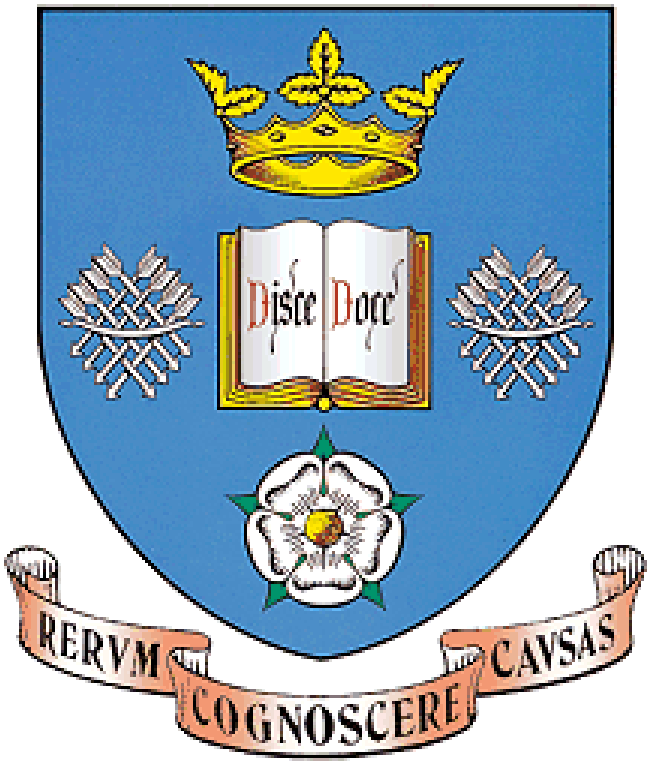

The interaction of *Staphylococcus aureus* with macrophages

Jamil Adib Jubrail

Department of Infection and Immunity,

University of Sheffield Medical School,

UK

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Table of Contents

Table of figures

Table of tables

Table of equations

Abbreviations

%: percentage

°: degrees

≤: Less than or equal to

~: approximately

>: Greater than

<: Less than

=: equals

+: plus

-: minus

ΔψM: Transmembrane potential

AAM: Alternatively Activated Macrophage

ABMs: Agent Based Models

Agr: Accessory Gene Regulator

AgrA: Accessory Gene Regulator A

AgrB: Accessory Gene Regulator B

AgrC: Accessory Gene Regulator C

AgrD: Accessory Gene Regulator D

AIP: Autoinducing peptide

AM: Alveolar Macrophage

AMP-PNP: Adenosine 5'-(β, γ imido) triphosphate lithium salt hydrate

Apaf1: Apoptosis activating factor 1

APC: Antigen Presenting Cell

Apo1/CD95: Fas

Arp 2/3: Actin related protein 2/3

ATCC: American Type Culture Collection

ATP: Adenosine Triphosphate

ATPγS: Adenosine 5′-(3-thiotriphosphate) tetralithium salt

ATPase: Adenosine Triphosphatase

Bak: Bcl-2 homologous antagonist/killer

Bax: Bcl-2 associated X protein

bp: base pairs

Bcl-2: B cell lymphoma 2

β: Internalisation of Se bacteria by Ma macrophages

BHI: Brain Heart Infusion

Bid: BH3 interacting domain death agonist

Bim: Bcl-2 interacting mediator of cell death

Bis: Basic Immune Simulator

BSA: Bovine Serum Albumin

C: Cytosine

C: Centigrade

CAM: Classically Activated Macrophage

CA-MRSA: Community Acquired-Methicillin Resistant *Staphylococcus aureus*

CBA: Columbia Blood Agar

ccrA: Cassette Chromosome Recombinase Gene A

ccrB: Cassette Chromosome Recombinase Gene B

CCL1: Chemokine (C-C) motif ligand 1

CCL17: Chemokine (C-C) motif ligand 17

CCR2: C-C chemokine receptor type 2

CCR5: C-C chemokine receptor type 5

cDNA: Complimentary deoxyribonucleic acid

$CD4^+$: Cluster of Differentiation 4 positive

CD11b: Cluster of Differentiation 11b

CD14: Cluster of Differentiation 14

CD16: Cluster of Differentiation 16

CD18: Integrin beta 2

CD36: Cluster of Differentiation 36

CDC42: Cell Division Control Protein homolog 42

c-fms: Colony stimulating factor 1 receptor gene

CFU: Colony forming units

CGD: Chronic granulatomous disease

CHIPS: Chemotaxis Inhibitory Protein of *Staphylococcus aureus*

Clf: Clumping factor

ClfA: Clumping factor A

ClfB: Clumping factor B

CO_2: Carbon Dioxide

COOH: Carboxyl terminal

CP5: Capsule polysaccharide 5

CP8: Capsule polysaccharide 8

C1q: Complement component 1q

C3b: Complement component 3b

C3d: Complement component 3d

C5: Complement component 5

C5aR: Complement 5a receptor

CR: Complement receptor

CR1: Complement receptor 1

CR3: Complement receptor 3

CRIg: Complement receptor Ig

CSF-1R: Colony Stimulating Factor 1 Receptor

CTD: C terminal domain

CXCL9: Chemokine (C-X-C) motif ligand 9

CXCL13: CXC motif chemokine 13

CX_3CR1: CX3C chemokine receptor 1

CXCR2: Chemokine (C-X-X) motif receptor 2

DCs: Dendritic cells

DAPI: 4’6, diamidino-2-phenylindole

dATP: deoxyadenosine 5'-triphosphate

DD: Death domain

dH_2O: distilled water

DIABLO: Direct inhibitor of apoptosis binding protein with a low isoelectric point

DIC: Differential interference contrast

DISC: Death inducing signalling complex

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

dn: Dominant negative

Eap: Extracellular Adherence Protein

Ecb: Extracellular complement binding protein

E. coli: *Escherichia coli*

Efb: Extracellular fibrinogen binding protein

e.g. Example

EGFP: Enhanced Green Fluorescent Protein

eNOS: Endothelial Nitric Oxide Synthase

ETs: Exfoliative toxins

ETA: Exfoliative toxin A

ETB: Exfoliative toxin B

ETC: Electron transport chain

FADD: Fas activated death domain

FasL: Fas Ligand

Fc: Fragment Crystallisable Region

FcγR: Fragment Crystallisable Gamma Receptor

FcγRI: Fragment Crystallisable Gamma Receptor 1

FcγRII: Fragment Crystallisable Receptor Two

FcγRIIA: Fragment Crystallisable Receptor Two A

FcγRIII: Fragment Crystallisable Receptor Three

FCS: Fetal Calf Serum

Fe-S: Iron-Sulphur

FITC: Fluorescein isothiocyanate

FIZZ1: Found in inflammatory zone 1

FLICE: FADD like IL-1β converting enzyme

FLIPr: Formyl peptide receptor like 1 inhibitor

fMLP: Formyl-Methionyl-Leucyl-Phenylalanine

FnBPs: Fibronectin Binding Proteins

FnBPA: Fibronectin binding protein A

FnBPB: Fibronectin binding protein B

FPLR1: Formyl peptide like receptor 1

FPR: Formyl peptide receptor

G: Guanine

g: grams

γ: Internalisation rate of Se by Mf macrophages

GEMM-CFU: Granulocyte, erythrocyte, monocyte, megakaryocyte colony forming unit

GFP: Green Fluorescent Protein

GM-CFU: Granulocyte, macrophage colony forming unit

GTPases: Guanosine triphosphates

HA-MRSA: Hospital Acquired-Methicillin Resistant *Staphylococcus aureus*

Hck: Haematopoietic cell kinases

HI: Heat inactivated

HIFCS: Heat inactivated foetal calf serum

HIV: Human Immunodeficiency Virus

HIV-1: Human Immunodeficiency Virus 1

Hla: Alpha haemolysin

Hlb: Beta haemolysin

Hld: Delta haemolysin

Hlg: Gamma haemolysin

Hmp: Haem metalloprotease

HOCl: Hyperchlorous Acid

ICAM-1: Intracellular Adhesion Molecule 1

iC3b: Inactive complement component 3b

IFNβ: Interferon beta

IFNγ: Interferon gamma

IgG: Immunoglobulin G

IL1: Interleukin 1

IL1β: Interleukin 1 beta

IL3: Interleukin 3

IL6: Interleukin 6

IL8: Interleukin 8

IL10: Interleukin 10

IL12: Interleukin 12

IL10R1: Interleukin 10 receptor

iNOS: Inducible Nitric Oxide Synthase

ITAMs: Immunoreceptor tyrosine activation motifs

ITIMs: Immunoreceptor tyrosine inhibition motifs

JNK: c-Jun N terminal kinase

k: Carrying capacity for Se bacteria

k: Production rate of WT virus by infected $CD4^+$ T helper cells

K: The average number of Si bacteria per cell

Kan^R: Kanamycin resistant

kDa: kilo dalton

k_m: Production rate of mutant viruses by infected $CD4^+$ T helper cells

l: litre

LAM: Lipoarabinomannan

LAMP: Lysosomal associated membrane protein

LAMP-1: Lysosomal associated membrane protein 1

LAMP-2: Lysosomal associated membrane protein 2

Ldh: Lactate dehydrogenase

ldh1: Lactate dehydrogenase 1 gene

ldh2: Lactate dehydrogenase 2 gene

LIMP-II: Lysosomal integral membrane protein 2

LK: Luria potassium broth

lm: Linear Model

LMP: Lysosomal membrane permeabilisation

logCFU: logarithmic colony forming units

LPS: Lipopolysaccharide

LTA: Lipoteichoic Acid

LukAB: Leukocidin AB

LukED: Leukocidin ED

LukF-PV: Leukocidin F Panton Valentine

LukS-PV: Leukocidin S Panton Valentine

Lyn: Lck/YES related novel protein

M: molar

M*: Infected cells population 2

M1: Classically Activated Macrophage

M2: Alternatively Activated Macrophage

Ma: Actively ingesting or active macrophage

Mac-2: Integrin alpha M beta 2

MACH: MORT-1 associated CED 3 homologue

MARCO: Macrophage receptor with collagenous structure

MBB: Molecular Biology and Biotechnology

Mbp: Mega base pairs

MBL: Mannose binding lectin

M-CFU: Macrophage colony forming unit

Mcl-1: Myeloid cell leukaemia sequence 1

Mcl-1_{Exon1}: Myeloid cell leukaemia sequence 1 exon 1

Mcl-1L: Myeloid cell leukaemia sequence 1 Long

Mcl-1S: Myeloid cell leukaemia sequence 1 Short

M-CSF: Macrophage Colony Stimulating Factor

MDMs: Monocyte Derived Macrophages

MDP: Muramyl dipeptide

mecA: Methicillin Encoded Cassette A

mecC: Methicillin Encoded Cassette C

Mf: Unexposed or free macrophage

µg: micrograms

mg: milligrams

MI: Mock infected

ml: millilitres

µM: micromolar

mM: millimolar

MMP12: Matrix metalloprotease 12

Mn: Manganese

MOI: Multiplicity of Infection

MOMP: Mitochondrial outer membrane permeabilisation

MORT-1: Mediator of receptor induced toxicity 1

MPO: Myeloperoxidase

MPS: Mononuclear Phagocyte System

MR: Mannose Receptor

mRNA: Messenger ribonucleic acid

MRSA: Methicillin Resistant *Staphylococcus aureus*

MSCRAMMs: Microbial Surface Components Recognising Adhesive Matrix Molecules

MSSA: Methicillin Sensitive *Staphylococcus aureus*

μ: Killing of Si bacteria by Ma macrophages

MyD88: Myeloid differentiation primary response gene 88

NADPH: Nicotinamide adenine dinucleotide phosphate

NETs: Neutrophil extracellular traps

NF-κB: Nuclear Factor kappa B

NF-Y: Nuclear factor Y

NHE: Sodium/hydrogen exchange

nM: nanomolar

nNOS: Neuronal Nitric Oxide Synthase

ng: nanograms

NK: Natural Killer Cell

NLRP3: NOD-like receptor family, pyrin domain containing 3

Nod: Nucleotide binding oligomerisation domain

Nod-2: Nucleotide binding oligomerisation domain containing protein 2

NO: Nitric Oxide

NOS: Nitric Oxide Synthases

NOX: NADPH oxidase

NOX2: NADPH oxidase 2

NOXA: phorbol-12-myristate-13-acetate-induced protein 1

NTD: N terminal domain

OatA: O-acetyltransferase A

OD: Optical Density

ODE: Ordinary Differential Equation

O/N: Overnight

ONNO$^-$: Peroxynitrite

ORFs: Open Reading Frames

p: Mutant virus propagation route

P2: Promoter 2

P3: Promoter 3

PAK1: p21 activated kinase 1

PAMP: Pathogen Associated Molecular Pattern

PBP: Penicillin Binding Protein

PBP2: Penicillin Binding Protein 2

PBP2a: Penicillin Binding Protein 2a

PBS: Phosphate buffered saline

PCD: Programmed Cell Death

PepG: Peptidoglycan G

PF: Paraformaldehyde

PGN: Peptidoglycan

pH: Potential hydrogen

PI3K: Phosphoinositol 3 kinase

PI3, 4, 5-P: Phosphoinositol 3, 4, 5 phosphate

PKC: Protein Kinase C

PKCε: Protein Kinase C epsilon

PLC: Phospholipase C

PMA: Phorbol 12-myristate acetate

PMN: Polymorphoneutrophil

PRRs: Pattern Recognition Receptors

PS: Phosphatidylserine

PSGL1: P-selectin glycoprotein ligand 1

PSMs: Phenol Soluble Modulins

PUMA: p53 upregulated modulator of apoptosis

PVL: Panton Valentine Leukocidin

r: Replication of Se bacteria

Rab-5: Ras associated protein 5

Rab-7: Ras associated protein 7

Rac1: Ras related C3 botulinum toxin substrate

Ras: Rat sarcoma

Rho: Ras homolog gene family member

Rho GTPases: Ras homolog gene family member guanosine triphosphate

RhoA: Ras homolog gene family member A

RhoG: Ras homolog gene gamily member G

RNA: Ribonucleic acid

RNAII: Ribonucleic acid two

RNAIII: Ribonucleic acid three

RNIs: Reactive Nitrogen Intermediates

ROI: Reactive Oxygen Intermediate

ROS: Reactive Oxygen Species

Rot: Regulator of transcription

RPMI 1640: Roswell Park Memorial Institute 1640

RT: Room temperature

Sak: Staphylokinase

SarA: Staphylococcal accessory regulator A

SarS: Staphylococcal accessory regulator S

SarT: Staphylococcal accessory regulator T

S. aureus: *Staphylococcus aureus*

SCCmec: Staphylococcal Cassette Chromosome Mec Element

SCIN: Staphylococcal complement inhibitor

SCV: Small Colony Variant

Se: Extracellular bacteria

SEM: Scanning electron microscopy

SERAM: Secretable Expanded Repertoire Adhesive Molecules

SH2: Src homology 2

Si: Intracellular bacteria

Smac: Second mitochondria derived activator of caspases

SOD: Superoxide Dismutase

SodA: Superoxide Dismutase A

SodM: Superoxide Dismutase M

Spa: Protein A

SPHK1: Sphingosine kinase 1

S. pneumoniae: *Streptococcus pneumoniae*

SR-I: Scavenger receptor type I

SR-II: Scavenger receptor type II

SRC: Sarcoma family kinases

SrtA: Sortase A

SrtB: Sortase B

SSL3: Superantigen like protein 3

SSL5: Superantigen like protein 5

SSL7: Superantigen like protein 7

SSSS: Staphylococcal Scalded Skin Syndrome

Syk: Spleen tyrosine kinase

T*: Latently infected T cell

T**: Actively Infected T cell

TAM: Tumour Associated Macrophage

TB: Tuberculosis

TCA: Tricarboxylic acid

TGN: Trans-golgi network

Th1: T helper 1 cell

Th 17: T helper 17 cell

TLRs: Toll like Receptors

TLR1: Toll like Receptor 1

TLR2: Toll like Receptor 2

TLR4: Toll like Receptor 4

TLR6: Toll like Receptor 6

TLR9: Toll like Receptor 9

TNF: Tumour Necrosis Factor

TNF-α: Tumour Necrosis Factor alpha

TNF-R1: Tumour Necrosis Factor Receptor Type 1

TRADD: Tumour Necrosis Factor Type 1 associated death domain

UoS: University of Sheffield

USA: United States of America

USA100: United States of America 100

USA300: United States of America 300

USA500: United States of America 500

V: Virus

V-ATPase: Vacuolar ATPase

VFs: Virulence Factors

VRSA: Vancomycin Resistant *Staphylococcus aureus*

v/v: Volume/volume

WT: Wild type

XIAP: X linked inhibitor of apoptosis protein

z: Scaled drug concentration

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This thesis is dedicated to my family. They have always given me support throughout my Ph.D and offered me advice and guidance. Our daily chats have helped to keep me sane throughout this process and I promise that in a short while I will get a proper job and finish with education.

Declaration

All the experiments performed in this thesis were my own. Help was appreciated from Dr. Alex Best on some aspects of the mathematical modelling element of my project. Katie Cooke and Jon Kilby helped with THP-1 cell line maintenance and differentiation.

Abstract

Macrophages are essential during the innate immune response to bacterial pathogens. They have a variety of roles during bacterial infections that extend beyond phagocytosis and intracellular killing. The interaction of *Staphylococcus aureus* with macrophages has not been completely characterised. Moreover, how *S. aureus* is able to manipulate the macrophage host defence has produced conflicting results. This thesis has examined the kinetics of phagocytosis and intracellular killing of *S. aureus* by macrophages, as well as identifying key features of these processes, and developed a mathematical model to describe the interaction of *S. aureus* with macrophages.

Exposure of macrophages to *S. aureus* at a range of doses demonstrated that macrophages accumulated intracellular bacteria but although they could kill fixed numbers of intracellular bacteria they showed a finite capacity to kill bacteria. There was an initial rapid phase of intracellular killing post phagocytosis and then a more gradual decline in intracellular, viable bacteria, with persistence of intracellular bacteria for extended periods of time. Macrophages maintained viability following *S. aureus* challenge over time and macrophage mediated apoptosis was not apparent. As well as this, at the point when macrophages had exhausted their ability to kill intracellular bacteria they still demonstrated an ability to phagocytose and accumulate further intracellular bacteria.

This thesis also demonstrated that intracellular bacteria were able to persist intracellularly for up to 3 days post infection and then macrophages were lysed by intracellular bacteria. The released bacteria were ingested by other macrophages and as a result an intracellular pool of bacteria was maintained. Examination of the cell death process revealed it was not apoptosis but probably necrosis. I also explored the intracellular compartment where *S. aureus* were residing and was able to show that the majority of intracellular bacteria were in a phagolysosome that was not appropriately acidified but that the majority of the bacteria colocalised with the late endosomal markers lysosome associated membrane protein 1 or 2 (LAMP-1/2). In contrast the majority of *Escherichia coli* and *Streptococcus pneumoniae* were found to be in phagolysosomes that were appropriately acidified.

This thesis describes a novel mathematical model for the interaction of *S. aureus* with macrophages. The mathematical model showed that macrophages had the ability to

phagocytose *S. aureus* at a rate that depended on the extracellular bacterial concentration. However, the model revealed that macrophages showed a finite capacity to clear intracellular bacteria which over time gave rise to a population of macrophages that were unable to kill all of the bacteria they had phagocytosed.

Overall this thesis has shown that macrophages can phagocytose *S. aureus* and they accumulate intracellular bacteria over time efficiently. However, they show only a limited capacity to kill intracellular bacteria and in response to *S. aureus* macrophage apoptosis is not engaged. This thesis demonstrates that prolonged intracellular persistence of *S. aureus* in macrophages is beneficial to the bacterium and over time they lyse the macrophage and are released and re-ingested by other macrophages. This thesis suggests that the ability to break this release and re-ingestion cycle could lead to better therapeutic management of *S. aureus* disease.

Chapter 1 Introduction

1.1. *Staphylococcus aureus*

1.1.1. Brief history

A long held view in microbiology, has always been that to be classed as a pathogen, an organism must cause a specific clinical infectious disease, but over recent years it has become evident that many pathogenic organisms cause common clinical diseases of the western world that historically were not recognised as being caused by infectious agents such as gastrointestinal ulcers caused by *Helicobacter pylori* (Finlay and Cossart, 1997). As well as this, because of selection pressures certain "old diseases" from days gone by that had become less common have re-entered the modern world e.g. *Mycobacterium tuberculosis* (Finlay and Cossart, 1997). Thus the field of infectious diseases is in constant flux with new pathogens being recognised and old ones being identified as having new roles.

The staphylococcal genus was first described by Alexander Ogston in the nineteenth century, in the form of micrococcus poisoning following his experiments with warm and cold abscesses (Lyell, 1989, Ogston, 1882). Today the identification of the species *Staphylococcus aureus* is attributed to Rosenbach, who on hearing of Ogston's research extended it (Lyell, 1989). For many years since this pioneering research *S. aureus* has been classified as an extracellular pathogen, but recent research from the past 50 years has provided strong evidence that the pathogen can survive intracellularly in a range of professional and non-professional phagocytes (Melly et al., 1960, Rogers and Melly, 1960, Gresham et al., 2000, Hess et al., 2003).

