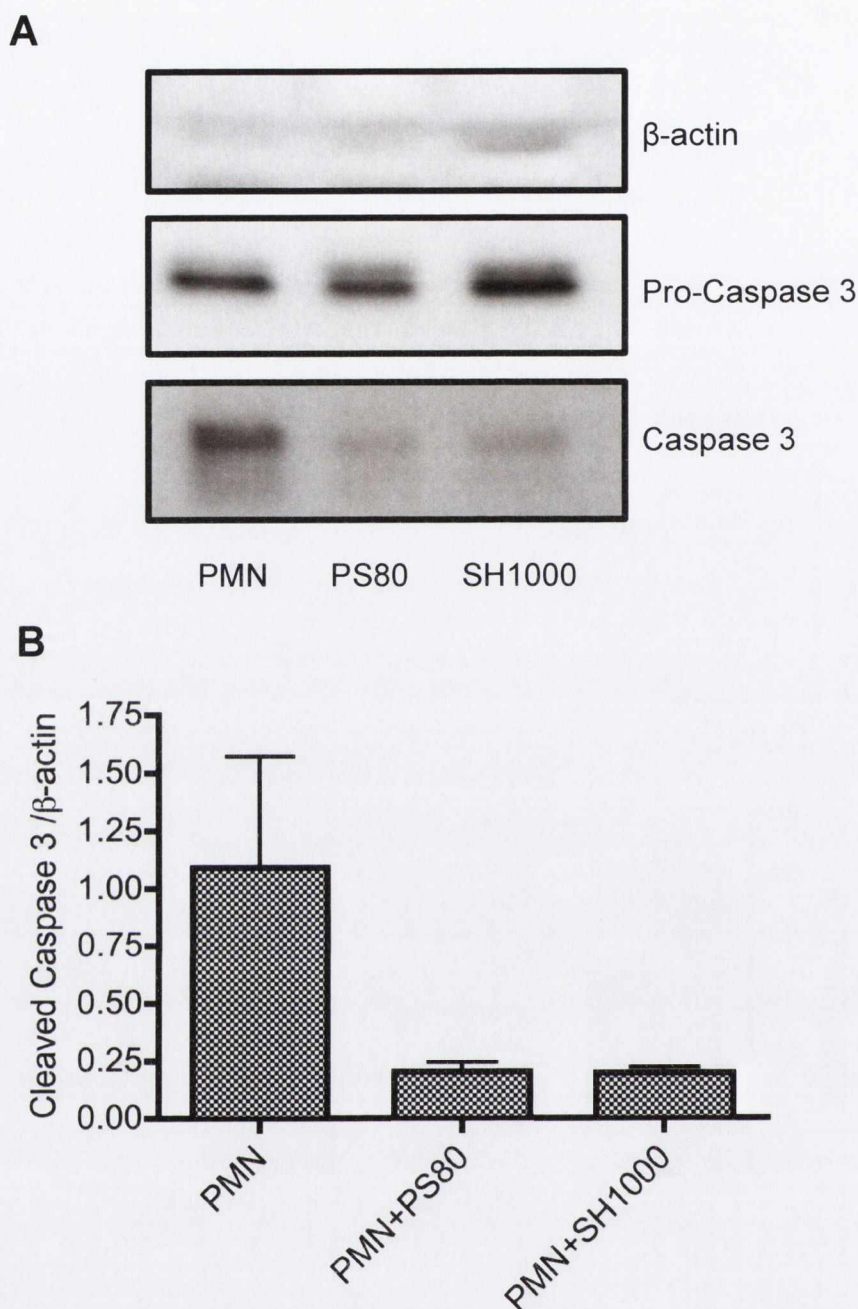


Figure 4.23: *S. aureus* strain SH1000 can also inhibit the spontaneous apoptosis of neutrophils *in vitro*, as measured by Caspase 3 Cleavage.

Human PMN were cultured with *S. aureus* strain PS80 or SH1000, or left uninfected. At 2 hours post infection cells were lysed, run on SDS-PAGE and transferred to PVDF membrane. This was probed with anti-Caspase 3. β -actin was probed for as a loading control. Results expressed as representative Western Blot (A) and plotted normalised to β -actin, mean $\pm$ SEM (B), n=3.

4.2.13 Role of autophagy in neutrophil response to *S. aureus*.

While *S. aureus* strains PS80 and SH1000 do not differ in terms of their ability to manipulate neutrophil apoptosis, based on the data presented in Chapter 3, they do exhibit differences in how they engage with autophagy in other phagocytes. Given that neutrophils can kill SH1000 and PS80 to different degrees, with no associated difference in the manipulation of apoptosis, the abilities of these strains to engage autophagy in neutrophils was then examined.

To investigate if subversion of autophagy represents a mechanism by which *S. aureus* can evade neutrophilic killing, human neutrophils were pretreated with the inhibitor of autophagy 3-MA prior to infection with *S. aureus* strains PS80 or SH1000 for 6 hours. In the presence of 3-MA, the ability of neutrophils to kill PS80 was substantially increased (22.5% increase in killing by 6 hours) as compared to the untreated infected cells (Figure 4.24A). Interestingly, SH1000 killing was not affected by the presence of 3-MA with almost 100% killing observed by 6 hours in both the presence or absence of 3-MA. These data suggest that *S. aureus* strain PS80 may be exploiting the autophagosome as a survival niche in neutrophils in the same way as it does in DCs, and macrophages, implicating this subversion of autophagy as a means by which PS80 evades killing by all phagocytic cells.