S. aureus is often referred to as a "golden-clustered grape" because of its characteristic cell division process which leads to the formation of irregular bacterial clumps or chains containing various numbers of bacteria (Tzagoloff and Novick, 1977). It is described as golden because of the production of a carotenoid pigment called staphyloxanthin (Marshall and Wilmoth, 1981) whose structure and biosynthesis has been described by Pelz and colleagues (Pelz et al., 2005) which gives the organism its characteristic colour. *S. aureus* is known to divide in three perpendicular planes and the sister cells

maintain their shape until the point of cell division; following this, the sister cells separate and become spherical again (Tzagoloff and Novick, 1977).

1.1.2. Summary of clinical manifestations

S. aureus has become one of the major causes of a range of clinical infectious diseases from milder infections such as skin abscesses to severe infections such as pneumonia, staphylococcal scalded skin syndrome (SSSS), skin and soft tissue infections, bone and joint infections, bacteraemia, sepsis and widespread abscesses when bacteraemia leads to the spread of infection to multiple sites (Lina et al., 1997).

30% of the general population are colonised with *S. aureus* , most of whom do not develop any serious infections (Archer, 1998). Research into carriage rates has elucidated that newborn babies have the highest rate of staphylococcal carriage (Williams, 1963). *S. aureus* has frequently been isolated from the nasal passages, but can also be found in other areas such as the throat, perineum and even the intestine (Williams, 1963).

Five stages have been identified in the pathogenesis of *S. aureus* (Figure 1.1). Following on from colonisation, infection can cause local infection or penetrate the skin and enter the blood, causing invasive disease. It can then spread systemically and cause more severe presentations including metastatic abscesses at sites remote from the initial site of infection (Archer, 1998). Systemic infection can result in the development of septic shock (Archer, 1998), a host response to infection mediated by cytokines which results in low blood pressure and poor blood flow to various organs that is associated with significant levels of mortality.

1.1.3. Antibiotic resistance

The emergence of antibiotic resistant *S. aureus* has been a huge healthcare burden for over 50 years (M.P., 1961). The reason behind this has been attributed to the over-use of antibiotics, as was demonstrated in the late 1940's with successive passage of *S. aureus* in penicillin rendering it able to grow in 4mg/ml penicillin, and successive passage in streptomycin allowing it to grow in 4mg/ml streptomycin (Klimek et al., 1948). This demonstrated for the first time that antibiotic pressure could select resistance in the organism. The emergence of methicillin resistant *S. aureus* (MRSA)

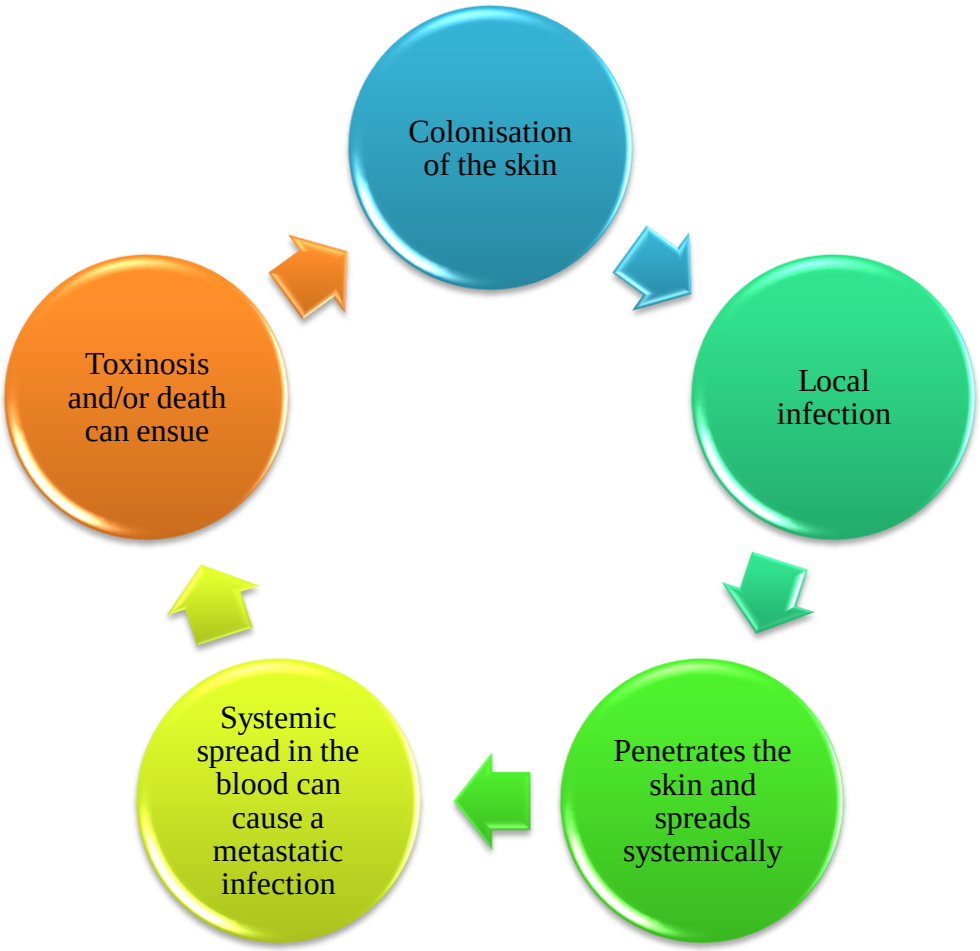

Figure 1.1: <u>The sequence of events in a typical *S. aureus* infection.</u>

Following colonisation, the infection can remain local, or if it manages to enter the blood, spread rapidly and cause severe destruction and in serious cases death.

and vancomycin resistant *S. aureus* (VRSA) has further complicated medical management increasing medical attention on this organism. MRSA has been classified as hospital acquired MRSA (HA-MRSA) or community acquired MRSA (CA-MRSA).

Associated with each of these are various strains, e.g. USA300 are typically found with CA-MRSA (Tenover et al., 2006) and USA100, and USA500 typically associated with healthcare outbreaks (McDougal et al., 2003). MRSA has become an increasing healthcare problem in the USA with CA-MRSA accounting for the majority of recorded skin infections (Moran et al., 2006). This is worrying because CA-MRSA can result in severe necrotising complications, including necrotising pneumonia with a high rate of mortality (Francis et al., 2005, Miller et al., 2005). There are various risk factors increasing an individual's chance of developing an MRSA infection including MRSA colonisation, a history of hospitalisation and/or surgery and living in a long term care home (Klevens et al., 2007).

Horizontal gene transfer appears to be responsible for the acquisition of antibiotic resistance within the staphylococcal family. For a methicillin sensitive *S. aureus* (MSSA) strain to be reclassified as an MRSA requires expression of the methicillin encoded cassette A (*mecA*) gene carried on the staphylococcal cassette chromosome mec element (SCCmec) which encodes the penicillin binding protein 2a (PBP2a) (Katayama et al., 2000, Chambers, 1997). This element is 52kB in size; it is known to carry the recombinase genes cassette chromosome recombinase gene A (*ccrA*) and cassette chromosome recombinase gene B (*ccrB*) which allow the SCCmec element to insert itself into the *S. aureus* chromosome (Katayama et al., 2000). PBP2a binds weakly to penicillin which contributes to the resistance (Utsui and Yokota, 1985). This means MRSA strains can keep synthesising peptidoglycan (PGN) even when penicillin is present (Hiramatsu, 2001).

Selection pressure has now led to the emergence of variant MRSA strains encoding variants of the *mecA* gene. An example of this can be seen in Garcia-Alvarez *et al.*, 2011. This study reported that the identified strain found in the United Kingdom (UK) and Denmark carried a novel *mecA* gene termed methicillin encoded cassette C (*mecC*) located chromosomally in the same location as found in other MRSA strains carrying *mecA* (Garcia-Alvarez et al., 2011). The SCCmec element was also novel but the variant encoded a penicillin binding protein (PBP) that failed to bind penicillin effectively, similar to other MRSA strains (Garcia-Alvarez et al., 2011).

In the United States of America (USA) and Japan, there have been reports of MRSA clones that have intermediate resistance to vancomycin (Hiramatsu et al., 1997b, Hiramatsu et al., 1997a, Tenover et al., 1998). This stimulated research to try and understand how vancomycin resistance might occur. For VRSA strain Mu50 it was found cell wall thickness determined its resistance profile (Cui et al., 2000). It was reported that allowing strain Mu50 to grow in media with an abundant level of factors required for cell wall synthesis, better supported resistance (Cui et al., 2000). For both strains Mu3 and Mu50, earlier research identified they overproduced penicillin binding protein 2 (PBP2) which supported resistance (Hanaki et al., 1998).

1.1.4. Genome sequence

S. aureus genomes have been estimated to be roughly 2.8Mbp and they contain a low percentage of guanine (G) and cytosine (C) nucleotides (Lowy, 1998, Baba et al., 2008). This section will focus on the sequence of *S.aureus* Newman because this is the strain principally used throughout this thesis.

The genome of Newman is 2, 878, 897bp and encodes for 2614 open reading frames (ORFs). The Newman strain contains a number of genes that have been identified as vital for pathogenesis (Baba et al., 2008). There are four prophages found in Newman designated ϕNM1-ϕNM4 which appear to be important for the strain's pathogenesis (Baba et al., 2008). Strains lacking either or all 4 of the prophages displayed reduced ability to form organ specific abscesses in a mouse model (Baba et al., 2008). These 4 prophages are unique to Newman; in addition, unlike some other staphylococcal strains, Newman has no major transposons and no SCCmec element (Baba et al., 2008).

S. aureus Newman contains 2 major pathogenicity islands designated vSaα and vSaβ, encoding the major virulence determinants and 2 minor pathogenicity islands designated vSaγ encoding exfoliative toxins and exotoxins and vSa4 which has an integrase and 3 proteins of unknown function (Baba et al., 2008). Newman expresses a range of major surface proteins. Its fibronectin binding protein A (FnBPA) and fibronectin binding protein B (FnBPB) are truncated at the C terminus meaning they lack sorting signals and are essentially non-functional (Grundmeier et al., 2004).

The genome lacks several major superantigen genes found in other strains and only encodes enterotoxin A (Baba et al., 2008).

1.1.5. *Staphylococcus aureus* virulence factors

S. aureus has been classed as a 'clever' pathogen because of the range of virulence factors (VFs) it produces. However, it is how the pathogen controls the production of its various VFs that makes it clever, as it only produces them when the population density is critical (Figure 1.2). As with most pathogens, *S. aureus* adheres to a range of tissues, and this is an essential first step in the pathogenesis of disease (Finlay and Cossart, 1997). Following adherence, the pathogen can be internalised by the cell it has adhered to (Finlay and Cossart, 1997). VFs produced by *S. aureus* include a range of surface proteins; structures present inside the bacterium and only released following structural damage to the bacteria and secreted proteins.

1.1.5.1. Sortase A

Sortase A (SrtA) aids the establishment of staphylococcal infections (Weiss et al., 2004). Sortases are localised in the bacterial membrane (Melvin et al., 2011). SrtA is important for anchoring certain bacterial surface proteins to the cell wall, the so called microbial surface components recognising adhesive matrix molecules (MSCRAMMs) (Mazmanian et al., 1999, Patti et al., 1994, Foster and Hook, 1998). SrtA cleaves target proteins with an LPXTG motif near the C terminus (Navarre and Schneewind, 1994),by cleaving between the threonine and glycine residue, liberating the carboxyl group of threonine to form an amide bond with the amine group of the pentaglycine in the cell wall linking the C terminus to the bacterial cell wall and hence the protein is attached to the bacterial surface (Navarre and Schneewind, 1994, Schneewind et al., 1995). It has been shown that SrtA is important early in staphylococcal infections (Weiss et al., 2004). Deleting the *srtA* gene severely affects the ability of *S. aureus* to infect and adhere to cardiac tissue presumably because the mutant fails to properly anchor its surface proteins that are required for attachment (Weiss et al., 2004). It was shown the mutant bacteria failed to form a complex with fibrinogen, most likely because clumping factor (Clf), the required factor for binding to fibrinogen was not properly anchored to the bacterial cell surface (Weiss et al., 2004). It has recently been shown that the srtA protein is resistant to inhibition by reactive oxygen species (ROS) (Melvin et al., 2011). However, earlier research challenges this because in a murine model of septic arthritis it was shown that there was no difference in the killing or phagocytosis of a wild-type (WT) or *srtA* mutant strain by macrophages (Jonsson et al., 2002).

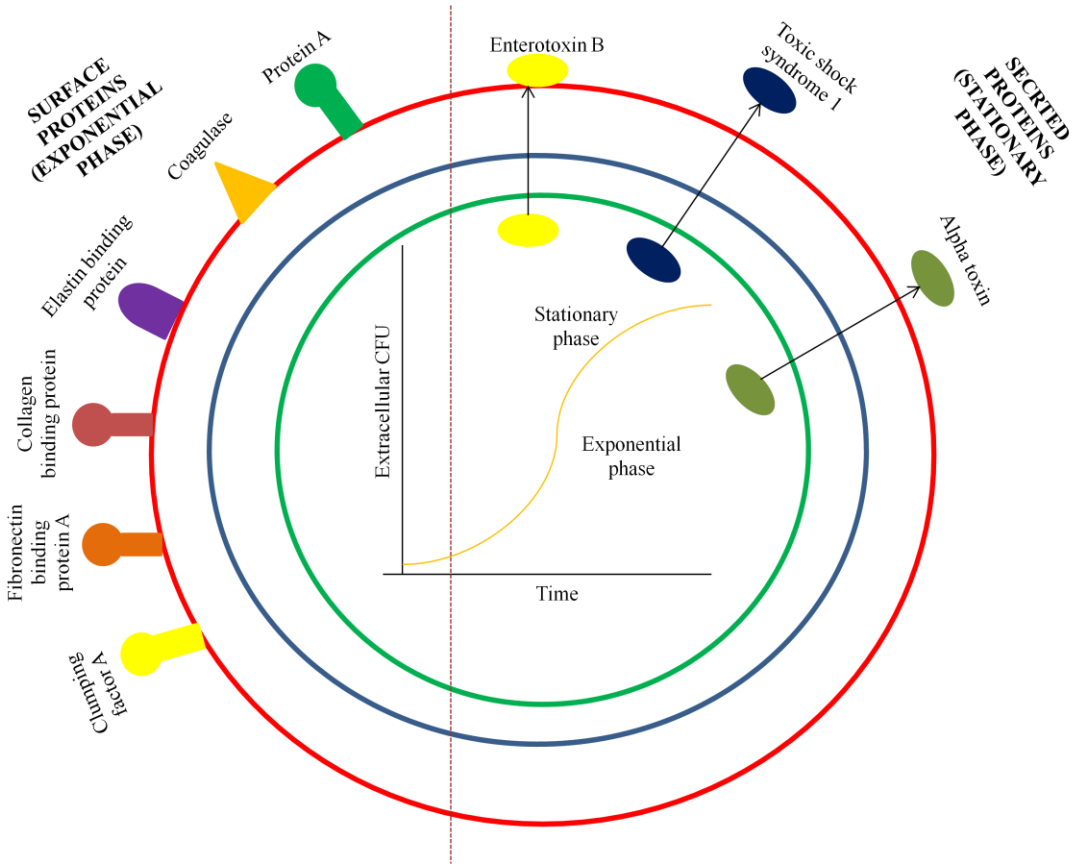

Figure 1.2: <u>The structure of *S. aureus*.</u>

The left hand side show surface proteins that are produced during the exponential phase of growth shown on the graph. The right hand side shows some secreted proteins produced during stationary phase as shown on the graph.

1.1.5.2. Fibronectin binding proteins

S. aureus fibronectin binding proteins (FnBPs) exist in two forms, FnBPA and FnBPB and both bind to fibronectin (Flock et al., 1987).When either FnBPA or FnBPB binds to fibronectin the staphylococci adhere better to host tissues and colonisation is enhanced (Vercellotti et al., 1984).

FnBPA and FnBPB appear to be necessary for initial infection. Peacock *et al.,* designed a study to analyse the genetic expression of FnBPA and FnBPB by various *S.aureus* strains (Peacock et al., 2000). Interestingly, no strains were discovered that contained 0 FnBPs, but, 25% were found to contain 1 and the remaining 75% contained both genes (Peacock et al., 2000). Expression of either was sufficient to mediate bacterial adherence to fibronectin and this did not differ between strains harbouring either 1 or 2 of the genes (Peacock et al., 2000).

Shinji *et al.,* has demonstrated that both FnBPs are required for the establishment of inflammatory responses required for optimal protective responses against bacteria; a mutation in either of the genes reduced phagocytosis of *S. aureus* by inflammatory macrophages, resulting in less release of factors from ingested bacteria to stimulate macrophage dependent production of inflammatory signals, which would in turn result in less activation of macrophage phagocytosis (Shinji et al., 2011).

1.1.5.3. Protein A

Protein A (Spa), is a cell bound protein and MSCRAMM that binds to immunoglobulin G (IgG) (Foster, 2005). Spa can bind the fragment crystallisable (Fc) portion of IgG preventing Fc receptor mediated phagocytosis because Spa deficient mutants are phagocytosed better by neutrophils and show decreased virulence in mice models of infection (Patel et al., 1987, Gemmell, 1991, Palmqvist et al., 2002). Once Spa has bound to IgG, the *S. aureus* cell surface is covered in IgG molecules in the wrong position to be recognised by the Fc receptor (Foster, 2005). The remarkable nature of this binding by Spa is evidenced by the number of Fc binding domains found in a Spa molecule. It has been found that 1 Spa molecule can contain between 2-5 Fc binding domains, allowing the protein to achieve a maximum inhibition of phagocytosis by this mechanism (Uhlen et al., 1984, Moks et al., 1986).

1.1.5.4. Peptidoglycan

Gram-positive and Gram-negative bacteria synthesise peptidoglycan (PGN) as part of their cell wall, and the hallmark of Gram positive bacteria is the overall content of the cell wall PGN. For example, the *S. aureus* cell wall has been found to be roughly 50% PGN by weight (Lowy, 1998).

PGN is important not only for maintaining the structure of the bacterium but because it mediates many of *S. aureus'* clinical manifestations when acting in association with other components of the bacterium. This can be seen in *S. aureus* induced septic shock (De Kimpe et al., 1995). It was shown that incubating macrophages with a component of PGN known as peptidoglycan G (PepG) had little effect on nitrite production, inducible nitric oxide synthase (iNOS) induction and very modest effects on cytokine production e.g. tumour necrosis factor alpha (TNF-α) production increased only slightly (De Kimpe et al., 1995). This led to the hypothesis that there must be other co-stimulatory factors required to act in combination with PGN to produce the phenotype, for example, lipoteichoic acid (LTA), because while incubating the macrophages with LTA alone caused an increase in nitrite production concomitant with activation of iNOS the administration of both components together led to TNF-α production and interferon gamma (IFNγ) release and iNOS activation in the macrophages, showing bacterial cell wall components can synergise to produce a clinical phenotype (De Kimpe et al., 1995).

The PGN polymer is important for activating macrophages, and the macrophage receptor cluster of differentiation 14 (CD14) was suggested to bind both membrane bound and non-membrane bound PGN (Pugin et al., 1994, Gupta et al., 1996, Dziarski et al., 1998) and the glycan part of the PGN chain was suggested as essential for CD14 binding and the muramyl dipeptide (MDP) was implicated as the minimal structure needed to bind to CD14 (Dziarski et al., 1998). With the identification of Toll-like receptors (TLRs) it became apparent that it was toll-like receptor 2 (TLR2) that played an important role in the identification of *S. aureus* and although numerous publications suggested TLR2 recognised PGN it became apparent that reports implicating TLR2 as recognising PGN were probably only the result of TLR2 recognition of LTA or lipoproteins that contaminated PGN preparations (Volz et al., 2010). Instead it appears that the intracellular degradation of PGN into muramyl dipeptides results in recognition by nucleotide-binding oligomerisation domain-containing protein 2 (Nod-2).

1.1.5.5. Lipoteichoic acid

Macrophages and monocytes can be activated by LTA to produce pro-inflammatory cytokines such as TNF-α, interleukin 1β (IL-1β), interleukin 6 (IL-6) and interleukin 8 (IL-8) (Bhakdi et al., 1991, Mattsson et al., 1993, Standiford et al., 1994). LTA has been shown to induce ROS in a range of phagocytic cells (Levy et al., 1990) and to activate iNOS in murine macrophages (Cunha et al., 1993).

Kuo *et al.,* using the human acute monocytic leukaemia cell line, THP-1 showed iNOS expression and subsequent nitric oxide (NO) production was stimulated by LTA and was prevented if protein kinase C (PKC) activation was inhibited (Kuo et al., 2003). In addition they showed that nuclear factor kappa B (NF-κB) was important for the response because preventing its activation prevented iNOS activation and nitrite release (Kuo et al., 2003).

LTA activates TLR2 but LTA from different bacteria, despite comparable TLR2 activation, have differential effects on the proteome of the THP-1 macrophage cell line (Zeng et al., 2010). For example, *S. aureus* LTA but not that from *Lactobacillus palntarum* was found recently to increase manganese (Mn) dependent superoxide dismutase (SOD) (Zeng et al., 2010).

The lipid anchor of LTA is sufficient to mediate cytokine release but it does so at a level that is a lot lower than the entire LTA molecule (Morath et al., 2002). Therefore whilst parts of the LTA molecule have function that has been characterised, some need to be explored further.

1.1.5.6. Alpha toxin

S. aureus secretes toxins during the stationary phase of growth. The toxins alter cell functions and sometimes lead to cell death (Schmitz et al., 1997) by making the membrane permeable (Finlay and Cossart, 1997).

Alpha toxin (Hla) is a 34kDa pore-forming toxin (Bernheimer and Schwartz, 1963, Coulter, 1966). Hla has been shown to cause cell necrosis, especially at high concentrations, which is not inhibited by caspase inhibitors (Essmann et al., 2003). In one study it was demonstrated that at low concentrations Hla bound to cell membranes, inducing apoptosis; in contrast at high concentrations it was adsorbed to the lipid bilayer and caused cellular necrosis, by pore formation (Jonas et al., 1994, Bantel et al., 2001).

However, this finding was challenged when Essmann *et al.,* found that Hla caused necrosis at both low and high concentrations (Essmann et al., 2003).

Earlier research identified that Hla damaged the structure and integrity of rabbit macrophages because macrophages incubated with heat inactivated (HI) toxin or no toxin did not show any significant loss in cellular viability compared to macrophages incubated with a low concentration of Hla which induced a time and dose dependent loss in viability over 4-8 hours (McGee et al., 1983). A similar time and dose dependent loss in viability was recently shown for T cells, where Hla concentrations as low as 3ng/ml reduced T cell viability which increased up to doses ≥ 100ng/ml (Haslinger et al., 2003).

Hla has been shown to lead to IL1β processing in macrophages because it activates the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome which causes caspase 1 activation and pro-IL1β processing (Kebaier et al., 2012). Kebaier *et al.,* found that in NLRP3 deficient mice there was less neutrophil infiltration into the lungs, in response to HI *S. aureus* in combination with Hla administration, which lead to the conclusion that the neutrophilic lung inflammation phenotype seen with Hla is dependent on NLRP3 activation (Kebaier et al., 2012). As well as this they found WT mice administered with HI *S. aureus* resolved the infection but that those administered with just Hla showed a 40% mortality, and those given HI bacteria and Hla showed 80-100% mortality. However, mortality was diminished in NLRP3 deficient mice, suggesting that the activation of NLRP3 and the resultant inflammatory response to Hla was responsible for the lung damage (Kebaier et al., 2012). Thus Hla can compromise the host response through induction of both cell death and through harmful cytokine driven inflammatory responses.

1.1.5.7. Panton-Valentine Leukocidin

Panton Valentine Leukocidin (PVL) is a pore forming toxin (Panton PN, 1932) found in CA-MRSA strains, and has been suggested to account for the high virulence of some CA-MRSA strains. However, it is now thought that the ability of CA-MRSA to synthesis large quantities of PVL rather than the presence of the *pvl* gene may be responsible for the higher virulence of some strains (Hongo et al., 2009). However, PVL is also expressed in some MSSA strains (Varshney et al., 2010).

PVL is comprised of two independent subunits termed leukocidinS-PV (lukS-PV) and leukocidinF-PV (lukF-PV) (Genestier et al., 2005) and causes a range of clinical presentations including necrotising pneumonia (Labandeira-Rey et al., 2007). The lysis of immune cells induced by PVL doesn't require binding of the entire toxin, forming a pore composed of both subunits to cause lysis but instead requires sequential binding of the components with recruitment of the S component to the neutrophils first, which then allows recruitment of the F subunit (Colin et al., 1994).

Like Hla, it has been suggested that at low concentrations PVL causes cell apoptosis and high concentrations cell necrosis, as seen in PMN (Genestier et al., 2005). Treatment with PVL resulted in 60% of neutrophils demonstrating deoxyribonucleic acid (DNA) fragmentation which was caspase dependent because in the presence of a caspase inhibitor only 1% of neutrophils treated with PVL showed DNA fragmentation (Genestier et al., 2005). PVL was also shown to target mitochondria because after 5 minutes, it was detectable in mitochondrial fractions (Genestier et al., 2005).

Despite PVL's capacity to induce cell death, strains lacking PVL did not display any noticeable reduction in virulence and compared to WT strains they demonstrated significantly larger abscesses associated with higher mortality (Bubeck Wardenburg et al., 2008). As well as this, for 2 CA-MRSA strains, lukS-PV or lukF-PV deletion did not seem to affect lung bacterial growth (Bubeck Wardenburg et al., 2007). Based on this it is thought that as well as PVL having a destructive effect on the host, it might prime the immune system to recognise and respond to the bacterium (Bubeck Wardenburg et al., 2008). This was also shown because PVL expressing strains were seen to reduce TNF-α production in the lung (Yoong and Pier, 2012). The authors speculated PVL activates the immune system that on one level causes a protective host response but that this if excessive also predisposes to host-mediated tissue injury due to inflammation, and that by also inducing cellular death PVL also helps reduce an excessive inflammatory response (Yoong and Pier, 2012).

A selection of other important VFs is given in Table 1.1.

1.1.6. *S. aureus* regulation of virulence factors

The accessory gene regulator (Agr) is the best characterised global regulator of *S. aureus* VF gene expression, controlling the synthesis of exoproteins and down-regulating surface adhesin production (Recsei et al., 1986) (Figure 1.3). The Agr system

VF	Type of molecule	Role in pathogenesis	References
Sortase B (SrtB)	Iron binding protein	Iron acquisition; thought to have a role in staphylococcal persistence in tissues.	(Mazmanian et al., 2002)
Capsule	Polysaccharide	11 serotypes, capsule polysaccharide 5 (CP5) and capsule polysaccharide 8 (CP8) most common; antiphagocytic; nutrient availability affects capsule production and virulence of capsule producing strain	(Arbeit et al., 1984) (Sompolinsky et al., 1985) (Nilsson et al., 1997) (Thakker et al., 1998) (O'Riordan and Lee, 2004) (Foster, 2005)
Clumping factor (Clf)	Fibrinogen binding protein	Exists as clumping factor A (ClfA) and clumping factor B (ClfB); reduces macrophage phagocytosis.	(Hawiger et al., 1982) (Ni Eidhin et al., 1998) (Palmqvist et al., 2004)
Coagulase	Thrombin activating protein	Secreted protein; activates thrombin converting fibrinogen to fibrin; three types; acts in concert with prothrombin and both form staphylothrombin which causes plasma clotting.	(Rammelkamp et al., 1950) (Hemker et al., 1975) (Kawabata et al., 1985) (Lowy, 1998) (Chavakis et al., 2005)
Extracellular adherence protein (Eap)	Secretable Expanded Repertoire Adhesive Molecules (SERAM)	Prevents leukocyte migration; binds to a range of plasma and matrix proteins like fibronectin; interacts with intracellular adhesion molecule 1 (ICAM-1) and diminishes acute inflammatory response because interferes with leukocyte-endothelial cell interactions dependent on ICAM-1	(Chavakis et al., 2002) (Foster, 2005)
Beta toxin (Hlb)	Toxin	Triggers immune cell death e.g. lyses monocytes; lyses erythrocytes to liberate iron in iron limiting environments; *Hlb* deficient mutants demonstrate decreased adherence; degrades keratinocytes.	(Walev et al., 1996) (Cifrian et al., 1996) (Huseby et al., 2007) (Katayama et al., 2013)

Gamma toxin (Hlg)	Toxin	2 component leukocidin; lyses leukocytes and erythrocytes; targets neutrophils.	(Foster, 2005) (Malachowa et al., 2011)
Delta toxin (Hld)	Toxin	Synthesised by RNAIII; inserts and disrupts cell membranes; binds to neutrophils, monocytes and erythrocytes; causes *S. aureus* phagosomal escape which is maximal when expressed with Hlb.	(Bhakoo et al., 1982) (Janzon et al., 1989) (Schmitz et al., 1997) (Giese et al., 2011)
Exotoxins	Toxins	Produced during stationary phase and released into surrounding fluid; exfoliative toxins (ETs) can disrupt skin e.g. exfoliative toxin A (ETA) and exfoliative toxin B (ETB) target desmoglein-1, a cadherin helping join cells together; can activate macrophages causing massive nitrite production and TNF-α release; can function as superantigens.	(White et al., 1989) (Fleming et al., 1991) (Kawabe and Ochi, 1991) (Amagai et al., 2000) (Amagai et al., 2002)
Leukocidin AB (LukAB)	Toxin	Targets phagocytes; interacts with integrin beta-2 (CD18) and cluster of differentiation molecule 11b (CD11b) and integrin αM/β2 (Mac-2)	(Dumont et al., 2011) (Dumont et al., 2013)
Leukocidin ED (LukED)	Toxin	Targets neutrophils; produced *in vivo* with similar effects to PVL; binds C-C chemokine receptor type 5 (CCR5) on macrophages, T cells and dendritic cells potentially modulating recruitment and activation.	(Gravet et al., 1998) (Alonzo et al., 2012) (Alonzo et al., 2013)

Table 1.1: List of several virulence factors produced by *S. aureus.*

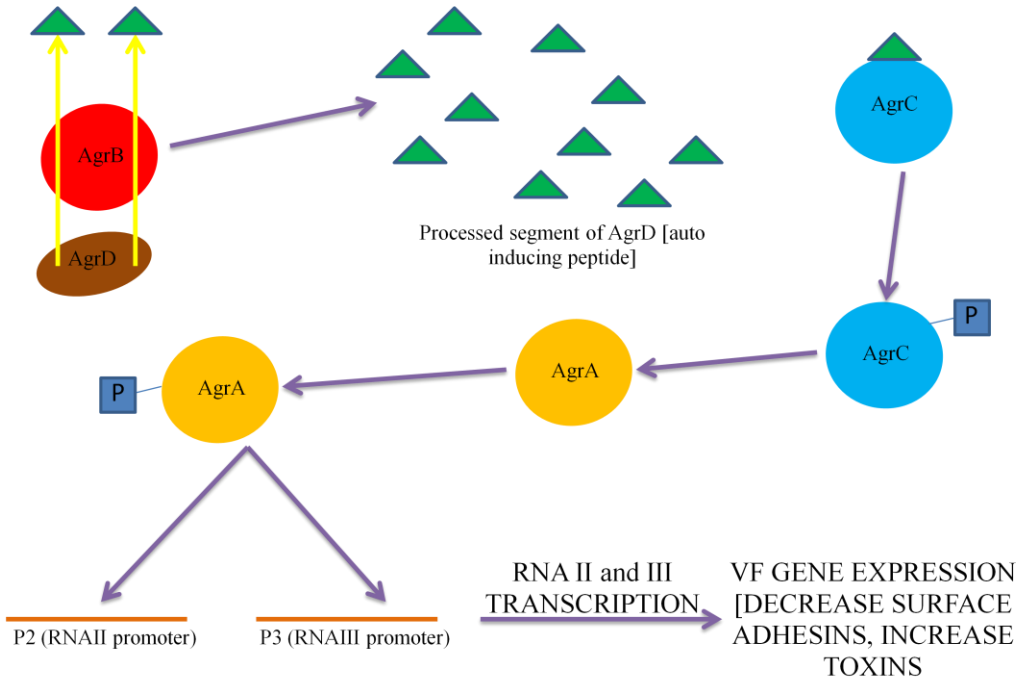

Figure 1.3: <u>A summary of the Agr system in *S. aureus* controlling virulence factor expression.</u>

AgrD is exported via AgrB and acts as the autoinducing peptide (AIP) released into the extracellular milieu that acts as a quorum sensing signal. The autoinducing peptide binds to the histidine sensor kinase AgrC leading to its phosphorylation. AgrC then phosphorylates AgrA which binds to promoter regions in the Agr system (P2 for RNAII and P3 for RNAIII) and leads to transcription of RNAII and RNAIII encoded genes which then control virulence factor gene expression and increases secreted protein production at critical bacterial densities. RNAII encodes the two component signal transduction system comprising AgrC and AgrA the AgrD precursor of the AIP signal and its maturation/export protein AgrB. RNAIII controls target gene expression.

contains 2 different operons with 2 different promoters classified as promoter 2 (P2) and promoter 3 (P3) (Novick, 2003). Ribonucleic acid III (RNAIII) regulates protein expression at both the transcriptional and translational level (Novick et al., 1993). The small ribonucleic acid (RNA) encoded by RNAIII regulates gene transcription either by direct base pairing with target messenger ribonucleic acid (mRNA) or by base pairing to a negative regulator of gene transcription rot, a member of the staphylococcal accessory regulator A (SarA) family of transcription factors (McNamara et al., 2000, Said-Salim et al., 2003, Boisset et al., 2007). As well as RNAIII, the Agr system contains a quorum sensing system (Novick, 2003). Ribonucleic acid II (RNAII) controls the synthesis of accessory gene regulator A-D (AgrA-AgrD); when a quorum sensing signal, or the autoinducing peptide (AIP) is received by accessory gene regulator C (AgrC). AgrC is the sensor histidine kinase, it then activates the response regulator accessory gene regulator A (AgrA) which binds to the P2 and P3 promoter regions (Novick et al., 1993, Novick et al., 1995, Novick, 2003). In this system the AgrD is the precursor protein for the extracellular auto-inducing peptide that functions as the quorum sensing signal and accessory gene regulator B (AgrB) functions as its export protein.

One model states that the AgrD octapeptide concentration is important, with low levels causing the expression of cell wall associated proteins and higher levels being responsible for the shift to exoprotein production (Projan, 1997). So, when *S. aureus* enters the host, and the population density is low, bacteria express cell wall associated proteins as the initial octapeptide concentration is low, allowing the pathogen to escape host defence and bind to host tissues (Projan, 1997). Then bacteria multiply and form an abscess protecting them further from host defences. As the bacterial population density increases, so does the concentration of the octapeptide, which then activates the Agr system to shift to exoprotein production, allowing the bacterium to escape the abscess with systemic spread and development of metastatic foci of infection where there can be further bacterial growth (Projan, 1997). Wesson *et al.*, suggested a modification to this model based on their observations that Agr deletion mutant strains accumulated to greater levels within host cells rather than the extracellular environment and also induced less apoptosis, which could be because of less toxin production or greater cell surface protein production leading to greater adherence and so internalisation (Wesson et al., 1998). Their model states that when *S. aureus* enters a new host they express surface proteins not because AgrD is produced less but because the octapeptide is diluted and therefore present at a lower concentration, leading to binding and

internalisation of bacteria. Once internalised, bacteria are within a membrane bound organelle causing the AgrD octapeptide to increase in concentration, activating Agr and exoprotein synthesis allowing the bacteria to escape confinement when exotoxins cause phagosomal lysis (Wesson et al., 1998).

Once in the cytoplasm, the AgrD octapeptide is again diluted, meaning the bacteria are now better adapted to cytoplasmic survival (Wesson et al., 1998). Once within the cytoplasm, the bacteria are postulated to be able to do one of three things. Either, they produce a peptide causing apoptosis, encasing the bacteria in an apoptotic body which is engulfed by macrophages, but protects the bacteria from any host response (Wesson et al., 1998). Secondly, the bacteria could become a small colony variant (SCV) and cause a more chronic and persistent disease (Wesson et al., 1998). Finally, if the octapeptide concentration becomes high, then exoprotein production would lead to cell lysis and allow the bacteria to spread throughout the body (Wesson et al., 1998).

The most studied member of the staphylococcal accessory regulator (Sar) family is the SarA transcription factor. SarA controls the expression of a range of proteins including Hla, Hlb, Hld, fibronectin and fibrinogen binding proteins (Cheung et al., 2004). SarA expression is found to be maximal towards the end of exponential phase, which interestingly coincides with the activation of Agr, leading to the prediction there may be a specific SarA-Agr interaction especially as both of these are responsible for controlling the transition from exponential to post-exponential growth (Rechtin et al., 1999). SarA is a DNA binding regulatory protein responsible for activating the Agr operon (Rechtin et al., 1999). The SarA protein was established to be important in VF regulation because a mutant lacking SarA demonstrated reduced RNAIII expression showing SarA to be required for WT RNAIII levels (Dunman et al., 2001).

Staphylococcal accessory regulator S (SarS) has been demonstrated to control Spa expression (Cheung et al., 2001). It was also shown that reconstituting SarS to the mutant restored Spa expression (Cheung et al., 2001). It is now also known that the Agr RNAIII regulates Spa expression by repressing SarS expression (Cheung et al., 2001). Staphylococcal accessory regulator T (SarT) has been found to be important in down-regulating the expression of Hla (Schmidt et al., 2001). SarT has also been found to interact with the Agr system (Schmidt et al., 2001). It is thought that when SarA is present and functional, SarT is not expressed increasing Hla transcription. A decrease in

SarA increases expression of SarT and this subsequently leads to a decrease in Hla transcription (Schmidt et al., 2001).

1.2. The innate immune system

1.2.1. The epithelial barrier

The epithelial barrier is the first line of defence for the human body shown in figure 1.4. The airway epithelium within the lungs is an example of this protecting against respiratory pathogens in accordance with other measures such as mucus (Vareille et al., 2011). The mucus component is important because it not only allows the efficient exchange of nutrients, water and gas but is rather good at trapping approximately 90% of inhaled pathogens that enter the lung (Vareille et al., 2011). Airway epithelial cells are also important because they sense the inhaled pathogens. As mentioned above, this occurs through receptor expression including pattern recognition receptors (PRRs) like TLRs (Vareille et al., 2011).

1.2.2. Neutrophils

Neutrophils recognise, phagocytose and kill a range of pathogens through the production of ROS and digestive enzymes (Hampton et al., 1996). Neutrophils begin their life in the bone marrow, equipped with granules containing various enzymes which cannot be replenished and hence neutrophils are classed as end cells ready to intervene for short periods with the capacity to respond quickly, following activation in response to stimuli they encounter after migration into the tissues (Baggiolini, 1984, Borregaard and Cowland, 1997).

Neutrophil granules are subdivided into 3 categories known as the azurophilic granules, secondary and tertiary granules (Borregaard and Cowland, 1997). Azurophilic or primary granules are classified based upon their myeloperoxidase (MPO) content, and can be split even further into “defensin-rich” and “defensin-poor” (Borregaard and Cowland, 1997). Azurophilic granules also contain the serine proteases, neutrophil elastase and cathepsin G. Secondary (specific) and tertiary granules are split depending on the concentration of lactoferrin (high in secondary granules) and gelatinase (high in tertiary granules) within them (Borregaard and Cowland, 1997). As well as granules the neutrophil also comes equipped with an nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the plasma membrane capable of generation reactive oxygen products such as superoxide (Baggiolini, 1984). The superoxide can be broken down

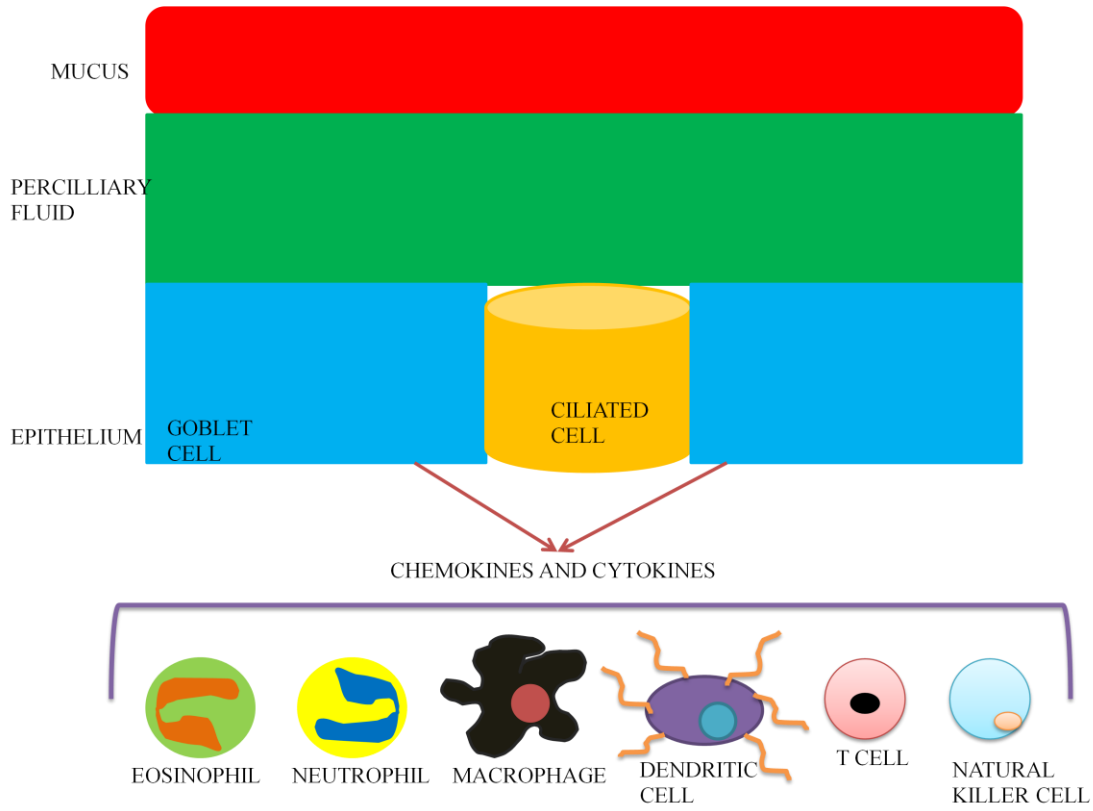

Figure 1.4: <u>An overview of the airway epithelium.</u>

The epithelium is made of a range of layers which in themselves form a protective barrier. In the epithelium there are a range of cells such as goblet cells and ciliated cells. These cells secrete a range of cytokines and chemokines which lead to the recruitment of a range of immune cells including macrophages.

into hydrogen peroxide by spontaneous dismutation and hydroxyl radicals which kill bacteria and other invading microorganisms (Baggiolini, 1984, Hampton et al., 1998). The hydrogen peroxide can also react with chloride ions in an enzymatic reaction mediated by MPO and form hyperchlorous acid (HOCl) which is extremely bactericidal and can inactivate a range of proteins (Hampton et al., 1998).