To investigate whether *S. aureus* strain PS80's ability to subvert autophagy in neutrophils was associated with its inhibition of apoptosis, neutrophils were pretreated with 3-MA, infected with *S. aureus* strain PS80 for 6 hours and then stained with TUNEL to determine the levels of apoptosis. It was found that inhibition of autophagy through treatment with 3-MA resulted in significantly increased levels of apoptosis in PS80 infected neutrophils (Figure 4.24B).

Taken together these data suggest that the processes of subversion of autophagy by *S. aureus* PS80, and its inhibition of apoptosis may be somehow linked, and that both work together to facilitate the survival of the bacteria within its intracellular niche. Inhibition of autophagy and increasing apoptosis resulted in enhanced killing of PS80 by neutrophils. These preliminary data suggest that although both SH1000 and PS80 are capable of inhibiting neutrophil apoptosis, by engaging with the autophagic machinery shortly after phagocytosis PS80 may increase its own survival in a way that SH1000 cannot.

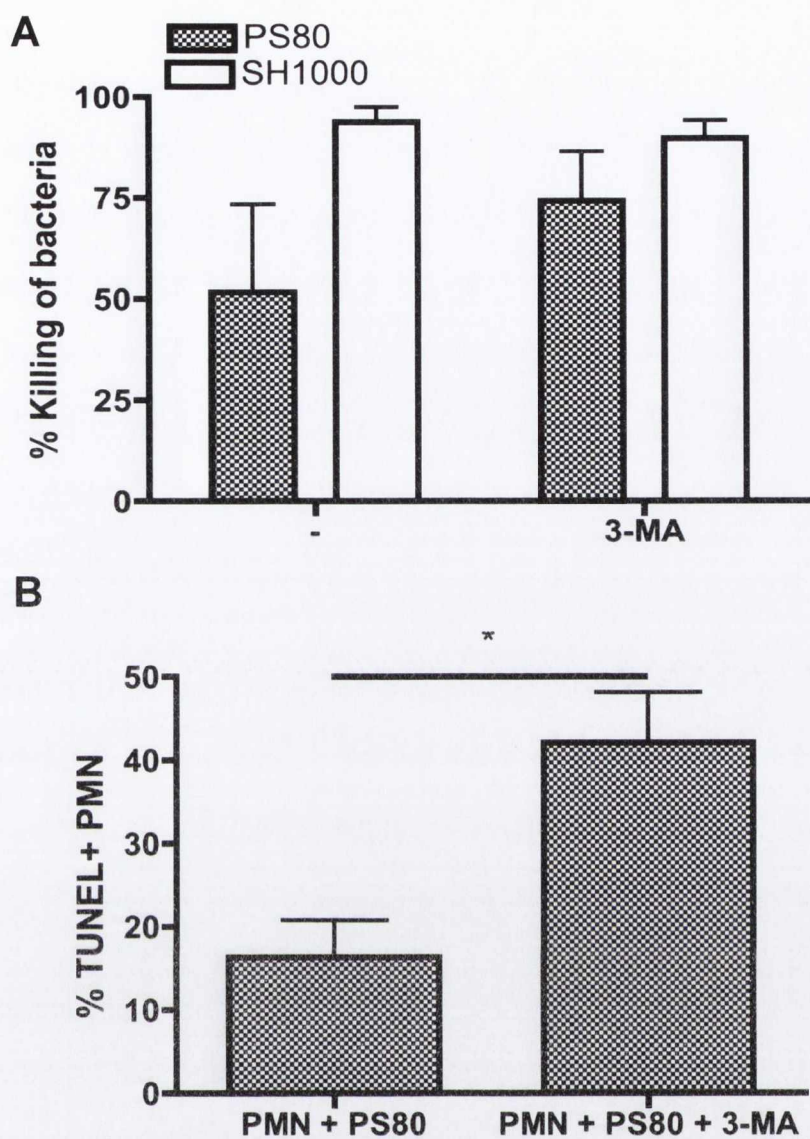


Figure 4.24: Blocking autophagy in neutrophils increases killing of PS80 and is associated with increased apoptosis.

Human PMN were pretreated with 3-MA for 30 min and infected with either *S. aureus* strain PS80 or SH1000 for 6 hours. % killing of bacteria was determined by comparing the total CFU in the presence of phagocytes to bacteria in media only. Alternatively, cells were then fixed, permeabilised and stained with fluorescein-conjugated TUNEL to be analysed by flow cytometry. Results expressed as mean $\pm$ SEM at each time point, $n=3/5$, * $p<0.05$.