Neutrophils are very important in the innate immune response to *S. aureus* and patients with defects in neutrophil numbers or in neutrophil function are at risk of *S. aureus* infection (Lekstrom-Himes and Gallin, 2000). Neutrophils have been shown to be essential in controlling pulmonary infections caused by the bacterium (Kohler et al., 2011). Using a mouse pneumonia model, it was suggested that a rapid influx of neutrophils into the lungs was responsible for protection during staphylococcal pneumonia (Kohler et al., 2011). The neutrophils use their oxidative mechanisms to destroy *S. aureus* and this is largely dependent on the MPO system since it was shown to be faster with an active MPO (Hampton et al., 1996). It must be noted however, that certain *S. aureus* strains prime neutrophil oxidative responses upon phagocytosis not seen with other staphylococcal species (Nilsdotter-Augustinsson et al., 2004). The relative importance of ROS in killing bacteria directly has been challenged; Reeves and colleagues proposed a model in which the neutrophil serine proteases mediated the microbial killing (Reeves et al., 2002). In this model the generation of ROS results in accumulation of anionic charge in the endocytic vacuole which necessitates an influx of potassium ions. The accumulation of ionic strength results in the release of the cationic granule proteases (neutrophil elastase and cathepsin G) and it is these that kill *S. aureus*. Although there is still debate as to whether this model completely explains the basis of bacterial killing when ROS are generated it has challenged the view that ROS directly mediate bacterial killing in neutrophils.

1.2.3. Eosinophils

Eosinophils are similar to neutrophils in that they are formed in the bone marrow and also have a range of granules containing various enzymes (Baggiolini, 1984). In a similar fashion to neutrophils, they are classified as end cells and they can migrate to infection sites in response to activation signals and are designed to respond rapidly (Baggiolini, 1984). Eosinophil granule stores also cannot be replenished, contributing to their end cell nature (Baggiolini, 1984). Eosinophils are not viewed as having a major role in antibacterial host defence but contribute to allergic diseases and *S. aureus*

exacerbates allergic disease, possibly via induction of eosinophil necrosis and release of eosinophilic granules in a process mediated by Hla (Prince et al., 2012).

1.2.4. Monocytes

Unlike neutrophils or eosinophils, monocytes have the potential to become longer lived cells but only a minority will differentiate into long lived tissue cells. They are synthesised from a precursor in the bone marrow and are released into the circulation where they persist for about 20 hours before in some cases migrating into peripheral tissues (van Furth and Cohn, 1968). At some stage in the monocyte's life cycle, usually quite early on, intracellular granules become discharged and the monocyte now functions as a macrophage (Baggiolini, 1984). In addition, some monocyte populations may take a different differentiation path and divide to form dendritic cells (DCs) (Serbina et al., 2008). In a similar fashion to other phagocytic cells, monocytes bind to, phagocytose and kill invading microorganisms. This killing is mediated through the production of reactive nitrogen intermediates (RNIs), ROS and enzymes present within the phagolysosome (Amer and Swanson, 2002, Fang, 2004).

1.2.5. Natural killer cells

Natural killer (NK) cells represent a small population of lymphocytes, in the range of about 5% (Herberman, 1986). The NK cell recognises and kills its targets in a different fashion to some other cells previously described, in that phagocytosis is not utilised. The NK cell recognises its target and induces a programme of lysis. The NK cell recognises a structure on its target and binds to it, which activates intracellular signalling within the NK cell (Herberman, 1986). This rearranges the NK cell granules and some other cytoplasmic organelles migrate towards where the NK cell has bound the target cell and the granules are released (Herberman, 1986). NK cells cytolytic granules contain the pore forming protein perforin and cytolytic proteases such as granzymes with which they induce target cell lysis (Bots and Medema, 2006).

1.2.6. Macrophages

1.2.6.1. Overview

Macrophages arise from monocytes and are long lived cells. They are relatively resistant to various apoptotic stimuli (Liu et al., 2001). Macrophages are found within a range of host tissues in low numbers, but a range of signals can lead to an increase in

macrophage number (Randolph, 2011). Macrophages are very important in immune responses against a range of infections and have been demonstrated to be the first line of defence against a range of microorganisms, being the resident phagocytes found in tissue (Green and Kass, 1964, Goldstein et al., 1974). Macrophages have various roles within the host including phagocytosis and killing of pathogens as well as coordinating the inflammatory response through the release of mediators and through the clearance of apoptotic cells (Geske et al., 2002). They also link with adaptive immune responses presenting peptides to T-helper cells (Oh and Swanson, 1996, Shinji et al., 1998). They detect and respond to infectious stimuli and if required elaborate the signals leading to the recruitment of other cells such as neutrophils (Nguyen et al., 2012). This can be demonstrated by studying the alveolar macrophage (AM) which is the first line of defence by which the lung defends itself against *S. aureus* (Goldstein et al., 1974) for example by maintaining the airspace as a sterile environment (Jonsson et al., 1985).

1.2.6.2. The mononuclear phagocyte system

The mononuclear phagocyte system (MPS) is defined as the population of cells that come from a universal progenitor in the bone marrow, differentiate into monocytes via monoblasts and promonocytes, enter the blood and finally enter resident tissues becoming primarily tissue macrophages and dendritic cells (van Furth et al., 1972). Cells within the MPS were often considered to be similar based on functional and biochemical characteristics (Hume et al., 2002). However, the relationships between the various populations always remained difficult to determine until monoclonal antibodies became available.

The F4/80 antigen was found to be expressed on all mature cells within the MPS and can be used as a marker of macrophage differentiation and to identify a subset of macrophage precursors (Hirsch et al., 1981). There is a variation in F4/80 expression on monocytes differentiating into macrophages which could be related to either differences in development or the activation state (Hirsch et al., 1981). Not all macrophages express F4/80 and it is only those that derive from the later stages of development linked to liver haematopoiesis and beyond that give rise to the F4/80 positive subset. As well as this, F4/80 antigen is unique to mouse macrophages and not expressed by human macrophages.

AM express lower levels of the F4/80 antigen compared to other cells of the MPS lineage (Hume et al., 2002, Gordon et al., 1986). However, creation of a transgenic mouse that expressed an enhanced green fluorescent protein (EGFP) driven by the colony stimulating factor 1 receptor (CSF-1R) (the macrophage colony stimulating factor (M-CSF) receptor) promoter, showed the product was expressed in a similar fashion to F4/80 though the lung macrophages had much lower levels of F4/80 staining this could have been in part due to difficulty in detection due to the high autofluorescence of lung macrophages (Sasmono et al., 2003). As well as this, EGFP was found on pro-monocytes, circulating monocytes and lung macrophages (Sasmono et al., 2003).

1.2.6.3. Differentiation within the mononuclear phagocyte system

Cells within the MPS originate from a universal myeloid progenitor. A universal stem cell gives rise to the pluripotent myeloid precursor, the granulocyte, erythrocyte, monocyte, megakaryocyte-colony forming unit (GEMM-CFU) which can then form the granulocyte/monocyte precursor, the granulocyte, macrophage-colony forming unit (GM-CFU). This then goes on to form the monocyte precursor, the macrophage colony forming unit (M-CFU) which gives rise to the monoblast. A monoblast then forms a promonocyte and the promonocyte eventually becomes a monocyte (Valledor et al., 1998). Monocytes begin their life cycle within the bone marrow, before moving into the blood to differentiate some more and then finally migrating into tissues to become resident macrophages and sometimes DCs (Valledor et al., 1998, Tacke and Randolph, 2006).

Differentiation of the progenitor cells into specialised types is a committed process with a range of stages that can eventually lead to the formation of the macrophage dendritic cell progenitor for example. This differentiation is a very controlled process and is carried out by a range of transcription factors. One of these transcription factors is PU.1 which controls expression of the M-CSF receptor (Zhang et al., 1994, Anderson et al., 1998, Valledor et al., 1998, Mosser, 2003). This receptor is limited to the monocyte/macrophage lineage (Sherr, 1990). As well as this PU.1 has been shown to control expression of genes encoding fragment crystallisable gamma receptor II (FcγRII) and fragment crystallisable gamma receptor IIA (FcγRIIA) and CD14 as well as scavenger receptors (Valledor et al., 1998). PU.1 null mice were found to have blockages in monocyte/macrophage development including reduced monocyte numbers

(Anderson et al., 1998). As well as this it is known monocytes express PU.1 at high levels indicating it is important for differentiation in the monocyte/macrophage lineage (Chen et al., 1995). PU.1 is also known to be important for neutrophil differentiation because there was PU.1 mRNA detectable in neutrophil extracts (Chen et al., 1995).

PU.1 is a myeloid B lymphocyte specific transcription factor produced by macrophages in development at the stage of hepatic haematopoiesis. In the absence of PU.1 F4/80 macrophages are dramatically reduced in number but phagocytes expressing colony stimulating factor gene (*c-fms*) RNA, which encodes for the M-CSF receptor, produced in the yolk sac at an earlier stage of development are still capable of producing F4/80 negative tissue macrophages (Lichanska et al., 1999).

Therefore, it appears PU.1 is essential at later stages of development more than at earlier stages. This was also shown by studies looking at the lack of PU.1 in the developing mouse embryo. What was found was the lack of PU.1 did not affect early gene expression such as MPO but did have an effect on later gene expression (Olson et al., 1995). Despite this, it is also known that although PU.1 is critical in the commitment to differentiation, it can occur in the absence of PU.1 (Henkel et al., 1999), as also evidenced by Lichanska *et al.*,(Lichanska et al., 1999). PU.1 also plays important roles after the development stage of hepatic haematopoiesis and is clearly essential for the development of monocytes into macrophages.

The control of differentiation is also under the control of other growth factors e.g. by interleukin-3 (IL-3) or IL-1β (Lopez et al., 1992). Stimulation of the universal progenitor with these interleukins can lead to the development of GEMM-CFU (Valledor et al., 1998). Stimulation of GEMM-CFU with these interleukins forces the progenitor to develop into the GM-CFU which is the progenitor of both macrophages and granulocytes (Valledor et al., 1998). Stimulation of this with M-CSF commits the monocytic precursor to become a monoblast and promonocyte before finally becoming a monocyte which will then leave the blood and enter tissues, becoming a macrophage (Valledor et al., 1998). In humans two main populations of monocytes exist (Strauss-Ayali et al., 2007). The $CD14^{++}$ $CD16^{-}$ monocyte makes up about 90% of monocytes and is called the ‘classical’ monocyte. In humans these play important roles in inflammatory responses and are important sources of IL-1β and T helper 17 (Th-17) responses (Smeekens et al., 2011). A separate population constitutes 5-10% of monocytes and is $CD14^{+}$ $CD16^{+}$ and is referred to as the ‘non classical’ monocyte, these

have important roles patrolling the blood vessels and in maintenance of tissue repair but may also be expanded during infection and may give rise to greater pro-inflammatory cytokine expression (Ziegler-Heitbrock, 2007). More recently an 'intermediate' monocyte population has been described which is $CD14^{++}$ $CD16^{+}$ which has a gene expression profile and phenotype that is for most characteristics at a level somewhere between the levels of the other two monocyte populations (Wong et al., 2011). In mice the populations are more evenly divided both giving rise to about 50% of monocytes. The cell ortholog of the classical monocyte is the C-C chemokine receptor type 2 ($CCR2^{+}$) monocyte which is often called an 'inflammatory' monocyte in mice as it is more rapidly recruited to inflamed tissues while the ortholog of the non classical monocyte analogue is the CX3C chemokine receptor 1 (CX_3CR1^{+}) monocyte. Although morphological appearance and receptor characteristics allow this comparison the functions of each group seem to differ between mice and humans (Serbina et al., 2008). When the monocyte differentiates into the macrophage it increases its hydrolytic enzyme content, lysosomal content, mitochondrial number and mechanisms of energy metabolism (Valledor et al., 1998). Macrophage differentiation and function is dependent upon the tissue it resides in, which imprints its programme of development (Valledor et al., 1998).

There are other transcription factors controlling differentiation that are classified as either essential for differentiation or as repressing or activating gene expression controlling differentiation at different stages (Valledor et al., 1998). The former group includes PU.1 and the latter group c-Myc. As examples c-Myc was shown to effect macrophage differentiation in 2 ways. Firstly it induced immature myeloid cell proliferation and had a negative effect on genes involved in the later stages of differentiation (Valledor et al., 1998). Nuclear Factor Y (NF-Y) was shown to induce ferritin synthesis and control major histocompatibility complex II (MHCII) expression forcing the progenitor towards a macrophage phenotype (Borras et al., 1995, Marziali et al., 1997). It is also important to note that repression of certain transcription factors is important for establishing the macrophage lineage (Valledor et al., 1998). Figure 1.5 summarises some of the major differentiation steps.

1.2.6.4. Macrophage classification

Macrophages have been divided into classically activated macrophages (CAM or M1) or alternatively activated macrophages (AAM or M2). It is thought that the macrophage

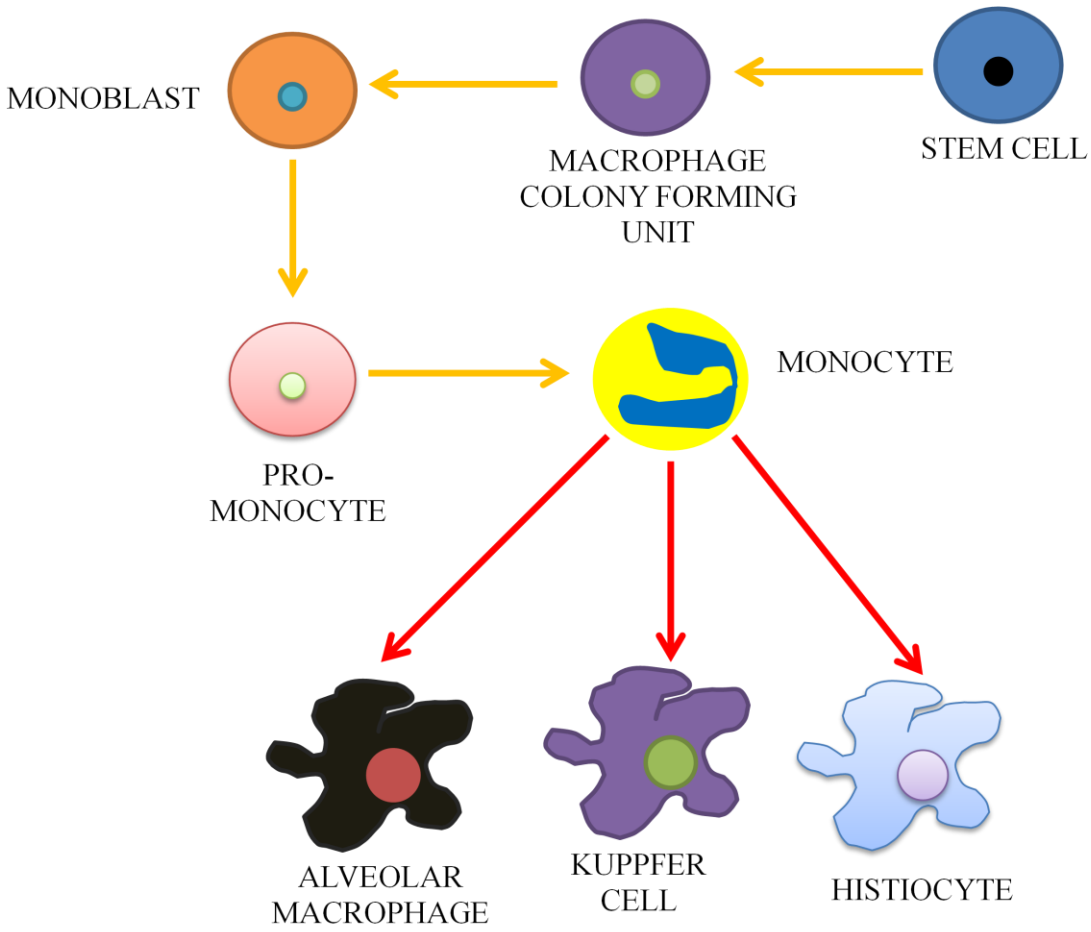

Figure 1.5: <u>A summary of macrophage development from a universal progenitor cell.</u>

Stimulation of a hematopoietic stem cell with a range of cytokines and chemokines results in the formation of the macrophage colony forming unit. This then matures via a range of intermediate steps to eventually form a monoblast. This then gives rise to a pro monocyte which then forms a monocyte. The monocyte moves into the blood forming a peripheral blood monocyte which then migrates into the tissues to form tissue macrophages e.g. alveolar macrophage in the lungs, Kupffer cells in the liver and histiocytes in the spleen.

environment is responsible for determining the type of macrophage that is programmed, and that monocytes recruited from the bloodstream form resident tissue macrophages that can convert to either an M1 or M2 phenotype and that once polarised macrophages can switch polarity dependent on prevailing conditions (Stout et al., 2005). More recently an alternative model relating to the source of M1 and M2 macrophages has been put forward. This states that different precursors give rise to M1 or M2 macrophages e.g. in mice monocytes expressing Lys6C are thought to give rise to M1 macrophages and to be recruited from the blood while the M2 macrophages arise from local replication of a subset of tissue macrophages (Auffray et al., 2007, Jenkins et al., 2011) . M1 macrophages are important in antimicrobial defences and are produced during cell mediated immune responses and are classified as being interluekin-12 (IL-12) positive and interleukin-10 (IL-10) negative (Mosser, 2003, Mantovani et al., 2004, Edwards et al., 2006). Recently using thermally injured mice, Asai *et al.*, demonstrated that following MRSA challenge, the macrophages present in the skin of normal mice were IL-12 positive, IL-10 negative, Chemokine C-X-C ligand 9 (CXCL9) positive and mannose receptor negative (Asai et al., 2010). In addition they also expressed iNOS mRNA (Asai et al., 2010). The macrophage polarisation of burned mice was typical of M2 macrophages, and M1 polarisation did not occur following infection, leading to failure to form abscesses and control infection, leading to the hypothesis that M1 macrophages are important in the formation of an MRSA abscess.

Further stimulation gives programmes associated with M2 macrophages that could inhibit abscess formation during MRSA (Asai et al., 2010). Following PRR stimulation by a pathogen associated molecular pattern (PAMP) a resident tissue macrophage can convert itself into a M1 macrophage. This conversion is important for host responses during acute infections but excessive M1 polarisation can also promote sepsis and tissue damage (Benoit et al., 2008). M1 macrophages kill invading microorganisms using a range of mechanisms and utilise a large volume of oxygen and express iNOS (Mosser, 2003, Houghton et al., 2009). M1 macrophages are pro-inflammatory secreting a range of cytokines and chemokines associated with a T helper 1 (Th1) response e.g. TNF-α, IL1β and IL-12 (Mosser, 2003). M1 macrophages were initially thought to require both TNF-α and IFNγ to activate them and certainly it was demonstrated that M1 macrophages stimulated with both tumour necrosis factor (TNF) and IFNγ before exposure to an invading pathogen was introduced lead to the macrophage efficiently clearing the infection (Mosser and Edwards, 2008). However, it is now accepted that

certain TLR agonists can bypass this requirement if they lead to the production of both TNF and interferon beta (IFNβ) (Mosser and Edwards, 2008).

M2 macrophages have historically been used to group all other types of AAMs including wound healing macrophages and tumor-associated macrophages (TAMs). It is increasingly recognised that M2 polarised macrophages come in many different subtypes and probably demonstrate functional diversity; the role of many AAM are unknown but some subsets play a role in tissue repair (Mosser and Edwards, 2008). It is also thought that a chronic infection can reprogram a tissue macrophage and push it towards an M2 phenotype (Benoit et al., 2008). M2 macrophages are anti-inflammatory (Brissette et al., 2012). AAM are generally associated with different responses compared to M1 macrophages. These include response to parasitic infections, angiogenesis, and wound healing (Randolph, 2011). It has been shown that AAM have a much reduced ability to kill many bacterial pathogens compared to M1 macrophages and that factors released by them can prevent pathogen stimulation of tissue macrophages that would push them towards a M1 macrophage phenotype (Katakura et al., 2004). AAM in mice express mannose receptors and found in inflammatory zone 1 (FIZZ1) mRNA, and release a range of cytokines including IL-10 and Chemokine C-C motif ligand 17 (CCL17), both of which prevent the conversion of resident macrophages towards an M1 phenotype (Mantovani et al., 2004, Edwards et al., 2006). Within the M2 family there are further subdivisions of macrophages which is determined by their gene expression and the range of synthesised chemokines. If an M2 macrophage produces CCL17 and expresses the FIZZ1 gene it is classified as M2a macrophage (Mϕ), an M2 macrophage producing Chemokine C-C motif ligand 1 (CCL1) and expresses the sphingosine kinase 1 (SPHK1) gene is called an M2b Mϕ and an M2 macrophage producing C-X-C motif chemokine 13 (CXCL13) and carrying the FIZZ1 gene is known as M2c Mϕ (Mosser, 2003, Mantovani et al., 2004, Benoit et al., 2008). The relevance of these three different subtypes is unknown, but all three can prevent resident macrophages becoming M1 macrophages. However, there are many variations between these particular types, many with features shared between 2 or more of these macrophage types (Mosser and Edwards, 2008).