Discussion

In recent years it has been postulated that *S. aureus* is capable of surviving inside neutrophils (133). In order to maintain this intracellular niche, this thesis proposes the theory that the bacteria must inhibit apoptosis of the neutrophils to prevent them rapidly dying and being cleared from the site of infection. From the bacterium's perspective, using the neutrophil as an intracellular niche will only be effective if the neutrophil can survive for an appreciable length of time at the infection site. The results of these studies illustrate that *S. aureus* can manipulate apoptosis of the neutrophil directly, both *in vitro* and *in vivo* and suggests a putative, strain dependent link between autophagy upregulation and the inhibition of apoptosis. Furthermore, it provides evidence that the T cell cytokine IFN γ , which is produced in response to *S. aureus* infection can inhibit neutrophil apoptosis both *in vitro* and *in vivo*, leading to an accumulation of neutrophils in which the bacteria can survive.

Neutrophils are notoriously short lived cells that die rapidly upon phagocytosis of bacteria. Their value as an intracellular survival niche is questionable as the energy expended by the bacteria to thwart killing by the cells and establish themselves intracellularly is in vain if the cell itself quickly dies. As other bacterium have been shown to delay neutrophil apoptosis to extend their survival window within these cells, it was prudent to establish whether *S. aureus* could also enhance neutrophil survival to propagate its niche. Indeed it was found using *in vitro* assays that infection with *S. aureus* delayed apoptosis of neutrophils.

Any studies into the manipulation of neutrophil apoptosis by bacteria have thus far been conducted in human neutrophils isolated from the peripheral blood. The effects of bacterial inhibition of neutrophil apoptosis *in vivo* have yet to be elucidated. This

study, for the first time, shows that *S. aureus* is capable of inhibiting neutrophil apoptosis *in vivo*, and that in this neutrophil-rich environment, there was significant intracellular survival of *S. aureus*. Using a peritoneal infection model it was established that neutrophils could harbour significant levels of bacteria intracellularly and that these neutrophils containing bacteria were significantly non-apoptotic.

Previous work by McLoughlin et al. used a clinically relevant surgical wound infection model to demonstrate that a high number of neutrophils present at the site of infection can have a detrimental effect on the clearance of bacteria, (89). The authors illustrated that blocking neutrophil recruitment can improve infection outcome, and suggest that this is as a result of the reduced availability of a bacterial survival niche within the neutrophils. Thus, it stands to reason that not only would increasing the recruitment of neutrophils allow the propagation of infection, but in the same vein, preventing the apoptosis and clearance of the neutrophils would increase the longevity of *S. aureus*' survival niche within the neutrophils. Consequently, the ability of *S. aureus* to manipulate neutrophil apoptosis in the surgical wound infection model was assessed. It was found that neutrophils were significantly less apoptotic in the presence of *S. aureus* than in uninfected mice, underlining the importance of *S. aureus*' ability to manipulate neutrophil apoptosis as a means of propagating its own survival at this site.

The role of IFN γ in the context of *S. aureus* infection is a contentious issue (See Section 1.2.3.2) with published studies declaring it both protective and deleterious. The publication by McLoughlin et al. discussed above implicated the cytokine IFN γ as a means by which neutrophil recruitment (and therefore intracellular bacterial survival) was enhanced in response to *S. aureus* surgical wound infection. However

that study did not take into account any effects IFN γ has on neutrophil apoptosis. Interestingly, IFN γ has been shown to be capable of delaying neutrophil apoptosis in a murine model of acute peritoneal inflammation (91). The results presented here show that IFN γ can directly inhibit neutrophil apoptosis *in vitro* independently of infection and there was some evidence of a cumulative effect when neutrophils were cultured in the presence of both *S. aureus* and IFN γ . The observed inhibition of neutrophil apoptosis by IFN γ also played a significant role *in vivo*. There was a significant reduction in the numbers of neutrophils present at the site of infection in IFN $\gamma^{-/-}$ mice compared to WT mice 7 days post infection and these neutrophils were substantially more apoptotic than those isolated from WT mice, illustrating that IFN γ is capable of inhibiting apoptosis in the surgical wound infection model *in vivo*.

Cumulatively, the data presented here illustrates that *S. aureus* is capable of direct inhibition of neutrophil apoptosis both *in vitro* and *in vivo*, and that it exploits this to propagate an intracellular survival niche. Additionally, it demonstrates that IFN γ can manipulate neutrophil apoptosis. Taken together these results suggest that striking the balance between sufficient neutrophil recruitment to facilitate clearance without the accumulation of excessive cells to provide a survival niche may be the key for a successful outcome in *S. aureus* infection.

The results discussed to this point have pertained exclusively to the interaction between *S. aureus* strain PS80 and neutrophils. However, as discussed at length in Chapter 3, strains of *S. aureus* differ in their abilities to manipulate phagocytic cells, consequently it was important to establish the effects of different *S. aureus* strains on neutrophils. Neutrophils were shown to be more successful at killing *S. aureus* strain SH1000 as compared to PS80. Interestingly, SH1000 had the same capacity

to inhibit neutrophil apoptosis as PS80. Given that *S. aureus* strains SH1000 and PS80 do not differ in their abilities to manipulate apoptosis, this suggests that factors facilitating inhibition of neutrophil apoptosis must be widely expressed among strains of *S. aureus*. Indeed, lipotechoic acid (LTA), a component of the bacterial cell wall of *S. aureus* has been shown to inhibit the spontaneous apoptosis of human neutrophils (221) and may therefore be the factor that is inhibiting apoptosis in this instance. It is important to bear in mind that this study included only 2 strains and therefore conclusions should be drawn with caution.

Chapter 3 detailed the novel finding that differences in phagocyte response to strains of *S. aureus* were due to the ability of specific strains to subvert autophagy. Thus, the effects of *S. aureus* strains PS80 and SH1000 on neutrophil autophagy was examined. To date, there is only one paper published that examines the subversion of autophagy in neutrophils by bacteria (specifically Adherent Invasive *E.coli*). This study showed that *E. coli* could induce autophagy and then prevent the acidification of the autolysosome, allowing intracellular survival (222). Interestingly the data presented in this chapter show that treatment with the autophagy inhibitor 3-MA enhances the ability of neutrophils to kill PS80. This result suggests that PS80 can act in a similar manner in neutrophils as it does in DCs and macrophages, and subvert autophagy as a survival niche. Future work will now be undertaken to definitively prove this hypothesis.

It has previously been shown that inhibition of autophagy in human neutrophils leads to an increase in apoptosis by a number of mechanisms illustrating the link between these two important pathways. Inhibition of autophagy prevents the degradation of damaged mitochondria, which initiate intrinsic apoptosis through the release of death

inducing compounds such as cytochrome c (176). Inhibition of autophagy in neutrophils infected with *S. aureus* strain PS80 led to an increase in apoptosis levels while simultaneously increasing the ability of neutrophils to kill PS80. This suggests that PS80 may be engaging with the autophagosome in order to inhibit apoptosis thus allowing it to survive intracellularly within the autophagosomal niche while preventing the death of its host cell.

The mechanism by which *S. aureus* controls apoptosis and autophagy in neutrophils has yet to be elucidated. *A. phagocytophilum* is known to parasitise neutrophils and inhibit neutrophil apoptosis to promote its own survival. This bacterium inhibits neutrophil apoptosis through interference with the p38 MAPK pathway, and maintenance of high levels of the antiapoptotic protein Mcl-1 (217). Intriguingly, *S. aureus* has been shown to upregulate Mcl-1 in macrophages to prevent cell death and allow for persistence and dissemination (157), so it stands to reason that a similar mechanism may be at play in neutrophils. Furthermore, Mcl-1 has been shown to regulate the balance between autophagy and apoptosis (223), making it a likely candidate to be targeted by *S. aureus* to manipulate these pathways. Consequently the interactions between strains of *S. aureus* and Mcl-1 to modulate the apoptosis/autophagy pathways warrants further study.

Overall, this study illustrates that *S. aureus* can inhibit apoptosis both *in vivo* and *in vitro* in order to propagate an intracellular survival niche within neutrophils (Figure 4.25). Furthermore, it was observed that although there was no difference in inhibition of apoptosis, certain strains of *S. aureus* had a survival advantage, likely due to its interaction with the autophagic pathway. These novel findings add to the growing collective knowledge of the intricacies of the interplay between *S. aureus* and the

host, and the support the hypothesis that neutrophils may play a deleterious role in this interaction.

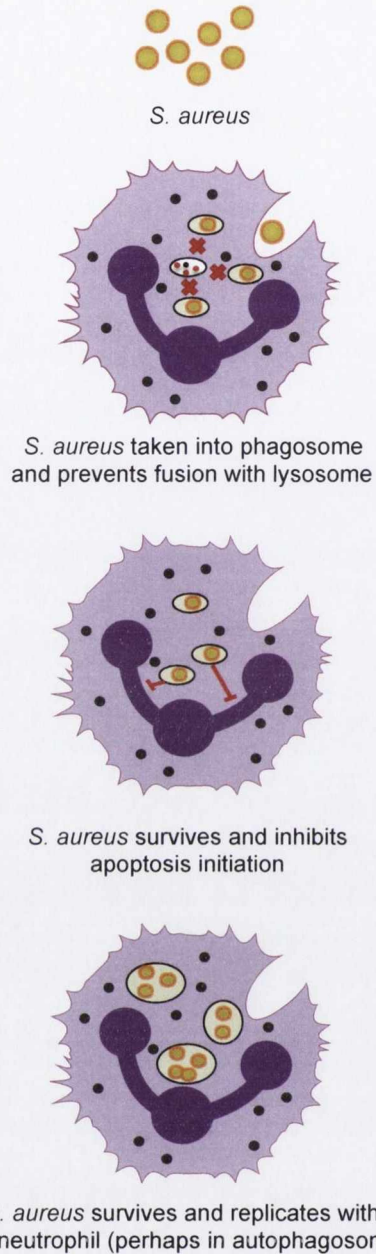


Figure 4.25: *S. aureus* inhibits neutrophil apoptosis to facilitate its survival.

S. aureus is phagocytosed by the neutrophil but is not killed. *S. aureus* prevents the initiation of apoptosis of the neutrophil, preventing it from dying. The bacterium then survives and replicates within the living cell – potentially within the autophagosomal compartment.

Chapter 5.