Going further it has recently been shown that IL-10 acts as a switch guiding monocyte to macrophage differentiation during infection. In the normal peritoneum there were 2 macrophage populations found. One was a minor population $CD11b^{int}$ expressing MHCII ($MHCII^{hi}$) and F4/80 while the major population was $CD11b^{hi}$ ($MHCII^{lo}$)

(Nguyen et al., 2012). Both populations were phagocytic but only the MHCII^{lo} population could phagocytose apoptotic cells (Nguyen et al., 2012). When IL-10 expression was high the MHCII^{lo} population of macrophages was formed. IL-10 was confirmed as a developmental switch for this major population as it was not formed in mice lacking IL-10 signalling or the IL-10 receptor (IL-10R1) (Nguyen et al., 2012). The hypothesis from this is IL-10 activates monocytes recruited to a site of inflammation to differentiate into MHCII^{lo} monocytes and these "signalling competent monocytes" cause "signalling incompetent monocytes" to differentiate into further MHCII^{lo} monocytes (Nguyen et al., 2012). Interestingly the introduction of signalling competent monocytes into IL-10R1 deficient mice allowed donor monocytes to convert resident macrophages into MHCII^{lo} (Nguyen et al., 2012). So, early on in infection it is thought IL-10 concentration is high making monocytes differentiate into the major population and engulf apoptotic neutrophils (Nguyen et al., 2012). IL-10 levels then decrease giving rise to the minor population which could activate the adaptive immune system, similar to an antigen presenting cell (APC) (Nguyen et al., 2012).

1.2.6.5. Macrophage receptors

1.2.6.5.1. Toll like receptors

TLRs are transmembrane glycoproteins found on the cell surface and within the endosomes of many cell types including immune cells such as macrophages (Bowie and O'Neill, 2000). Different TLRs recognise different PAMPs, for example lipopeptides are recognised by toll like receptor 2 (TLR2), lipopolysaccharide (LPS) is recognised by toll like receptor 4 (TLR4) and bacterial DNA is recognised by toll like receptor 9 (TLR9) but each receptor has multiple ligands (Chang, 2010). In the case of *S. aureus* for example it is proposed that LTA activates TLR2/toll like receptor 6 (TLR6) heterodimers (Han et al., 2003) and internalisation into the phagolysosome with subsequent digestion will liberate the DNA and engage TLR9 (Hemmi et al., 2000). In this manner TLRs can very quickly cause immune activation. Once the TLR has sensed the presence of the microorganism, an inflammatory response is initiated which helps stimulate pathogen eradication (Gruenberg and van der Goot, 2006). The activation of TLRs was shown to be through dimerisation upon ligand binding, which recruits myeloid differentiation primary response 88 (MyD88) and leads to intracellular signalling (Jin and Lee, 2008). For most TLRs, ligand binding induces homodimer formation, but in the case of TLR2, it can form a heterodimer with either toll like

receptor 1 (TLR1) or TLR6, allowing it to respond to a wider array of ligands (Jin et al., 2007). TLR dimerisation activates signalling leading to NF-κB activation, which in turn stimulates gene expression, which will lead to pathogen eradication (Takeda and Akira, 2004). I am going to focus on TLR2 in this section because it has been shown to play an important role during *S. aureus* infections and LTA from *S. aureus* appears to be a more potent TLR2 stimulus than that of some other Gram positive bacteria (Han et al., 2003).

Research identified that mice lacking TLR2 or MyD88 were highly susceptible to *S. aureus* infection (Takeuchi et al., 2000). As well as this the macrophages from these mice produced less pro-inflammatory cytokines in response to HI *S. aureus* (Yokoyama et al., 2012). The mice cleared the infection a lot slower than WT mice and the macrophages were insensitive to LTA (Takeuchi et al., 2000). The importance of TLR2 to host responses has been demonstrated for a range of clinical presentations by *S. aureus*. Another study using a brain abscess model demonstrated that animals lacking TLR2 had more bacteria persisting and took longer to control the infection; a similar effect was not seen in animals lacking TLR4 (Stenzel et al., 2008). Also TLR2 deficient mice demonstrated larger influxes of neutrophils and $CD11b^{+}$ and $F4/80^{+}$ and $F4/80^{-}$ macrophages to the abscess which persisted for up to 72 days post infection (Stenzel et al., 2008). In this model TLR4 deficient mice had a worse outcome than WT mice, though significantly better than TLR2 deficient mice. Based on these observations, it is proposed that TLR2 provided the major role with TLR4 providing a minor role in host defence against brain infection with *S. aureus* (Stenzel et al., 2008). This protective effect of TLRs will only be beneficial if the resultant inflammatory response maximises pathogen clearance and minimises dysregulated inflammation and tissue injury and highlights the fact that anti-bacterial responses usually require the combined activation of multiple PRRs (Anand et al., 2012).

Another role for TLR2 during *S. aureus* infection is responding to phenol soluble modulins (PSMs). It was shown that PSMs signal through TLR2, with TLR2 heterodimerisation with TLR1 enhancing the TLR2 response to PSM and TLR6 impeding this response (Hajjar et al., 2001). Interestingly, cells expressing TLR2 without the intracellular domain became unresponsive to LTA supporting the role of this receptor for recognition of Gram positive cell wall constituents (Schwandner et al., 1999). As well as this, the expression of TLR2 in cells lacking TLR2 caused them to respond to putative TLR2 ligands (Yoshimura et al., 1999).

1.2.6.5.2. Nod Like Receptor 2

The nucleotide binding oligomerisation domain (Nod) receptors respond to and detect bacterial constituents within the cytoplasm. Nucleotide binding oligomerisation domain containing protein 2 (Nod2) has been shown to be important during infections caused by *S. aureus*. Early research showed Nod2 signalling increased pro-IL-1β production, and increased the production of antimicrobial peptides as well as other host defence mechanisms, implicating their importance in the host defence to microorganisms (Ting et al., 2010).

It is now known that Nod2 is required for bacterial clearance *in vivo*. Nod2 was found to be the receptor for muramyl dipeptide (MDP) which is released from PGN found in Gram positive and Gram negative bacteria (Girardin et al., 2003). In mice models, it was found that a lack of Nod2 meant the mice responded less well to *S. aureus* pneumonia but also were a lot more sensitive to intraperitoneal and cutaneous *S. aureus* infections (Kapetanovic et al., 2010). Nod2 was needed to elicit an optimal IL-6 response to *S. aureus* (Hruz et al., 2009). Based on these observations a model was put forward that stated that lack of Nod2 signalling would result in delayed bacterial recognition, leading to larger bacterial loads and more severe and dysregulated later stages of inflammation as described for TLR2 deficiency above (Hruz et al., 2009). The importance of IL-6 in this model is because it is a central stimulator of the immune response (Hruz et al., 2009).

Other research found that chronic Nod2 stimulation enhanced bacterial killing and there was less pro-inflammatory cytokine production overall when Nod2 was stimulated by live bacteria (Hedl and Abraham, 2013). Also it was shown that p40phox, p47phox and p67phox were upregulated, explaining the link between Nod2 stimulation and the activation of killing pathways within macrophages (Hedl and Abraham, 2013).

1.2.6.5.3. Cluster of differentiation 14

CD14 acts as a co-receptor during interaction of TLRs with their ligands (Sabroe et al., 2003). The importance of CD14 in *S. aureus* infections is still unclear. In some studies it was demonstrated that CD14 did not affect the outcome of infection or play a huge role in *S. aureus* clearance (Haziot et al., 1999). When CD14 was co-expressed with TLR2, an enhanced response to *S. aureus* was observed in other studies, which has led to the suggestion that a range of Gram positive micro-organisms may require CD14 for

optimal recognition by TLR2 (Yoshimura et al., 1999). It has been found that when triacylated lipopeptides bind to CD14 it enhances their interaction with the TLR1/2 complex and although CD14 does not actually bind the lipoprotein it enhances their interaction with the TLR1/2 heterodimer (Nakata et al., 2006). CD14 is also required to enhance the interaction of other *S. aureus* ligands with TLR2. For example it has been found to enhance the interaction of Panton Valentine Leukocidin (PVL) and its LukS subunit with TLR2 and be required for optimal cytokine production by alveolar macrophages and pulmonary inflammation in response to PVL (Zivkovic et al., 2011).

1.2.6.5.4. Macrophage Fc gamma receptors

Fragment crystallisable gamma receptors (FcγR) belong to a family of immunoreceptors whose functional activity can be regulated by the phosphorylation status of the cytoplasmic tail (Swanson and Hoppe, 2004). They recognise IgG opsonised particles and several have immunoreceptor tyrosine based activation motifs (ITAMs) in their cytoplasmic tails (Ravetch and Bolland, 2001, Underhill and Ozinsky, 2002). While some FcγR contain ITAM motifs, a second class have immunoreceptor tyrosine based inhibition motifs (ITIMs) (Ravetch and Bolland, 2001). FcγR ITAMs are phosphorylated and in turn regulate kinases that causes the phosphorylation of a range of downstream targets, regulating actin polymerisation and ultimately phagocytosis (Swanson and Hoppe, 2004); those containing ITIMs recruit phosphatases inhibiting the signalling (Ravetch and Bolland, 2001). When an IgG opsonised particle binds to the FcγR the receptor clusters by patching (Kwiatkowska and Sobota, 1999) causing phosphorylation of tyrosine residues in the ITAM motifs (Kwiatkowska and Sobota, 1999). This results in the formation of a protein complex around the receptor (Booth et al., 2002) and causes internalisation through actin polymerisation, membrane movement towards the site of binding, pseudopod formation, extension around the particle and finally engulfment (Aderem and Underhill, 1999). FcγR phagocytosis is known to trigger inflammatory responses and activate the macrophage NADPH oxidase (Sakata et al., 1987, Gresham et al., 1988, Ravetch and Clynes, 1998).

1.2.6.5.5. Macrophage complement receptors

Complement receptors (CR) such as complement receptor 3 (CR3) recognise complement opsonised particles (Ross, 2000). There are a range of complement receptors such as complement receptor 1 (CR1) found on B cells and monocytes and

CR3 found on macrophages and dendritic cells (Underhill and Ozinsky, 2002). CR1 recognises a range of microbial opsonins including complement component 1q (C1q) and mannose binding lectin (MBL) (Klickstein et al., 1997, Ghiran et al., 2000). CR3 recognises iC3b which is formed following cleavage of the complement component 3b (C3b) (Underhill and Ozinsky, 2002). These receptors cannot mediate internalisation without additional signals; in the case of CR3 these signals increase receptor number at the membrane surface, increase the affinity of the receptor and allows them to phagocytose the bound particle (Pommier et al., 1983, Berger et al., 1984, Wright and Griffin, 1985, Sengelov et al., 1993**)**. An old view of CR phagocytosis suggested that the particle bound to the receptor and by a distinct mechanism sank into the cell (Aderem et al., 1985, van Lookeren Campagne et al., 2007, Kaplan, 1977), but now it is accepted that there are intermediate steps in the process governed by molecules that include Ras homolog gene family member guanosine triphosphate (Rho GTPases) and the spleen tyrosine kinase (Syk) (Caron and Hall, 1998, Shi et al., 2006). Another complement receptor, complement receptor Ig (CRIg), has been demonstrated to be important in CR mediated phagocytosis because endosomes containing CRIg were recruited to the site of binding providing the membrane for the phagosome (van Lookeren Campagne et al., 2007) with microscopy verifying the presence of CRIg in the membrane of the maturing phagosome (van Lookeren Campagne et al., 2007). In contrast to Fc receptor mediated phagocytosis, CR mediated phagocytosis is non-inflammatory and does not activate the NADPH oxidase complex (Wright and Silverstein, 1983, Yamamoto and Johnston, 1984).

1.2.6.5.6. Scavenger receptors

There are a range of scavenger receptors including scavenger receptors type I and type II, macrophage receptor with collagenous structure (MARCO) -both type A scavenger receptors, as well as the cluster of differentiation 36 (CD36), also known as a type B scavenger receptor. They recognise Gram positive bacterial components, most notably recognising LTA (Dunne et al., 1994, Thomas et al., 2000).

Type I and II macrophage scavenger receptors are membrane proteins interacting with and phagocytosing a range of targets including apoptotic thymocytes (Platt et al., 1996). As stated above these scavenger receptors bind to LTA. It was demonstrated that mice lacking both type I and type II receptors (SR-I/II deficient) were more susceptible to *S.*

aureus infection with reduced bacterial clearance and phagocytosis leading to the idea that these receptors are important in the host defence (Thomas et al., 2000). Despite this, a lack of these receptors did not affect leukocyte recruitment and indeed, mice lacking them recruited similar numbers of leukocytes to the site of infection as WT mice (Thomas et al., 2000).

CD36 is a transmembrane glycoprotein (Baranova et al., 2008). It recognises and binds to Gram positive bacteria, including *S. aureus*, but will also with weaker affinity bind to Gram negative bacteria. CD36 has been demonstrated to be important in bacterial clearance because it facilitates phagocytosis and concentrates *S. aureus* and LTA in the phagosome/endosome and engages TLR signalling (Stuart et al., 2005). CD36 recognises a range of species and cell wall products and can activate both phagocytosis and c-Jun N terminal kinase (JNK) signalling pathways resulting in cytokine release (Baranova et al., 2008). During *S. aureus* phagocytosis it has been suggested to have a role to play as a TLR independent signalling receptor (Miller et al., 2011). This was shown because upon receptor activation sarcoma family kinases (Src) were upregulated which lead to JNK dependent pro-inflammatory signalling independent of TLR2/4 (Stuart et al., 2005, Baranova et al., 2008). Macrophages lacking CD36 showed about 50% less ability to phagocytose *S. aureus* and there was 60% less LTA binding to the macrophages implicating LTA as the CD36 ligand (Stuart et al., 2005). Based on this there are 2 hypotheses for CD36 action. Mechanism 1 states CD36 at the cell surface clusters *S. aureus* released LTA leading to TLR2/6 engagement (Stuart et al., 2005) whilst mechanism 2 states CD36 works independently of TLRs and some of the above models have shown CD36 driven phagocytosis and cytokine production occurs in the absence of TLRs (Baranova et al., 2008). Research has also shown that whilst CD36 is a phagocytic receptor, only certain parts of the receptor are essential for phagocytosis; these are residues in the COOH domain (Stuart et al., 2005).

Another scavenger receptor implicated in *S. aureus* host defence is the type II SRA-II receptor MARCO. This is a receptor found on a range of macrophages (Elomaa et al., 1998). In mice it was demonstrated that several tissue populations expressed the receptor in response to various inflammatory stimuli (van der Laan et al., 1999). MARCO is increased in expression in response to bacterial infection (Elomaa et al., 1998). On AMs, MARCO was demonstrated to be the most dominant receptor for binding bacteria (Arredouani et al., 2005) and has a role in binding bacteria in the blood

(van der Laan et al., 1999). As well as binding bacteria MARCO can bind unopsonised particles (Palecanda et al., 1999). Using a tuberculosis (TB) model it was shown that tissue macrophages display an increased expression of MARCO and recruited macrophages also expressed it (van der Laan et al., 1999). However, in the newly recruited macrophages the MARCO receptor was only seen on a few of the cells suggesting its expression is not universally expressed but under tight and strict control resulting in expression by a subset of cells (van der Laan et al., 1999). However the role of scavenger receptors in mediating internalisation of *S. aureus* is not completely clear as inhibitors of scavenger receptors that have not blocked internalisation of several strains of *S. aureus*, even though they blocked uptake of a heat killed commercial strain of *S. aureus* suggesting that strain heterogeneity may influence results and that only some strains may utilise uptake by scavenger receptors (DeLoid et al., 2009).

1.2.6.5.7. The mannose receptor

The mannose receptor (MR) was initially identified on AMs as a receptor for the clearance of objects containing glycoproteins (Gazi and Martinez-Pomares, 2009). It is now known to be expressed by a subpopulation of macrophages and DCs, and its expression in non-phagocytic cells renders them phagocytic (Ezekowitz et al., 1990, Underhill and Ozinsky, 2002). Expression of MR complementary deoxyribose nucleic acid (cDNA) in Cos-1 cells led to them phagocytosing unopsonised *Candida albicans* (Ezekowitz et al., 1990). The MR has been demonstrated to be important in the phagocytosis of a range of pathogens such as *M. tuberculosis* and *Francisella tularensis* (Kang et al., 2005, Schulert and Allen, 2006). Insights from the TB studies have given us a lot of information about how the MR functions. Virulent TB displays lipoarabinomannan (LAM) which has terminal mannose residues and the MR binds mannosylated LAM and can phagocytose mannosylated LAM beads leading to increases in intracellular calcium in monocyte derived macrophages (MDMs) (Schlesinger et al., 1994, Kang and Schlesinger, 1998, Bernardo et al., 1998). This demonstrates how the MR can phagocytose a pathogen and activate intracellular signalling pathways (East and Isacke, 2002). MR phagocytosis can elicit inflammatory responses, and when internalised mannose coated beads or chitin caused the production of TNFα, IFNγ and IL1 by murine spleen cells not seen with cells unable to perform phagocytosis (Shibata et al., 1997). As well as this it has been suggested that MR interact with other PRRs such as TLR2 and transfecting a cell line with both TLR2 and

MR cDNA led to IL-8 secretion in response to *Pneumocystis carinii* not seen with either alone (Tachado et al., 2007). Co-precipitation studies showed the pathogen induced an interaction between both receptors leading to the suggestion that the MR bound the pathogen/pathogen component and formed a complex with TLR2 leading to intracellular signal transduction pathways being activated (Tachado et al., 2007). MR expression in cell lines that do not normally express MR resulted in enhanced uptake of *S. aureus* (Vigerust et al., 2012). This suggests that AAM may also be able to phagocytose *S. aureus* since MR is enriched on these cells as described above (Mantovani et al., 2002).

1.2.6.6. Macrophage phagocytosis

Macrophage phagocytosis is split into opsonin dependent and opsonin independent phagocytosis (Shinji et al., 1998). In opsonin dependent phagocytosis, it can be through IgG binding to the Fc receptor, the C3 complement component iC3b binding to CR, or potentially other opsonins such as surfactant proteins binding to their receptors, whereas in opsonin independent phagocytosis bacterial molecules usually are directly interacting with cell receptors to be recognised (Ross and Medof, 1985, Ravetch, 1994, Ofek et al., 1995).

Fc receptor mediated phagocytosis is an important mechanism in macrophages, that occurs when the receptors ligate particles opsonised with IgG (Aderem and Underhill, 1999). When this happens F-actin is seen to be present on the phagosome membrane demonstrating its importance in phagocytosis and the mechanism requires functional tyrosine kinases such as phosphoinositol-3-kinase (PI3K) and the activation of PI3K dependent Ras homolog gene family member (Rho) family of guanosine triphosphatases (GTPases) such as Ras-related C3 botulinum toxin substrate (Rac1) and the PI3K-independent cell division control protein 42 homolog (Cdc42) as well as the activation of downstream kinases such as p21 activated kinase 1 (PAK1) to allow F-actin polymerisation (Manser et al., 1994, Araki et al., 1996, Cantrell, 2001). It has also been demonstrated that some mechanisms of Fc receptor mediated phagocytosis e.g. those involving fragment crystallisable gamma receptor III (FcγRIII) is calcium dependent (Edberg et al., 1995). Once the particle has been phagocytosed by this mechanism, intracellular processes lead to reactive oxygen intermediate (ROI) production and particle digestion. Looking at complement mediated phagocytosis, it is noted that there are a range of proteins distributed over the phagosome during the

process and protein kinase C (PKC) activation is required during the process (Allen and Aderem, 1996). Unlike Fc receptor phagocytosis CR mediated phagocytosis does not lead to ROI production (Wright and Silverstein, 1983, Aderem et al., 1985).

Once the particle has been taken up by either opsonin dependent or independent mechanisms, it is enclosed in a membrane bound vacuole termed the phagosome which cannot kill the pathogen alone (Gruenberg and van der Goot, 2006). Next it undergoes a maturation process where it exchanges molecules with the cytoplasm, fuses with lysosomes, its pH falls, and microbicidal molecules become activated in what is now termed the phagolysosome (Gruenberg and van der Goot, 2006).

Initially it was thought a single phagosome fused with a single lysosome, but it is now accepted that the phagosome changes and then fuses with multiple lysosomes (Desjardins et al., 1994). Early on in maturation you see the loss of receptors like the Fc receptor, MR and the acquisition of membrane bound proteins such as lysosome associated membrane protein 1 (LAMP-1) and lysosome acquired proteases such as cathepsin D and L (Pitt et al., 1992, Oh and Swanson, 1996). The lysosome associated membrane proteins (LAMP) were first identified in the 1980's on lysosomal membranes (Chen et al., 1985). They are type I integral membrane proteins that are highly glycosylated and found on the plasma membrane (Rohrer et al., 1996, Lichter-Konecki et al., 1999). They can also be distributed on endosomes (Griffiths et al., 1988). LAMP-1 and LAMP-2 are similar proteins (Sarafian et al., 2009) and the phagolysosome is enriched in LAMP-1 and LAMP-2 (Huynh et al., 2007). There have been 2 pathways described for how LAMP proteins reach their target. The first states the proteins are transported from the trans- golgi network (TGN) to the endosomes and finally the lysosomes (Rohrer et al., 1996). The second pathway states the proteins are delivered from the golgi to the cellular surface, internalised and transported along the endocytic pathway to lysosomes (Rohrer et al., 1996). This requires some LAMP to be recycled to the plasma membrane (Rohrer et al., 1996).