General Discussion

The rampant evolution of antimicrobial resistance is currently a global health threat and *Staphylococcus aureus* is the forerunner of this epidemic. Its infective potential has been exacerbated by the emergence of strains that have quickly acquired resistance to treatment by antibiotics. In order to manage this often fatal infection, alternatives to antibiotics such as vaccines or immunomodulatory therapies are urgently needed. However, before attempts to develop these therapies can be successful, there are two challenges that need to be overcome.

Firstly, the correlates of protective immunity to *S. aureus* need to be thoroughly understood. The interactions between *S. aureus* and the host are continuously becoming more clearly defined. For example, it has recently been acknowledged that a robust T cell response is as important, if not more so, than a B cell response in protection against *S. aureus* (23) and consequently vaccines will be required to elicit both a robust antibody response and an appropriate T cell response to direct the immune system to facilitate the efficient clearance of infection with this bacterium. Furthermore the position of phagocytes as the quintessential *S. aureus*-killing cell has been questioned in recent years with the realisation that the bacterium is capable of surviving and replicating within these normally bactericidal cells (133). Before there is success in the generation of new therapies, the mechanism behind these interactions between *S. aureus* and the host's immune system need to be more thoroughly understood to allow us to manipulate them.

Secondly, the generation of effective vaccines or immunomodulatory treatments that target *S. aureus* is complicated by the rapid evolution of the bacterium. As it has evolved concomitantly with a human host as part of the natural microbiome, *S. aureus* has an intricate knowledge of the workings of the immune system and thus

has developed a vast arsenal of immune evasion strategies. Its rapid rate of evolution means that this bacteria is continuously evolving to evade any killing mechanisms directed against it (25). It seems as though as soon as a new immune mechanism that is important for protection against *S. aureus* infection is defined, a means of evasion of this mechanism by the bacteria is unearthed!

This thesis aimed to unravel the mechanisms by which *S. aureus* manipulates phagocytes in order to facilitate its intracellular survival within these normally bactericidal cells.

S. aureus' immense capacity for immune evasion is exemplified by the number of mechanisms that this bacterium has evolved to evade practically every facet of the immune response. The innate humoral response can be evaded through the degradation of AMPs (116) and the destabilisation of complement depositions (32). All stages of the phagocytic response can be evaded, from recognition being hindered by cloaking with fibrinogen (107) to inhibition of recruitment by the expression of CHIPs (103). If the phagocytes are recruited to the site of infection, *S. aureus* can evade phagocytosis (109) and even if phagocytosed, the bacterium possesses many mechanisms by which it can evade killing (117-119). Once safely within the host cell, it has been shown in non-phagocytic cells that *S. aureus* may then subvert autophagy, which is a key cell protection mechanism, giving the bacterium control over the fate of the cell (175). Given this multitude of evasion mechanisms, with hindsight it now seems inevitable that *S. aureus* would survive within the phagocytic cells. The "Trojan Horse" theory presented by Thwaites et al (133) combines *in vitro*, *in vivo* and clinical data to propose that *S. aureus* can

propagate its own survival within neutrophils as a means of dissemination around the host.

An inherent shortcoming of this immune evasion strategy however, is that neutrophils are notoriously short-lived cells. This raises the question of the benefit that survival within neutrophils affords *S. aureus* if the energy expended by the bacteria to evade killing results in only a very short window of survival before the host cell dies. Thus, this thesis proposes that *S. aureus* is capable of deliberate manipulation of neutrophil apoptosis in order to propagate an intracellular niche in which it can survive. This phenomenon was identified *in vitro* using human neutrophils and importantly, was shown to be relevant *in vivo*, as neutrophils isolated from *S. aureus* infected mice were less apoptotic than those from PBS-treated control mice. Furthermore, neutrophils isolated from *S. aureus* infected animals that contained viable bacteria were shown to be non-apoptotic, confirming that in order to prolong their survival, viable intracellular *S. aureus* were actively inhibiting the apoptosis of the neutrophil. Previous work has demonstrated that *S. aureus* infection induces significant amounts of IFN γ production by T cells and that this IFN γ can be either protective or injurious, perhaps dependent upon the site of infection (See Section 1.2.3.2.1). The data in this thesis suggest that during *S. aureus* surgical wound infection IFN γ may have a deleterious effect by inhibiting neutrophil apoptosis, thus contributing to infection by promoting the accumulation of neutrophils which provide an intracellular survival niche.

While it is well established that an effective neutrophil response is indispensable for the efficient clearance of *S. aureus* (as highlighted in patients that lack neutrophil killing mechanisms and suffer from recurrent *S. aureus* infections (45)) emerging

data from the field, supported by results discussed in this thesis, suggest that an appropriately controlled neutrophil response is key, as the accumulation of too many neutrophils at the site of infection can actually facilitate bacterial survival by providing an intracellular niche for survival. Work published by McLoughlin et al. illustrated that enhanced neutrophil recruitment to the site of *S. aureus* surgical wound infection resulted in exacerbated infection, while limiting neutrophil recruitment promoted bacterial clearance (89). Using this same model of infection, the results presented here illustrate how the inhibition of neutrophil apoptosis and clearance also facilitates neutrophil accumulation, resulting in increased bacterial survival. Taken together these results support the theory that a balance needs to be struck between proficient recruitment and timely apoptosis and clearance of neutrophils in order to ensure effective resolution of infection.