Whilst LAMP-1 acquisition peaked at 8 hours (Pitt et al., 1992), LAMP-2 was seen to steadily increase over time (Desjardins et al., 1994). It is also seen that a member of the rat sarcoma (Ras) superfamily of monomeric G protein GTPases, Ras associated protein Rab-5 (Rab-5) decreases over 15 hours, whereas Ras associated protein Rab-7 (Rab-7) increases within 2 hours and then decreases suggesting that the Ras associated protein guanine triphosphatases (Rab GTPases), known coordinators of vesicle traffic are

coordinating the maturation of the phagolysosome (Desjardins et al., 1994). Following phagolysosomal fusion events, the phagosome needs to become acidified to about pH5 which is mediated by an adenosine triphosphatase ATPase in the membrane translocating protons into the phagolysosome (Lukacs et al., 1990). This process of acidification is dependent upon adenosine triphosphate (ATP) hydrolysis because non-hydrolysable ATP analogues such as Adenosine 5'-(β, γ imido) triphosphate lithium salt hydrate (AMP-PNP) or Adenosine 5′-(3-thiotriphosphate) tetralithium salt (ATPγS) did not give rise to acidification (Lukacs et al., 1990). Once all of these processes of phagolysosomal maturation and acidification are complete the phagosome is now the mature phagolysosome.

This complexity within the process is evident when studying *S. aureus*. Early on in an *S. aureus* challenge pathways for cytoskeletal rearrangement are activated during phagocytosis (Miller et al., 2011). As well as this signalling pathways which culminate in a change in calcium concentration were activated (Miller et al., 2011). Phagocytosis of *S. aureus* was also found to activate PI3K which is important in pseudopod extension and phagocytic cup closure leading to engulfment and later on phagosome maturation (Miller et al., 2011). The role of the phagolysosome in *S. aureus* phagocytosis has also been explored. It is not only required to kill the pathogen but also to release PAMPs from the cell wall. Without this occurring, then *S. aureus* ligands that stimulate the immune system remain inaccessible and cannot activate many TLR or other PRR dependent responses (Ip et al., 2010). To confirm this, Ip *et al.*, showed that phagocytosis of *S. aureus* and then acidification of the phagosome must occur before maximal TLR signalling in response to the bacterium (Ip et al., 2010).

1.2.6.6.1. Macrophage phagocytic signalling

FcγR ITAMs are phosphorylated by members of the Src family of tyrosine kinases called Lck/YES related novel protein (Lyn) and hematopoietic cell kinases (Hck) and this results in recruitment of Syk which causes the phosphorylation of a range of downstream targets including a regulatory subunit of PI-3K and a range of factors regulating actin polymerisation and ultimately phagocytosis (Swanson and Hoppe, 2004). Some signalling molecules are involved in phagocytosis from several different receptors. Examples include PI-3K and PKC. Pharmacological inhibition of PI-3K blocked both FcγR and CR mediated phagocytosis (Araki et al., 1996, Cox et al., 1999). The inhibition of PI3K did not affect the binding of the ligands to each receptor or actin

polymerisation suggesting the initial phagocytic signalling was not interrupted; but membrane fusion was inhibited (Araki et al., 1996). Araki *et al.*, also demonstrated that PI-3K inhibition prevented the phagosome closing (Araki et al., 1996). The inhibition of PI-3K also did not prevent F-actin dependent pseudopod extension suggesting it occurred in a PI-3K independent manner (Cantrell, 2001). PI-3K inhibition led to prolonged activity of the PI-3K dependent Rho GTPase, Rac1 and the PI3K independent Cdc42 (Beemiller et al., 2010).

Early research found that dominant negative (dn) alleles of Rac1 and Cdc42 prevented actin polymerisation at nascent phagosomes during Fc receptor mediated phagocytosis suggesting they were important for actin assembly (Caron and Hall, 1998). They inhibited particle uptake but not the initial binding (Caron and Hall, 1998). Despite this effect being seen with both if only one of the proteins was inhibited there was still some actin polymerisation supporting their complimentary roles during phagocytosis whereby both proteins act at different times during the phagocytic process **(**Hoppe and Swanson, 2004). It was demonstrated that Cdc42 acted early in the process and was recruited to phagosomes because it was found at the ends of the pseudopodia (Hoppe and Swanson, 2004). Rac1 on the other hand was found to be distributed throughout the phagocytic cup and to be important during the closing of the phagocytic cup around the particle (Hoppe and Swanson, 2004). Recently during Fc receptor phagocytosis it was shown that when activated Cdc42 stimulated PI-3K leading to increased levels of phosphoinositol 3, 4, 5 phosphate (PI3, 4, 5-P) in phagocytic cups. This then led to a PI3, 4, 5-P dependent deactivation of Cdc42 which was necessary to complete phagocytosis (Beemiller et al., 2010).

PKC inhibition prevented phagocytosis by both Fc and CR receptors (Zheleznyak and Brown, 1992, Allen and Aderem, 1995). PKC is involved in the early stages of the phagocytic process because PKC inhibition prevented actin polymerisation occurring underneath the area where the particle was bound (Allen and Aderem, 1995). A similar role has been documented for phospholipase C (PLC). PLC is recruited to maturing phagosomes that contain particles that have been opsonised by IgG and its inhibition blocked particle internalisation (Botelho et al., 2000). More detailed analysis revealed PLC inhibition prevented actin filaments forming underneath the area of particle contact (Botelho et al., 2000).

In general there are subtle differences in the initial signalling between FcγR and CR phagocytosis. During FcγR receptor phagocytosis there is a phosphorylation of tyrosine residues within the ITAM domains which function as binding sites for Src homology 2 (SH2) domain containing proteins such as Syk and inhibition of Syk kinase prevented internalisation of IgG opsonised particles (Matsuda et al., 1996, Kiefer et al., 1998). For example FcγRI or IIA cross-linking in monocytes resulted in receptor association with Syk and Syk phosphorylation results in its activation (Kiener et al., 1993). Activated Syk stimulates tyrosine phosphorylation in a range of downstream proteins such as PAK1 involved in actin dynamics (Groves et al., 2008). PAK1 is found to be localised to forming phagosomes, and inhibiting PI-3K prevents its loss from phagocytic cups rather than its recruitment and association to the forming phagosome (Diakonova et al., 2002). CR3 mediated phagocytosis does not depend on tyrosine phosphorylation (Groves et al., 2008). Actin remodelling during CR3 phagocytosis depends on Ras homolog gene family member A (RhoA) because a dn form of Rho and specific Rho inhibitors prevented CR internalisation (Caron and Hall, 1998). The Rho family GTPases work by recruiting and interacting with a number of downstream proteins in an active GTP dependent manner; these regulators can then activate the actin related protein 2/3 (Arp 2/3) complex important for actin remodelling at phagocytic cups (May et al., 2000). Recent research has shown Ras homolog gene family member G (RhoG) also to be important for both Fc and CR phagocytosis; downstream signalling through both receptors required different Rho GTPases but both required the presence of functional RhoG (Tzircotis et al., 2011).

1.2.6.7. Macrophage killing

1.2.6.7.1. Reactive oxygen species

Macrophages have a range of mechanisms to destroy phagocytosed material. Phagocytosis and killing by AM is an energy dependent process with the energy being generated by glucose metabolism and pyruvate formation (McGee et al., 1983).

ROS are formed in various ways within the cell. For example, they can be generated by the mitochondria where electrons leak as they are transferred between complexes and through NADPH oxidase reducing oxygen into superoxide and other ROS (Boveris and Chance, 1973, Boveris and Cadenas, 1975). The release of ROS is not random and is stimulated following microbial exposure. Early research suggested that ROS generation

could be mediated through arachidonic acid production upon Fc receptor crosslinking (Sakata et al., 1987). It was thought arachidonic acid could activate the NADPH oxidase complex, by possibly helping it assemble and hence activating it (Sakata et al., 1987).

Six homologs of the cytochrome subunit of the phagocyte NADPH oxidase complex exist and are referred to as the NOX family of NADPH oxidases (Bedard and Krause, 2007). It is now accepted that the generation of ROS depends upon NOX's existing in one of several forms within the cell, of which NOX2 or $gp91^{phox}$ is the major isoform in phagocytes, forming a dimer with $p22^{phox}$ which is essential for its activity (Morgan et al., 2008). The membrane associated $p22^{phox}$/ $gp91^{phox}$ (NOX2) complex recruits additional subunits from the cytosol for activity. These are $p47^{phox}$ and $p67^{phox}$ (Morgan et al., 2008). Mechanistically, when $p47^{phox}$ becomes phosphorylated it binds to phospholipids, interacts with $p22^{phox}$ and then recruits $p67^{phox}$ to the complex; the $p67^{phox}$ then binds and stabilises Rac which interacts with this whole complex and the complex is now active (Morgan et al., 2008). It was shown that the phosphorylation of $p47^{phox}$ was an essential part of ROS generation and required for recruitment of $p47^{phox}$ to the phagolysosomal membrane (DeLeo et al., 1999). Further phosphorylation of $p47^{phox}$ results in loss of $p47^{phox}$/$p67^{phox}$ from the phagolysosomal membrane and loss of the complex into the cytosol terminates ROS generation (DeLeo et al., 1999).

It has been shown that the opsonisation status of *S. aureus* does not affect ROS generation and both opsonised and unopsonised bacteria generated a similar ROS response (Devalon et al., 1987). The role of ROS is to inactivate and destroy invading microorganisms. ROS damages cellular proteins, lipids and nucleic acids and can activate signalling cascades and cause cell death (Morgan et al., 2008).

1.2.6.7.2. Nitric oxide

It was demonstrated from *in vitro* research that mouse macrophages could generate nitrate and nitrite when activated and microbial products could lead to RNI generation (Nathan and Hibbs, 1991). It was also shown that macrophages released a compound that was thought to be nitric oxide (NO) or a related compound like nitrate (Stuehr et al., 1989). NO is formed in an enzymatic reaction from L-arginine through the action of nitric oxide synthases (NOS) (Kuo et al., 2003). There are 3 NOS isoforms of which iNOS/NOS2 is involved in immune responses, while endothelial NOS (eNOS) and

neuronal NOS (nNOS) are the two other forms (Kuo et al., 2003). The difference between these is iNOS is transcriptionally regulated in response to stimuli (Kuo et al., 2003). Following the initial generation of NO other intermediates can be formed. Superoxide can combine with NO forming peroxynitrite ($ONNO^-$).

It has been shown that protein kinase C epsilon (PKCε) is important for iNOS activation and NO production by macrophages and macrophages lacking PKCε produced reduced levels of NO (Castrillo et al., 2001). As well as this, LPS induction of NOS2 activity was reduced in macrophages lacking PKCε (Castrillo et al., 2001). Treatment of macrophages with *S. aureus* LTA enhanced the expression of PKC and NO showing that signal transduction as a result of LTA stimulation can also lead to iNOS expression (Kuo et al., 2003).

Following its generation, NO and its intermediates have various roles to play in killing intracellular bacteria. RNI can attack iron-sulphur (Fe-S) clusters including those within the mitochondrial complex I and II and can attack cis-aconitase in the tricarboxylic (TCA) cycle (Lancaster and Hibbs, 1990). NO can also bind mitochondrial enzymes and as a result cellular respiration becomes inhibited and $ONNO^-$ can nitrosylate a range of cellular proteins or further react to form hydroxyl radicals which are bactericidal (Wizemann et al., 1994, Laskin et al., 1994).

NO production is important in the host response against *S. aureus* infections, and is stimulated in response to the bacterium in phagocytes; infection with *S. aureus* in a murine model increased iNOS mRNA and inhibiting NO production led to increased mortality in this model (Sasaki et al., 1998). Also it was demonstrated using an arthritis model, that administering NOS inhibitors worsened the outcome of infection because the macrophages could not kill *S. aureus* (Sakiniene et al., 1997). However, the organism also expresses lactate dehydrogenases (Ldh) which help it survive under conditions of nitrosative stress (Richardson et al., 2008). The expression of Ldh is found with *S. aureus* but not with several other staphylococci such as *S. epidermidis* or *S. saprophyticus* (Richardson et al., 2008). Ldh expression is essential for *S. aureus* to resist nitrosative stress and although inactivating *ldh2* had no effect on virulence, inactivating *ldh1* attenuated the virulence and a double mutant was avirulent (Richardson et al., 2008). As well as lactate dehydrogenases, it is known *S. aureus* produces haem metalloproteases (Hmp) which detoxify the NO (Richardson et al., 2006). Thus the overall picture suggests NO may play an important role in controlling

S. aureus but the microorganism possesses several strategies to partially resist NO-mediated killing. In addition although human macrophages appear to generate NO they appear to do so at lower levels than mice so the implications of these murine studies to man require further clarification (Schneemann and Schoeden, 2007).

1.2.6.8. Macrophage-associated apoptosis

1.2.6.9. Brief overview

Apoptosis is a form of programmed cell death (PCD). It is characterised by DNA fragmentation, chromatin condensation, cell shrinkage and the redistribution of phosphatidylserine (PS) to the outer leaflet of the plasma membrane (Kerr et al., 1972). It is ATP dependent and a block on ATP pushes the death mechanism towards necrosis, and it appears the ATP is needed before the morphological changes associated with apoptosis occur, showing that these changes are the result of a bioenergetic requiring process (Eguchi et al., 1997, Leist et al., 1997). Apoptosis is usually an ordered event and not random. It can be used by macrophages to contribute to pathogen control which can remove the intracellular niche and complement other intracellular killing strategies (Dockrell et al., 2001, Dockrell et al., 2003). As apoptosis does not lead to the release of cytoplasmic contents it is not inflammatory (Labbe and Saleh, 2008).

1.2.6.9.1. The intrinsic pathway

This pathway makes use of the mitochondria, cytochrome c and a family of cysteine dependent aspartate directed proteases called caspases. During activation of the intrinsic pathway release of cytochrome c from the mitochondria results in formation of a complex containing cytochrome c, dATP, the apoptosis activating factor 1 (Apaf-1) and pro-caspase 9 to form the apoptosome which then activates pro-caspase 9 and cleaves downstream caspases (Bantel et al., 2001). There are 3 families of caspases, initiator caspases (2, 8, 9 and 10), which initiate the cascade, executioner caspases (3, 6 and 7), which destroy the cell and inflammatory caspases (1, 4, 5 and 12) (Stroh and Schulze-Osthoff, 1998). Newly formed caspases are inactive and are then proteolytically modified activating them. Initiator caspases 8 or 9 activate the executioner caspases 3, 6 or 7, which cleave a range of substrates leading to cell death (Stroh and Schulze-Osthoff, 1998, Porter and Janicke, 1999). Activation of caspase 3 has been shown to be important for DNA fragmentation seen with apoptosis and also other biochemical and morphological changes. When caspase 9 binds to Apaf-1, caspase 9 becomes cleaved

and caspase 9 is now active, which cleaves caspase 3 activating it and the execution phase of apoptosis begins (Li et al., 1997). Caspase 9 activation can also lead to the cleavage and activation of caspases 6 and 7 leading to apoptosis.

Another family of proteins important in the intrinsic pathway is the B cell lymphoma 2 (Bcl-2) family comprising 3 subgroups known as antiapoptotic family members including Bcl-2 and myeloid cell leukaemia sequence 1 (Mcl-1), proapoptotic multi domain channel forming proteins including Bcl-2 associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak) and BH3 only proapoptotic proteins including p53 upregulated modulator of apoptosis (PUMA), phorbol-12-myristate-13-acetate-induced protein 1 (Noxa), BH3-interacting domain death agonist (Bid) and Bcl-2 interacting mediator of cell death (Bim) (Bae et al., 2000). Within a cell there is balance of Bcl-2 family anti- and proapoptotic proteins and it is this concentration that can determine the fate of the cell. Anti-apoptotic Bcl-2 family proteins prevent mitochondrial pore formation by Bax and Bak preventing cytochrome c release (Kluck et al., 1997). To promote the antiapoptotic role of Bcl-2 it has been suggested it must interact with Bax, which was shown to occur *in vivo* (Oltvai et al., 1993, Hirotani et al., 1999). However there are two main theories of how BH3 only Bcl-2 proteins induce mitochondrial outer membrane permeabilisation (MOMP) to allow cytochrome c release and initiate the intrinsic pathway of apoptosis. In the first, the BH3 proteins neutralise the anti-apoptotic Bcl-2 proteins, releasing Bax and Bak to induce MOMP while in the second they directly activate Bax and Bak (Galonek and Hardwick, 2006, Kim et al., 2006, Willis et al., 2007, Chipuk and Green, 2008, Lovell et al., 2008).

An example of direction activation of Bax is the interaction between Bid and Bax which can trigger apoptosis. Bid gets cleaved by caspase 8 and moves to the mitochondrion where it induces cytochrome c release (Luo et al., 1998, Gross et al., 1998) This is Bax dependent and when Bax binds to Bid there is a conformational change in Bax leading to cytochrome c release (Desagher et al., 1999). This leads to caspase activation and apoptosis. Bid also interacts with other Bcl-2 family proteins at the mitochondria inducing cytochrome c release (Akgul et al., 2004).

Built into this pathway are also proteins that either promote or prevent the apoptotic process occurring. One of these proteins is Mcl-1 which is important in preventing macrophages from undergoing apoptosis in health and disease (Liu et al., 2001). There are splice variants of Mcl-1 e.g. Mcl-$1_{Exon1,}$ which is pro-apoptotic with a mitochondrial

location and can induce a loss of inner mitochondrial transmembrane potential ($\Delta\psi M$) and MOMP (Marriott et al., 2005). Other splice variants include Mcl-1S (Bae et al., 2000). Mcl-1L interacts with a range of apoptotic proteins whereas Mcl-1S only interacts with Mcl-1L leading to the hypothesis that the splicing mechanism of Mcl-1 is regulated to give rise to Mcl-1S which induces cell death by taking up some of the binding capacity of Mcl-1 for other pro-apoptotic Bcl-2 family members (Bae et al., 2000). Mcl-1 is thought to sequester the pro-apoptotic protein Bak on the mitochondria so it cannot induce apoptosis but more recent studies suggest it has little capacity to directly interact with Bax (Willis et al., 2005, Zhai et al., 2008). Also it interacts with several BH3 only Bcl2 proteins such as Bim, reducing the concentration of Bim that can interact with Bax and move to the mitochondria to induce apoptosis (Opferman et al., 2003). It also interacts with NOXA (Zhang et al., 2011).

1.2.6.9.2. The extrinsic pathway

This pathway involves the ligation of death receptors such as Fas, that can recruit the Fas activated death domain (FADD) and pro-caspase 8 into what is termed the death inducing signalling complex (DISC), which can activate caspase 8 and lead to downstream effects (Bantel et al., 2001).

There are various receptors involved in this pathway such as the TNF receptor type 1 (TNFR-1) and Fas (Apo-1/CD95) which have a common motif called the death domain (DD) (Itoh and Nagata, 1993, Tartaglia et al., 1993). Ligand binding causes the receptors and therefore the DD to aggregate activating a signalling cascade leading to cell death (Song et al., 1994, Boldin et al., 1995, Vandevoorde et al., 1997). TNFR-1's DD clusters and recruits tumour necrosis factor type 1 associated death domain (TRADD) forming a site for FADD and mediator of receptor induced toxicity 1 (MORT-1) binding whereas with Fas, the FADD DD associates directly with the Fas DD (Hsu et al., 1995, Hsu et al., 1996). In both of these processes, FADD then recruits FADD like IL-1β converting enzyme (FLICE), MORT-1 associated CED 3 homologue (MACH) and pro-caspase 8, which activates it leading to apoptosis (Boldin et al., 1995, Muzio et al., 1996). Fas clustering also activates pro-caspases 3 and 7 as both are caspase 8 substrates (Wallach et al., 1997). Fas induced cell death did not occur in a caspase 8 mutant and it was demonstrated that this was because there was no cleavage of downstream substrates and other caspases (Juo et al., 1998).

The components for Fas signalling are pre-assembled in the plasma membrane. When all the association and formation processes are complete, the level of caspase 8 and 3 in the DISC increases and caspase 3 becomes activated, with the caspase 3 containing DISC located in lipid rafts (Aouad et al., 2004). It is suggested that some cell types (so called type II cells) Fas activation induces relatively weak levels of caspase 8 activation and that engagement of apoptosis requires an amplification loop in which caspase 8 activates the BH3 only protein Bid (Scaffidi et al., 1998, Li et al., 1998). This then activates the intrinsic pathway and MOMP which leads not only to activation of caspase 9 to increase caspase 3 activation but also results in release of second mitochondria derived activator of caspases (Smac)/direct inhibitor of apoptosis binding protein with a low isoelectric point (DIABLO) from the mitochondrion which antagonises a factor which sequesters caspase 3 (Srinivasula et al., 2000). This factor X linked inhibitor of apoptosis protein (XIAP) is an inhibitor of caspase activation and its neutralisation by Smac/DIABLO removes caspase 3 from the constraints of XIAP. In type I cells stimulation of the DISC induces sufficient activation of caspase 8 and caspase 3 to induce apoptosis (Scaffidi et al., 1998).

1.3. *Staphylococcus aureus* evasion of the innate immune system

1.3.1. Overview

The ability of a pathogen to adapt to the immune mechanisms that aim to control it is a feature of evolution. Some bacteria prevent internalisation, some prevent phagosome: lysosome fusion and some even survive in this acidic compartment or escape from it (Gruenberg and van der Goot, 2006). *S. aureus* has evolved a range of mechanisms for avoiding the innate immune system, ranging from blocking leukocyte migration, preventing phagocytosis and inhibiting its killing in host cells. This section will describe a few mechanisms used by this "well armed pathogen" (Archer, 1998).