The vast bulk of the research on phagocytic killing of *S. aureus* has thus far focused on neutrophils and to some degree macrophages. In this thesis it is proven for the first time that dendritic cells are also capable of directly killing *S. aureus* and that significant DC interaction with the bacterium *in vivo* is observed, confirming that the killing observed was not an artefact of the bacterial infection of cells *in vitro*. Furthermore, this thesis describes a method of immune evasion that has not previously been described in phagocytes. Certain strains of *S. aureus* are shown to subvert autophagy within dendritic cells and macrophages by inhibiting autophagic flux, which allowed them to survive within the autophagosome thus propagating an effective intracellular survival niche. This capacity to subvert autophagy was dependent upon the levels of Agr – but not Hla – expression by the strain of *S. aureus*.

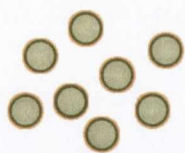
This thesis proposes a model for *S. aureus* survival within phagocytes which alters the cell death pathway of the host cell. It suggests that the processes of autophagy and apoptosis are interlinked and that both are manipulated by *S. aureus* (See Figure 5.1). In preventing their host cell from dying, the bacteria potentially drive the cell to induce the autophagic pathway, which they are capable of exploiting by preventing the fusion of the autophagosome to the lysosome (175), permitting the survival and replication of the bacteria intracellularly. Although it is beyond the scope of this thesis, it would be interesting to unravel the mechanism behind this process and to understand the molecular signalling undertaken by *S. aureus* that facilitates this apoptotic to autophagic switch. It is known that inhibition of autophagy induces apoptosis in the cell by a variety of methods, for example when autophagy is occurring normally, it clears damaged mitochondria from the cell but in the absence of autophagy, these accumulating aged or damaged mitochondria initiate intrinsic apoptosis. This autophagic to apoptotic switch could potentially be an interesting avenue of further study as a drug that inhibited autophagy would presumably alter the host cell to an apoptotic state (224), correcting the balance of phagocytes at the site of infection and facilitating efficient clearance of the bacteria.

Some recent, very preliminary results have shown interesting data that may influence the progression of this study in our lab. *S. aureus* is known to be detected by the innate immune system through interaction of its peptidoglycan and lipoteichoic acids with the PRR TLR2 on the surface of immune cells (225, 226). VIPER (viral inhibitor peptide of TLR4) is a compound which has been shown to prevent TLR2 signalling by impeding the adaptor proteins MAL and TRAM which are downstream of the receptor (227). Inhibition of TLR2 signalling in phagocytes by treatment with VIPER promoted more effective killing of *S. aureus* by phagocytes (Appendix Figure 2). TLR2 has been previously implicated in the induction of autophagy in macrophages

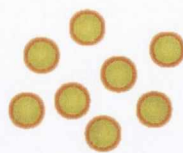
in response to *S. aureus*, as knocking down TLR2 expression reduced the levels of autophagy in response to infection (199). This suggests that inhibiting TLR2 signalling by *S. aureus* may inhibit the induction of autophagy by the cells, preventing the bacterial subversion of the autophagic pathway and allowing the cells to kill the bacteria. This very preliminary finding opens a fascinating avenue of future study, determining whether *S. aureus* can signal through TLR2 to induce autophagy and thus inhibit apoptosis, creating a survival niche for itself within the phagocyte.

The hypotheses presented above are based almost entirely on the interaction of *S. aureus* strain PS80 with phagocytes. A critical finding that emerged from this thesis is the variability in the immune response to different strains of *S. aureus*. Strains that expressed high levels of the global virulence regulator Agr –such as PS80– were much more adept at surviving within phagocytes than strains that expressed low levels of Agr. SH1000 was a representative strain that had low Agr activity levels and was killed by the phagocytes, without the involvement of autophagy. Fascinatingly, this phenomenon was not only observed in laboratory based strains but a similar divergence in immune response was observed when phagocytes were infected with clinical strains that had recently been isolated from bacteremic patients. This fact illustrates the clinical relevance of these findings and suggests that Agr activity may be a potential predictor of the invasiveness of infection in a clinical setting. However it also highlights a major problem in the field of host-pathogen interaction research. The majority of published studies on *S. aureus*' interactions with the immune system base their findings on a single strain of *S. aureus* but as is shown here, that is in no way reflective of every strain and one must be careful not to draw conclusions based on a single strain of the bacteria. The relevance of strain selection on the outcome of studies is a factor that must be taken into account in experimental design and reporting results.

In summary, this thesis significantly advances our understanding of *S. aureus*' ability to manipulate phagocytic cell response, and highlights the important role that apoptosis and autophagy can play in determining infection outcome. The answer to overcoming the epidemic of *S. aureus* infection that currently plagues healthcare systems worldwide no doubt lies in modulating the immune system to more efficiently kill *S. aureus* rather than targeting the bacteria directly with antibiotics. However the question still remains whether this will be possible or will this ever-adaptable bacterium be capable of continuing to evolve to remain one step ahead.

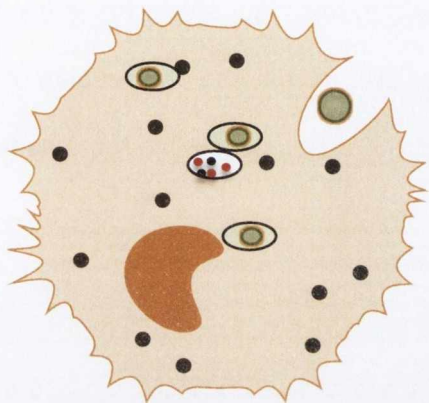


Classical extracellular bacteria

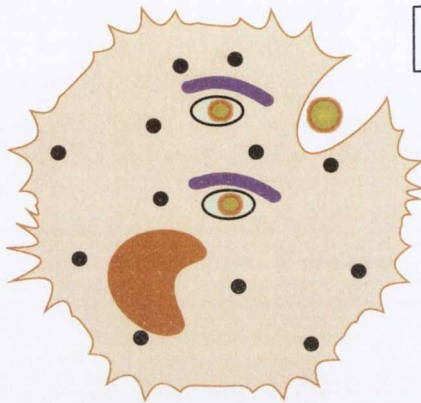


S. aureus

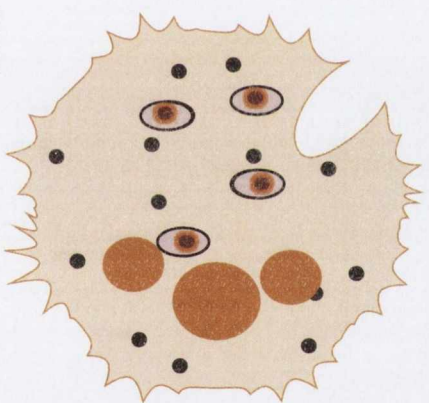
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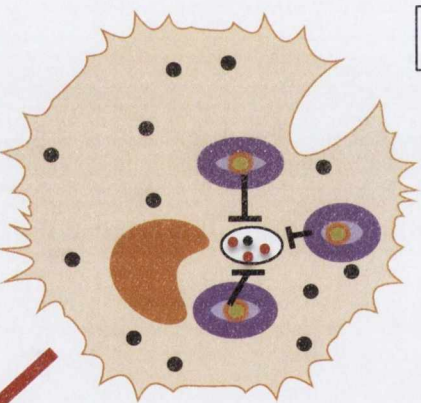
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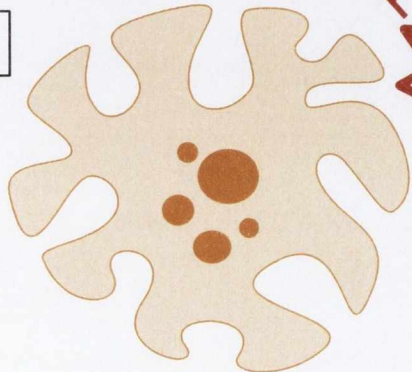
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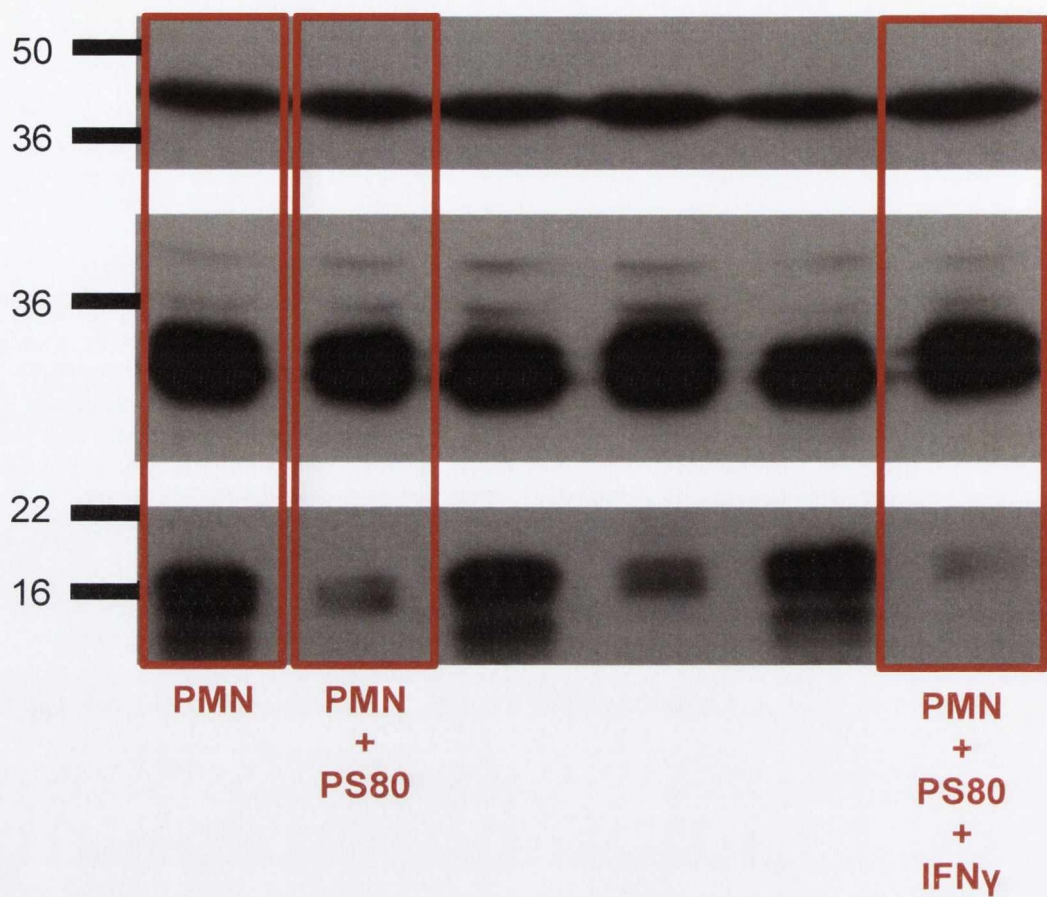
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Figure 5.1: *S. aureus* manipulates normal progression of phagocyte bacterial killing.