1.3.2. Chemotaxis inhibitory protein of *Staphylococcus aureus* and staphylococcal complement inhibitor

S. aureus produces a range of proteins that prevent leukocyte migration to the site of infection and phagocytosis. These include chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS), and staphylococcal complement inhibitor (SCIN). CHIPS bind to neutrophil chemoattractant receptors blocking the actual ligand binding (de Haas et al., 2004, Rooijakkers et al., 2005a).

CHIPS has been shown to bind to the formyl peptide receptor (FPR) and the complement 5a receptor (C5aR) preventing complement component 5a (C5a) and Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) induced neutrophil chemotaxis (Postma et al., 2004, de Haas et al., 2004). As well as this CHIPS has been shown to prevent activation of the innate immune system but as the bacteria multiply and reach sufficiently high numbers, it was demonstrated that host chemokines can overcome the inhibition caused by CHIPS and the bacteria get phagocytosed (de Haas et al., 2004). CHIPS is important in the very early stages of infection, allowing *S. aureus* to colonise a specific niche (de Haas et al., 2004).

The SCIN protein is found in 90% of *S. aureus* clinical isolates. It is an effective mechanism of preventing phagocytosis because it interacts with C3 convertases on the bacterial surface preventing C3b being deposited and hence blocking complement mediated phagocytosis (Rooijakkers et al., 2005a). SCIN has a varied mode of action, leading to either a failure of the C3 convertase to reassemble after its action on C3, a failure of the convertase to form initially or if it has formed a failure of the enzymatic activity to occur (Rooijakkers et al., 2005a).

Other molecules that affect early processes are described in Table 1.2.

1.3.3. Superantigen like protein 3

Superantigen like protein 3 (SSL3) is produced in all sequenced *S. aureus* strains. It was shown that SSL3 modulated neutrophil and monocyte responses by inhibiting TLR2 interactions with its ligands (Bardoel et al., 2012). It is also thought to prevent TLR2 interacting with other TLRs as a heterodimeric complex (Bardoel et al., 2012). When SSL3 bound the extracellular domain of TLR2 it blocked macrophage stimulation with HI *S. aureus*, and other TLR2 specific ligands which lead to reduced IL-12 production by macrophages, and it was shown that whilst SSL3 inhibited cytokine production (Yokoyama et al., 2012). Other SSLs are described in Table 1.2.

1.3.4. Adenosine and staphyloxanthin

Macrophages express adenosine receptors and depending on their activation they have been found to express up to 4 (Thiel et al., 2003). WT *S. aureus* were demonstrated to synthesise adenosine using *adsA* and an *adsA* deficient mutant was more rapidly cleared from blood and unlike WT bacteria could not produce abscesses (Thammavongsa et al., 2009). The adenosine produced by *S. aureus* was found to bind to macrophage

adenosine receptors and reduce the production of specific cytokines, most notably IL-12 and TNF-α, and attenuate macrophage responses to *S.aureus* (Hasko et al., 2000).

Staphyloxanthin is a carotenoid pigment responsible for giving *S. aureus* its golden colour and mutants with a decreased biosynthetic capacity for staphyloxanthin show growth defects (Olivier et al., 2009). Staphyloxanthin is an antioxidant which protects *S. aureus* from oxidative stress by scavenging oxygen radicals and protecting against ROS (Clauditz et al., 2006, Olivier et al., 2009). It was shown that mutants lacking the pigment were more effectively killed by ROS than WT strains and had reduced survival in neutrophils and whole blood (Liu et al., 2005). Staphyloxanthin is found in the bacterial cell membrane and this has led to the hypothesis that it might be preferentially protecting bacterial lipids against oxidative damage (Clauditz et al., 2006). Staphyloxanthin has now been accepted to not only allow *S. aureus* to evade killing but to maintain tissue infection and form characteristic abscesses, though it does not play a role in *S. aureus* colonisation of mucosal surfaces (Liu et al., 2008).

1.3.5. Catalase and superoxide dismutase

Catalase is another product made by *S. aureus* that can help it resist oxidative stress, having been first shown to be present in *S. aureus* in the late 1960's (Amin and Olson, 1968). Early research suggested the importance of catalase in destroying hydrogen peroxide and thus protecting phagocytosed microbes within the phagolysosome (Mandell, 1975). This destruction of hydrogen peroxide by *S. aureus* catalase breaks the compound into oxygen and water and thus offers the organism protection from its effects (Mandell, 1975). Interestingly, following incubation with *S. aureus* there was more catalase found inside the macrophages (Das and Bishayi, 2009). Specifically inhibiting catalase production by the macrophages confirmed bacterial production of catalase by *S. aureus* (Das and Bishayi, 2009). It is thought the catalase is most beneficial in high hydrogen peroxide concentrations because it enables *S. aureus* to scavenge oxygen radicals by breakdown of hydrogen peroxide, avoiding hydroxyl radical production (Das and Bishayi, 2009).

S. aureus produces 2 SOD enzymes classed as superoxide dismutase A (SodA) and superoxide dismutase M (SodM). SodA is the major SOD in *S. aureus* thought to respond to oxidative stress with SodM believed to have an additional role (Valderas and Hart, 2001). Both SodA and SodM contribute to survival in animal models of *S. aureus* infection. SOD enzymes allow the bacterium to resist oxidative stress within

phagolysosomes when phagocytosed. It has been found that the transcription of both genes is under tight control and depends upon the presence of a Mn cofactor when superoxide is present (Karavolos et al., 2003). Despite this research the reason for the bacterium producing 2 separate SOD enzymes, remains unknown. It was shown that during late exponential phase SodM levels increase in a SodA deficient mutant protecting the mutant from oxidative stress (Valderas and Hart, 2001). This suggested SodM may play a unique role at a distinct phase of bacterial growth. (Valderas and Hart, 2001). Table 1.2 summarises other immune evasion molecules produced by *S. aureus*.

1.4. Mathematical modelling of host: pathogen interactions

1.4.1. Why use mathematical models?

Research looking at host: pathogen interactions focuses around using biochemical and cellular techniques in order to understand the mechanisms behind observed phenomena (Kirschner and Marino, 2005); however, if we are to deepen our understanding of these behaviours we need to use studies at a much larger scale and this is where mathematics can help. Mathematical modelling can help with this understanding because host: pathogen interactions are complex with various components interacting with one another in a much larger biological system (Kirschner and Marino, 2005). Mathematical modelling gives us a different and interesting method to analyse and study these complex systems, and more importantly, the interactions between the various components within the system (Kirschner and Marino, 2005).

Mathematical modelling of biological systems can start with a simple diagram. This is then extended into a group of equations which is then extended further to make predictions about the system. These predictions are then tested experimentally to determine if the model holds up (Perelson, 2002, Kirschner and Marino, 2005). The next stage in the model is to define it by deciding what mechanisms are to be included and what hypotheses are to be tested; then parameters can be estimated from experimental data (Kirschner and Marino, 2005).

Mathematical models are useful because they allow the researcher to analyse large amounts of data in a quick way which can then help with planning decisive experiments to answer key questions and yield very important results which can be fed into the model (Kirschner and Marino, 2005). As well as this they allow the researcher to make predictions about the effect of inhibiting various pathways or processes on the

Molecule	Role in immune evasion	References
Formyl peptide like 1 inhibitor (FLIPr)	Binds formyl peptide receptor like 1 (FPLR-1) preventing leukocyte recruitment.	(Prat et al., 2006)
Staphopain A	Cleaves N terminal domain (NTD) of chemokine C-X-C motif receptor 2 (CXCR2) preventing proper IL-8 association, neutrophil recruitment and activation.	(Laarman et al., 2012)
Extracellular complement binding protein (Ecb)	Binds complement component 3d (C3d) inactivating C3b containing convertases in alternative complement pathway; affects C5 convertases.	(Jongerius et al., 2007)
Extracellular fibrinogen binding protein (Efb)	Binds C3d and blocks C3b containing convertases; binds to fibrinogen preventing neutrophil adhesion, activation and migration.	(Jongerius et al., 2007, Ko et al., 2011, Jongerius et al., 2012)
Superantigen like protein 5 (SSL-5)	Recognises P-selectin glycoprotein ligand 1 (PSGL-1) preventing neutrophil rolling; binds to receptors for C3a and C5a preventing neutrophil chemotaxis; binds and blocks matrix metalloprotease 9 (MMP-9)	(Bestebroer et al., 2007, Bestebroer et al., 2009, Itoh et al., 2010)
Superantigen like protein 7 (SSL-7)	Binds immunoglobulin A (IgA) and complement component C5; prevents IgA binding to fragment crystallisable alpha receptor 1 (FcαR1).	(Langley et al., 2005, Bestebroer et al., 2010)
Staphylokinase (Sak)	Forms complex with plasminogen converting it into plasmin; plasmin causes IgG and C3b cleavage from bacterial surface; interferes with Fc and CR phagocytosis; neutralises defensins.	(Parry et al., 2000, Molkanen et al., 2002, Jin et al., 2004, Rooijakkers et al., 2005b)
Nuclease	Breaks down neutrophil extracellular traps (NETs).	(Berends et al., 2010)

Table 1.2: Immune evasion molecules produced by *S .aureus*

behaviour of a system, and this can be translated clinically. Also they allow the researcher to test results and question how close to 'real' behaviour are they.

The first aim of any mathematical model is to determine if it can produce results similar to what is observed experimentally and when this has been achieved it can be modified to address new problems such as the rate at which certain events are occurring (Kirschner and Marino, 2005). The model can then be extended to, for example, begin to address what factors might be needed to control the infection in a biological system, and what parameter settings are going to be beneficial for the host and/or pathogen to succeed. Ultimately this outcome depends on which area of the system you are interested in (Kirschner and Marino, 2005). So, mathematical models are extremely interesting because they offer insights into hypotheses that have been generated and can allow you to compare and contrast conflicting arguments about what might be occurring biologically (Kirschner and Marino, 2005). But, these models do have drawbacks and the most obvious is that a lot of the time we are modelling individual cells or agents or the entire population. In such a fashion it can be difficult to distinguish a heterogeneous population from a homogeneous one; and it can be difficult in much larger models to follow the fate of an individual cell. But new technologies such as two photon imaging, will allow us to produce a time series of *in vivo* cellular dynamics which can both support and help validate mathematical models (Miller et al., 2002, Kirschner and Marino, 2005).

1.4.2. Mathematical modelling methods

1.4.2.1. Agent based models

Agent based models (ABMs) are generally computational models where the system is studied at the level of individual 'agents' (i.e. individual cells, bacteria, etc). These agents obey set probabilistic rules which, when simulated over a large number of time steps give rise to the population-level dynamics. In an ABM, the system is broken down into unique agents, which interact with each other and with other agents (Segovia-Juarez et al., 2004, Folcik et al., 2007). These models are useful in understanding infectious diseases because they are flexible and can help capture emergent issues (Bonabeau, 2002) and for example has demonstrated that during TB infection, the slow growth of the bacteria is a contributor to virulence (Segovia-Juarez et al., 2004).

A good example of an ABM is the basic immune simulator (BIS) which was created to examine how the immune system interacts (Folcik et al., 2007). Using a range of

parameters, the role of the immune system in infection control was examined. There were 3 scenarios identified from the model. Firstly, if immune cells cleared the infective agent and immune cells were replaced by further immune cells, it would win the battle, secondly, if bacteria persisted and killed off immune cells or immune cells were activated in insufficient numbers, the immune system would lose and finally if the immune cells proliferated an excessive response would result (Folcik et al., 2007). The agents in this system have imposed behaviour and have to behave in a specific, pre-defined way. An over vigorous immune response was seen when not only were there too few cells responding, but the cells were activated a lot later and interestingly the model observed that programmed cell death could avoid this scenario (Folcik et al., 2007). These models are useful in predicting, how one agent can potentially out-compete another.

Despite potential advantages with ABMs, they have some potential flaws. A non-trivial disadvantage is an ABM can be more expensive in terms of time and effort to implement than equation based models (Bonabeau, 2002). As well as this it can be difficult to analyse how an individual parameter within the entire system affects the output behaviour of the ABM (An et al., 2009). Linked into this is the difficulty of looking at a single agent's behaviour and beginning to analyse it in multiple environments and many ABM modellers expand the initial interaction space so they can look at how 'agents' communicate on one level (An et al., 2009). Finally, the nature of ABM model means the modeller does rely on very deep computational methods in order to generate statistically analysable data sets (An et al., 2009).

1.4.2.2. Ordinary differential equation models

Ordinary differential equation (ODE) models are used as a starting point for mathematical modelling because of their relative simplicity (Bauer et al., 2009) and have proved useful in describing host-pathogen interactions (Antia et al., 1996). ODE models are mathematical models where variables in the system e.g. cells, bacteria are described by differential equations. These differential equations contain various parameters which affect the behaviour of each variable or population, and when stimulated over time it produces mathematical dynamics and predictions at the population level. The essential building blocks of an ODE model are differential equations containing a range of various parameters based upon *in vitro* observations. Then these parameters are assigned values which are again based upon the experimental

results and then used within the model to solve the equations and finally the model is assessed for biological accuracy as seen in TB and Human Immunodeficiency Virus (HIV) studies (Antia et al., 1996, Di Mascio et al., 2004, Day et al., 2011, Hosseini and Gabhann, 2012). In order for an ODE model to be as accurate as it can be, it is important to decide which parameters it can estimate (Miao et al., 2011). This allows a simple model to be created which can offer basic results and information, and then more complex scenarios can be built in (Segovia-Juarez et al., 2004). Built into these models is one key assumption that the population is homogeneous and as a result the ODE model is not very good at estimating deviations away from this (Bauer et al., 2009). Having said this, ODE models can be set up to describe a heterogeneous population, but they are less adept at this than ABMs.

1.4.3. The Human Immunodeficiency Virus-1 mathematical model story

Human Immunodeficiency Virus 1 (HIV-1) is a very good example of where mathematical modelling has better informed our understanding of host: pathogen interactions. These models used very simple differential equations to describe the interaction between susceptible cells, infected cells and free virus (Di Mascio et al., 2004) and a lot of the early mathematical work was interested in answering how a single virus particle was able to infect a susceptible cell, and, how the various cellular populations changed throughout the time course of infection (Di Mascio et al., 2004, Dixit and Perelson, 2005).

Perelson *et al.*, 1993 is notable for producing the first extensive model examining how HIV-1 affects cluster of differentiation 4 positive ($CD4^+$) T helper cells (Perelson et al., 1993). The investigators used differential equations to examine the effect of HIV-1 on $CD4^+$ T helper cell numbers and HIV-1 disease progression. They found there were two possible infectious states, the uninfected state and the endemically infected state, in which the virus (V) level was constant so that it was affecting the $CD4^+$ T helper cell population and, the total number of $CD4^+$ T helper cells was lower than in an uninfected individual (Perelson et al., 1993). The investigators also examined $CD4^+$ T helper cell depletion. In order to observe increased $CD4^+$ T helper cell depletion there would have to be more rapid movement between T cell populations, and most important from latently infected T cells (T^*) to the actively infected T cells (T^{**}) (Perelson et al., 1993).

Analysis illustrated that HIV-1 disease starts with a lag phase where $CD4^+$ T helper cell numbers remain at a constant level, and then at a defined time there is a second phase of infection represented by a decline in the numbers of $CD4^+$ T helper cells (Perelson et al., 1993). They found latently infected T cells (*T**) and actively infected T cells (*T***) were initially similar and as $CD4^+$ T helper cells became infected with V, *T** decreased and *T*** increased (Perelson et al., 1993). V molecules initially decreased as they bound to $CD4^+$ T helper cells and were ingested, and then V showed exponential growth to a steady state (Perelson et al., 1993).

This basic model by Perelson *et al.,* has formed the basis for many further models in this area. The HIV-1 story has been extended by examining how mutant virions arise within an infected $CD4^+$ T helper cell. In these models, the investigators assumed that before any drug therapy was administered, mutant viruses existed alongside WT viruses in HIV-1 infected individuals. Nowak *et al.,* formed a simple set of equations outlining how mutant virions could be produced by infected $CD4^+$ T helper cells, and then examined how anti-retroviral therapy affected this production. The mathematical model formed by the researchers showed that if anti-retroviral therapy prevented replication of the WT virus, then the number of virus particles and the proportion of $CD4^+$ T helper cells in the T* population decreased (Nowak et al., 1997). However, if the production rate of WT virus by infected $CD4^+$ T helper cells termed k matched the production rate of mutant viruses by infected $CD4^+$ T helper cells termed k_m then mutant virus increased in number in both the virus and T* populations at the same time (Nowak et al., 1997). However, if $k > k_m$ mutant virus progeny increased in the T* population first, and if $k < k_m$ mutant virus increased in number in the virus population first (Nowak et al., 1997).

This initial model has proven useful for a range of researchers in beginning to inform our understanding of how mutant virus can arise and how treatment affects the balance between WT and mutant virus. The Kepler *et al.,* model was an extension of the Nowak model. In the first instance the model examined how mutant viruses arose in a cell with a single compartment for virus. To do this the investigators examined how a scaled drug concentration, termed z affected the production of mutant progeny. In this single compartment, the investigators stated the production of mutant virus depended on whether the mutant virus could propagate (p) and the overall mutant production rate (Kepler and Perelson, 1998).

Assumption 1 is primarily an increasing function of *z* such that below a positive *z* value *p* will be 0 (Kepler and Perelson, 1998), termed z_L. Factor 2 is primarily a decreasing function of *z* such that at some finite *z* value it will be 0 (Kepler and Perelson, 1998).

This is termed z_U. Incorporating this into the model and subsequent analysis revealed that the product of z_L and z_U generated a curve that peaked and reached 0 at either side (Kepler and Perelson, 1998), as shown in figure 1.6. The investigators state that it is between these two values, that mutant virus can be produced.

The model was extended by the investigators to examine the effect of different drug concentrations on mutant production. This was done in the context of a cell with 2 compartments. Compartment 1 is termed the bulk compartment which has a large volume and carries the higher drug concentration (Kepler and Perelson, 1998). Compartment 2 is termed the sanctuary which has a smaller volume and carries a lower drug concentration (Kepler and Perelson, 1998). The investigators assume that virus can move between these compartments but infected cells cannot move between the compartments (Kepler and Perelson, 1998). As there are different concentrations of drug present, term *z* is now termed z_1.

The investigators assume that a virus molecule in either compartment, termed 'i' has 3 fates:

1) It can productively infect a $CD4^+$ T helper cell, which can then produce *N* viral progeny (Kepler and Perelson, 1998).
2) The virus can perish and therefore no progeny are produced (Kepler and Perelson, 1998).
3) The virus can move to the other compartment (Kepler and Perelson, 1998).

However, for a provirus molecule in compartment 'i' to be able to propagate, then at least 1 of the progeny virions must have the ability to propagate. Taking compartment 1 as an example, a virion molecule here can either infect a $CD4^+$ T helper cell and propagate as a provirus or move to compartment 2 and propagate.

The investigators then analysed what happened to the chance of a resistant mutant virus arising in a cell with two compartments. In the sanctuary compartment, with a small drug penetrence partially resistant mutants arose, replicated and expanded in number (Kepler and Perelson, 1998). They then left the sanctuary and replicated in the bulk compartment where they had a selective advantage (Kepler and Perelson, 1998).

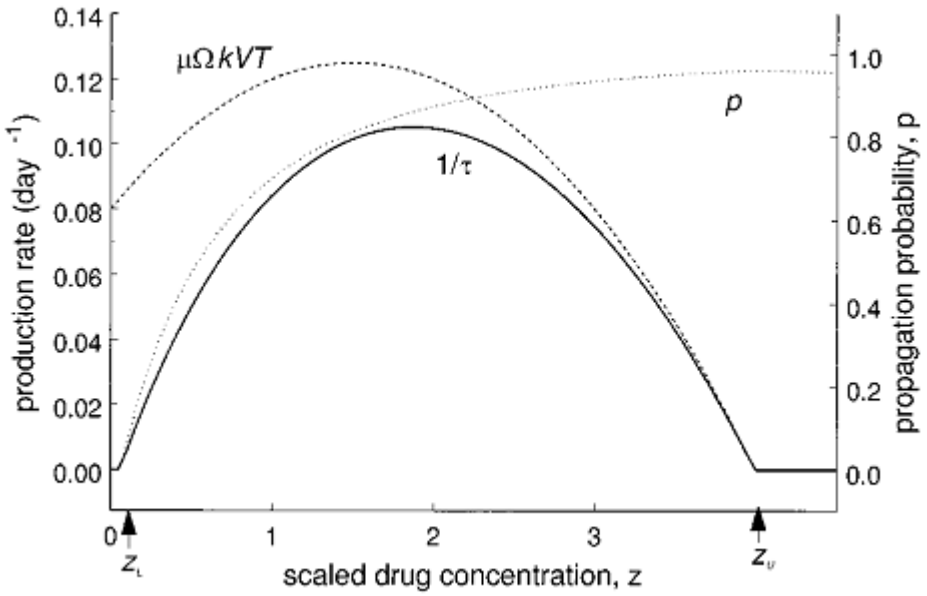

Figure 1.6: <u>The product of z_L *versus* z_U.</u>

The solid line represents the mean production rate of resistant virions which is equal to the inverse of the mean time to arrival of founding resistant virus ($1/\tau$) and remains positive over a finite window of drug concentrations, z between z_L and z_U. The dashed lines represent factors 1 and 2 (probability mutant propagates and mutant production rate). From the graph above z =4 the production rate of mutant virus reaches 0. The graph represents an acquisition of 2 independent mutations.