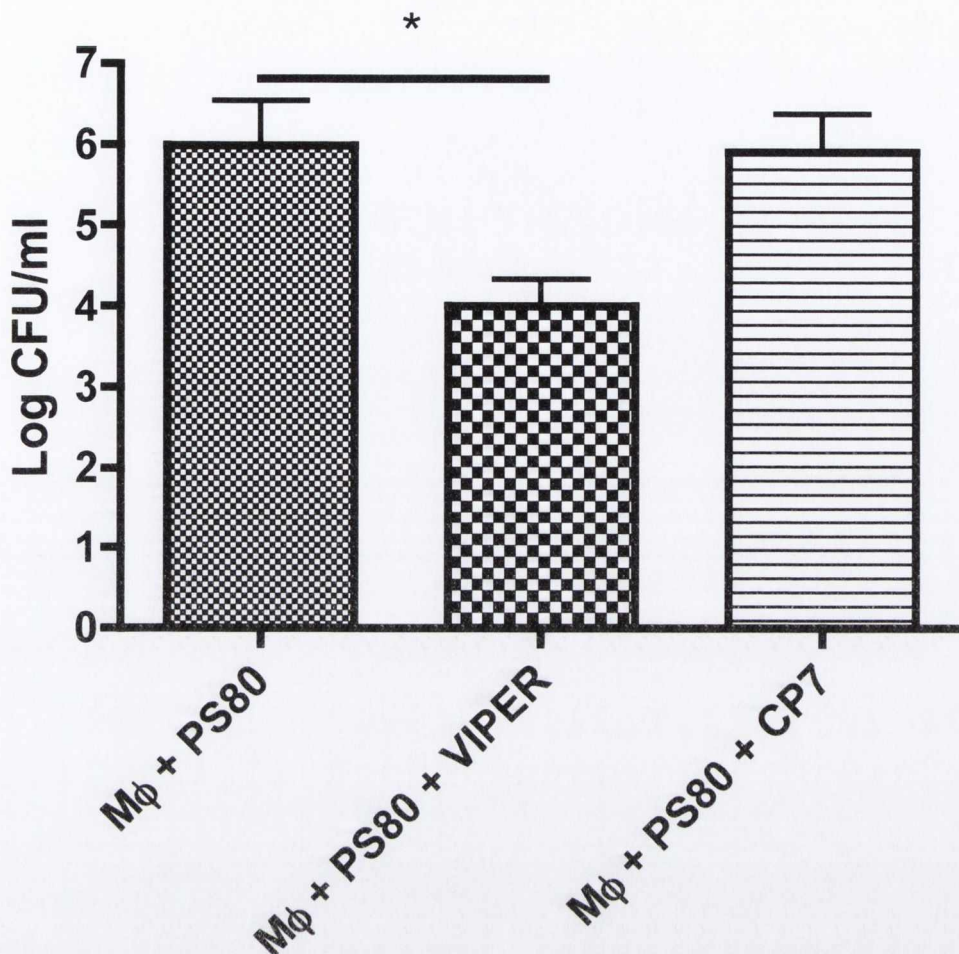
Under normal circumstances phagocytes phagocytose bacteria (1), kill the bacteria (2) and undergo apoptosis (3). Upon phagocytosis (4) *S. aureus* initiates autophagosome formation (5) and can survive within this intracellular niche (6). To maintain this intracellular survival niche *S. aureus* also inhibits the progression of apoptosis (7). The specific mechanisms by which *S. aureus* does this remains to be elucidated.

Chapter 6.

Appendices



Appendix 1: Uncropped blot from Fig 4.11



Appendix 2: Treatment with a TLR2 inhibitor can enhance PS80 killing by Macrophages

BMDMs were pretreated with VIPER (5μM) or the control peptide CP7 (5μM) for 1 hour. They were then infected with *S. aureus* strain PS80 (MOI 100) and CFUs determined 6 hours post infection by plating on TSA. n=3, *p<0.05. Experiment performed by Katie O'Brien

Chapter 7.

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