More recent modelling in this area has examined how multiply infected T cells can arise. Before the model was constructed a simple schematic was drawn to describe how a target cell was multiply infected with HIV (Dixit and Perelson, 2005). The initial model was designed to mimic the effect of adding HIV virions to $CD4^+$ target cells *in vitro* and then following over time the evolution of multiply infected cells. The model showed that multiply infected cells *in vitro* followed two phases characterised by an initial increase, which decreased as V increased and then a subsequent decline in numbers (Dixit and Perelson, 2005). Model calculations revealed that the number of multiply infected cells was always lower than the number of singly infected cells (Dixit and Perelson, 2005).

Other HIV-1 infection models have examined acute and long term infections and how these 2 states are controlled. The long term infection model revealed that there was a second population of infected cells, termed M* which were a source of virus, and died at a slower rate than productively infected cells or T* (Di Mascio et al., 2004). The investigators assumed that M* and T* were the only sources of virus in an infected individual. Analysis of the model by the investigators showed that anti-retroviral therapy reduced viral numbers by about 3 months post onset of treatment, but had to be maintained for at least 2-3 years, because the slower decay of virus without treatment took between 2-3 years (Di Mascio et al., 2004). The acute model by Smith *et al.*, found that during HIV-1 infection there were 2 phases. During phase I virus levels decreased and then showed exponential growth because virus was lost and there was a subsequent delay before infected cells produced virus (Smith et al., 2010). If $CD4^+$ T helper cell levels were constant, then the investigators found the virus grew exponentially, causing $CD4^+$ T helper cell decline as they became infected (Smith et al., 2010). Phase II was characterised by a much slower viral growth because $CD4^+$ T helper cell numbers were reduced and most susceptible $CD4^+$ T helper cells had become infected (Smith et al., 2010). The model also showed in phase II viral numbers peaked and began showing an exponential decrease. The investigators assume from phase II that the death rate of $CD4^+$ T helper cells influenced the decrease in viral numbers (Smith et al., 2010). This model revealed there were distinct phases in viral clearance and was useful in predicting how the length of the first phase could be used to determine the outcome of treatment for the patient.

1.4.4. Bacterial mathematical models

For bacterial infections, a lot of mathematical modelling has focussed on producing mathematical models that reliably predict the observed features of infection. For example, models of TB infection have been used to explain certain infectious phenotypes, e.g. in the case of TB why lung lesions are rare during the initial infection phase (Bru and Cardona, 2010). Also, these models can be extended to examine the impact of immune restriction on infection, and in the case of TB they demonstrated the importance of the innate immune response in controlling the spread of TB (Bru and Cardona, 2010). These models also demonstrated that macrophages struggled to control extracellular bacterial replication (Warrender et al., 2006).

An ODE model has been used to examine *Bacillus anthracis* by inhalation (Day et al., 2011). The mathematical model was designed to examine how effectively phagocytosis could contain the infection in the lung since it was not known how many spores a lung could contain (Day et al., 2011). The model was extended to analyse the effects of treatment. It defined a threshold level of exposure that the individual could tolerate and control with treatment, allowing survival but if this was exceeded the patient died (Day et al., 2011). These models show how a simple ODE model can be made more complex and effectively applied to investigation of infectious diseases.

A recent bacterial model examined carriage rates of *Streptococcus pneumoniae*. The investigators modelled a situation based on individuals who were bacterial carriers which they sub-divided further and also non-carriers i.e. a population level model (Erasto et al., 2012). The investigators defined their statistical model in the first instance and then used a Bayesian approach to estimate parameter values. This is an approach in which investigators estimate a range of parameter values and determine which set best fits the biological data. The reason for the use of this model was that the investigators could use Bayesian data augmentation to address incompletely observed data. Their data informed transmission predictions, for example it showed how the presence of one carrier in a family resulted in a twelve fold increase in the risk of acquiring the pneumococcus as compared to living n a family of non-carriers (Erasto et al., 2012). This Bayesian model has some assumptions and simplifications. Amongst these is the assumption that individuals are only carried with one strain of bacteria at a time and the model would require modification to factor in overlapping periods of colonisation with more than one strain, as occurs in high exposure settings.

Mathematical modelling of *S. aureus* infections has been very limited and most of the work has focussed on better understanding the mechanism of quorum sensing. The work surrounding quorum sensing and mathematical modelling has hinged on there being various *S. aureus* scenarios, each made of 2 populations with varying activity and that these could be present in varying combinations (Jabbari et al., 2012a). This model is based on a competitive exclusion principle whereby if 1 population is larger than the other, it will cause the other population to effectively fade out through suppression of its agr system (Jabbari et al., 2012a, Jabbari et al., 2012b). The model illustrates how when a *S. aureus* strain is present in larger numbers essentially it needs a "larger quorum sensing coefficient", and upregulates Agr to support its emergence as the "dominant" population (Jabbari et al., 2012b).

1.5. Hypothesis, aims and objectives

The interaction of *S. aureus* with the macrophage hasn't been explored in as much depth, as its interaction with neutrophils, which have been more thoroughly investigated. Since the macrophage is the resident tissue phagocyte I hypothesise that macrophage interactions with *S. aureus* are likely critical for pathogenesis. I believe that investigation of the interaction between the macrophage and *S. aureus* and the mechanisms used by *S. aureus* to avoid macrophage responses is likely to provide critical insights into the pathogenesis of *S. aureus* infection.

I hypothesise that *S. aureus* modifies macrophage responses in host defence to enhance its survival and aid its dissemination. More specifically I hypothesise that:

1) *S. aureus* modifies macrophage intracellular killing to enable persistence.
2) *S. aureus* blocks macrophage cell death processes preventing an important facet of macrophage killing of bacteria.
3) *S. aureus* uses the macrophage as a protective niche which allows it to persist and escape other aspects of host defence.

The main aim of my thesis was to characterise the kinetics of macrophage ingestion and killing of *S. aureus* and to develop mathematical ODE models to describe macrophage interactions with *S. aureus* informed by my own experimental data describing *S. aureus* phagocytosis and killing by macrophages, and then to explore whether the model predictions would hold true when these processes were modified experimentally.

More specifically I had 4 key aims:

1) Challenge THP-1 macrophages with *S. aureus* to describe phagocytosis, intracellular killing and apoptosis associated killing of *S. aureus* within macrophages.
2) Develop mathematical models to describe the above and use the mathematical model to make predictions about these cellular events when they are perturbed.
3) Manipulate phagocytosis and intracellular killing and test whether the model predictions are supported by the experimental data.
4) Use the combined experimental and modelling approach to provide novel insights as to how *S.aureus* subverts macrophage host defence.

Chapter 2 Materials and Methods

2.1. Materials

Roswell Park Memorial Institute 1640 (RPMI 1640) media was purchased from Lonza. Low endotoxin heat inactivated foetal calf serum (HIFCS) was from Promocell. 4% stock paraformaldehyde (PF, BDH Lab Supplies) was stored at -20°C and diluted 1:2 in sterile phosphate buffered saline (PBS) to make 2% working stock and stored at 4°C. 1% saponin was made by dissolving 0.1g powdered saponin (Sigma) in 10ml sterile PBS, filter sterilised and stored at 4°C. 3% bovine serum albumin (BSA) was diluted 1:10 in serum free RPMI 1640 from 30% v/v stock (Biowhitaker) and stored at 4°C. Brain heart infusion (BHI) media (Sigma) was made up according to the manufacturer's instructions, autoclaved and stored at room temperature (RT). Phorbol 12-myristate acetate (PMA, Sigma) was prepared in dimethyl sulfoxide (DMSO, Sigma) at 100μM and stored in single use aliquots at -20°C.

Lysostaphin was provided by Professor S.J.Foster, University of Sheffield. It was prepared from a solid form. 25mg of powder (stored at -20°C) was added to 20mM sodium acetate and dissolved giving a concentration of 5mg/ml. It was aliquoted as 100μl aliquots and stored at -20°C until needed. During experiments a vial was thawed and added to RPMI 1640 +10% v/v HIFCS to give the desired concentration. Kanamycin was prepared in ethanol as per manufacturer's instructions. Tetracycline was prepared in ethanol as per manufacturer's instructions.

LK broth was prepared by dissolving 10g Tryptone, 7g potassium chloride and 5g yeast extract in 1l distilled water (dH_2O). LK agar was made by adding 0.05% sodium citrate and 1.5% bacteriological agar to the previous mixture. Both were autoclaved and stored at RT until use. When prepared, appropriate antibiotics were added to the plates.

2.2. Cell line maintenance

2.2.1. THP-1 cell maintenance

THP-1 cells (ATCC) were maintained in RPMI 1640 + 10% HIFCS + 1% L-Glutamine and split on a weekly basis at a dilution of 1:3 in sterile T75 flasks by adding 20ml cell

suspension into 40ml RPMI 1640 + 10% v/v HIFCS. They were maintained at 37°C with 5% carbon dioxide (CO_2) and checked daily.

2.2.2. THP-1 cell differentiation

THP-1 cells were differentiated using 2nM PMA for 3 days followed by resting for 5 days (Daigneault et al., 2010). 1ml of cells was seeded in 24 well plates with or without coverslips at a concentration of 2 x 10^5/ml in the presence of PMA. Once seeded, the cells were placed at 37°C and 5% CO_2 for 3 days and then the media was removed and the cells left in RPMI 1640 + 10% v/v HIFCS without PMA for a further 5 days to complete differentiation. Adherence was checked after 24 hours and the cells were checked daily.

The THP-1 differentiation protocol has been characterised by the Dockrell research group and compared to differentiated monocyte derived macrophages (MDMs) to verify the cell line as a good model of differentiated tissue macrophages (Daigneault et al., 2010). A key feature of macrophage differentiation is an increase in cytoplasmic volume. It was found that similar to MDMs, PMA rested THP-1 cells showed a large increase in cytoplasmic volume and firm adherence (Daigneault et al., 2010). As wells as this, similar to MDMs, PMA rested THP-1 cells had increased granularity and autofluorescence on flow cytometry and showed increased lysosomal and mitochondrial staining (Daigneault et al., 2010). MDMs and PMA rested THP-1 cells showed similar TLR2 expression. A defining feature of macrophages is their inherent resistance to apoptosis. Both MDMs and PMA rested THP-1 cells were resistant to apoptosis induced by ultraviolet (UV) light or staurosporine (STS) and both cell types retained Mcl-1 expression following STS treatment (Daigneault et al., 2010). The capacity for bead phagocytosis was similar between both cell types. One key difference between MDMs and PMA rested THP-1 cells was in their activation profiles. PMA rested THP-1 cells produced more IL1β and TNFα in response to TLR 2 or 4 stimulation and exhibited lower cluster of differentiation 206 (CD206) levels than MDMs, a marker of AAM differentiation (Daigneault et al., 2010). Based on the above, PMA rested MDMs are suggested to represent MDMs and are useful as models of differentiated tissue macrophages, but might be more M1 than MDMs.

2.3. Monocyte derived macrophage isolation, differentiation and maintenance

Blood was collected from 2 donors and pooled. The blood was decanted aseptically into 2 sterile T75 cell culture flasks and 12.5ml Ficoll-plaque was transferred to 2 sterile 50ml falcon tubes. 25ml of blood was added to each falcon tube and centrifuged at 1500 *x g* for 23 minutes. The serum was discarded and the cell layers transferred to fresh 50ml tubes and centrifuged again at 1000 *x g* for 13 minutes. The supernatants were discarded and the pellets dislodged by gentle tapping. The cells from both tubes were combined and topped up with sterile PBS and centrifuged again at 1000 *x g* for 13 minutes. The supernatant was discarded and the pellet resuspended in 10ml fresh RPMI 1640 +10% v/v HIFCS + L glutamine. They were then diluted 1:20 and counted to give a cell concentration of 2 x 10^6/ml. The cells were then seeded in 24 well plates at 1ml volume and differentiated for 14 days with media being replaced every 2 days.

2.4. Bacterial preparation and maintenance

2.4.1. Bacterial growth

All strains used are listed in table 2.1. All *Staphylococcus aureus* strains were grown in BHI media and tested for growth in cell culture media (RPMI 1640 +10% v/v HIFCS). Bacteria were streaked overnight onto a Columbia Blood Agar (CBA) plate or a BHI plate with or without antibiotics and left at 37°C, 5% CO_2 overnight (O/N). The next day 10 colonies were added to 30ml BHI and the bacteria grown up to mid-log phase, measured by spectrophotometry using optical density (OD) 600. This typically took between 2-4 hours dependent on strain. At this point, 1ml aliquots were made and stored at -80°C. Following freezing, 2 aliquots were taken and plated out on CBA to work out the colony forming units (CFU)/ml of frozen stock.

Streptococcus pneumoniae was grown in BHI media supplemented with 10% FCS until mid-log phase was reached, measured by spectrophotometry using OD 600. At this point, 1ml aliquots were made and stored at -80°C. *Escherichia coli* was grown in BHI media until mid-log phase was reached as for all other strains and 1ml aliquots were made and stored at -80°C.

2.4.2. Preparation of nitrocellulose bead stocks

To prepare bead stocks for storage, strains were streaked out on CBA plates with or without antibiotics O/N and left at 37°C, 5% CO_2. The next day 10 colonies were taken

Strain	Sourced from	Growth conditions	Creation
S. aureus Newman	Professor S.J.Foster, UoS, MBB Department	BHI media	Wild type (WT)
S. aureus SH1000	Professor S.J.Foster, UoS, MBB Department	BHI media	WT
S .aureus kanamycin resistant (Kan^R)	Professor S.J.Foster, UoS, MBB Department, created by G.M^{c}Vicker	BHI media supplemented with 50µg/ml kanamycin	Bacteriophage transduction of a fragment bearing the kanamycin resistance cassette from strain SJF 3594* into strain Newman
S. aureus Newman GFP	This study	BHI media supplemented with 5µg/ml tetracycline	Bacteriophage transduction of a GFP bearing plasmid into strain Newman
S. pneumoniae D39	Dr Martin Bewley	BHI media with FCS	WT
E. coli strain C29, group 2 capsular serotype K54	Dr Helen Marriott	BHI media	WT

Table 2.1: List of bacterial strains used in this study.

*SJF 3594 is an RN4220 derivative containing a chromosomally integrated copy of pMUTIN4 designed to place a kanamycin resistance gene downstream of the *lysA* lysine biosynthesis gene. The resulting strain is both $LysA^+$ and $KanR^+$ and maintains antibiotic resistance without selective pressure.

and added to 30ml BHI and left growing O/N at 37°C, 5% CO_2 to allow the bacteria to reach stationary phase. Following this, they were centrifuged at 4400 *x g* for 10 minutes. The supernatant was discarded and the bacterial pellet added to nitrocellulose beads. These were left for 2 minutes, the fluid inside the bead tube was discarded and the beads stored at -80°C.

2.4.3. Newman-green fluorescent protein transduction

Green fluorescent protein (GFP) was transduced into strain Newman for live microscopy to have a fluorescent strain in the same background as all other experiments. Strain Newman was streaked out onto an LK agar plate and incubated O/N at 37°C. 1 colony was inoculated into 50ml LK and grown O/N at 37°C, 2500 *x g*. The O/N growth was centrifuged at 5000 *x g* for 10 minutes at RT and the pellet was resuspended in 3ml LK. 2 different set ups were prepared:

1) 500µl recipient cells, 1ml LK, 10µl 1M calcium chloride and 500µl lysate.
2) 500µl recipient cells, 1.5ml LK, 15µl 1M calcium chloride (control).

The above were incubated at 37°C for 25 minutes and then at 37°C, 2500 *x g* for 15 minutes. 1ml ice cold 0.02M sodium citrate was added to each and they were incubated on ice for 5 minutes and centrifuged at 5000 *x g* for 10 minutes at 4°C. The supernatant was removed and the pellets resuspended in 1ml ice cold 0.02M sodium citrate and incubated on ice for 1h. 100µl of each was spread onto LK + 5µg/ml tetracycline + citrate plates and incubated O/N at 37°C. The plates were checked for growth after 24 hours and resubcultured onto LK + 5µg/ml tetracycline + citrate plates and checked for growth after 24 hours. Frozen bead stocks were prepared and the bacteria checked for green fluorescence under the fluorescence microscope.

2.5. Bacterial infection of cell cultures

All infections were performed with *S. aureus* Newman unless stated. THP-1 cells were differentiated at 2 x 10^5/ml described in section 2.2.2. The media in the wells was replaced with 500µl fresh RPMI 1640 + 10% v/v HIFCS. A bacterial stock was thawed and centrifuged at 9300 *x g* for 1 minute (*S. aureus*) or 2 minutes (*E. coli*), the supernatant was discarded and the pellet resuspended in 1ml sterile PBS. This was centrifuged again as above, the supernatant was discarded and the pellet resuspended in 1ml sterile PBS. *S. pneumoniae* was first opsonised with immune serum containing anti-

serotype antibodies for 30 minutes at 37°C, 5% CO_2 shaking. The bacteria were then centrifuged at 13200 *x g* for 3 minutes, the pellet resuspended in 1ml PBS and centrifuged again. An aliquot of bacteria was taken and added to the differentiated macrophages to give the desired multiplicity of infection (MOI). The macrophages and bacteria was placed on ice for 1 hour to allow for bacterial adherence to the macrophages and then placed at 37°C, 5% CO_2 for the desired time course. At each time point the cells were washed 3 times with ice cold sterile PBS to stop internalisation and then 500µl fresh RPMI 1640 + 10% v/v HIFCS + 20µg/ml lysostaphin was added (*S. aureus)* or 20µg/ml gentamicin (*E. coli* and *S. pneumoniae*) and placed at 37°C, 5% CO_2 for 30 minutes to kill extracellular bacteria. Lysostaphin is *S. aureus* specific and cleaves the pentaglycine bridge in the peptidoglycan cell wall (Schindler and Schuhardt, 1964). The wells were then washed twice with sterile PBS and treated as appropriate for the various protocols.

2.5.1. Monocyte derived macrophage infection

Monocyte derived macrophages (MDMs) were differentiated at 2 x 10^6/ml for 14 days. *S. aureus* was prepared as outlined in 2.5 and MDMs were infected in a similar manner. Fresh media was added to the MDMs at the start of infection and then it proceeded in a similar fashion to THP-1 differentiated macrophages.

2.5.2. Infections with cytochalasin D

Infections were carried out as outlined in 2.5. Cytochalasin D (Sigma Aldrich) was added at the indicated time points at a concentration of 5µM in RPMI 1640 +10% v/v HIFCS.

2.6. Determination of viable intracellular bacteria using a lysostaphin protection assay

This method is similar to the gentamicin protection assay and allows enumeration of intracellular bacteria which are not affected by the extracellular lysostaphin (Baughn and Bonventre, 1975). Saponin was used because it recognises cholesterols present in the macrophage membrane but not in the bacterial membrane and therefore breaks down the macrophage membrane without affecting the intracellular bacteria.

Infections were carried out as in 2.5 at a range of MOIs. Following 30 minute treatment with lysostaphin, cells were washed twice in sterile PBS and 250µl 1% saponin (Sigma)

added and placed at 37°C, 5% CO_2 for 12 minutes. Following this, 750µl sterile PBS was added and the cells lysed by vigorously pipetting up and down. The mixture was diluted as appropriate and plated out on CBA plates using the Miles and Misra dilution technique (Miles et al., 1938) and left O/N at 37°C, 5% CO_2. The plates were counted the next day.

2.7. Lysostaphin pulse chase killing assay

The lysostaphin pulse chase killing assay or the lysostaphin protection assay is a method to enumerate intracellular surviving bacteria without the extracellular lysostaphin affecting the intracellular bacteria. It relies on the principal that the lysostaphin rapidly kills extracellular and cell surface adherent bacteria, does not penetrate the macrophages and is not rapidly taken up by macrophages (Easmon et al., 1978, Maurin and Raoult, 2001, Kumar, 2008).

This was done to allow enumeration of surviving bacteria following an initial internalisation period. The infections were set up by allowing the macrophages to internalise *S. aureus* for a certain time period. At this point the cells were washed with PBS and the remaining extracellular bacteria killed with lysostaphin. Following this the macrophages were incubated with low dose lysostaphin to prevent extracellular bacterial replication.

Macrophages were infected as outlined in 2.5 at varying MOIs and extracellular bacteria killed as described above. The time course for infection was initial infection 4-16 hours, followed by 0.5 hours with lysostaphin. Following this some wells were immediately treated with 1% saponin as outlined above to allow a starting number of viable surviving bacteria to be determined. To all other wells was added 500µl RPMI 1640 + 10% v/v HIFCS + 2µg/ml lysostaphin and left for up to 4 hours with measurements taken at various time points post lysostaphin. This low concentration was selected because it inhibited extracellular bacterial growth and did not affect intracellular viability. At each time point post lysostaphin, cells were lysed and plated out as previously described.

2.7.1. Interferon gamma pulse chase killing assay

Interferon gamma (IFNγ) is a pro-inflammatory cytokine (Denis et al., 2005). IFNγ was chosen for stimulation because it has been shown to enhance reactive nitrogen intermediate (RNI) production by macrophages (Denis, 1991). Also, during diseases

such as chronic granulamatonous disease (CGD) where patients are unable to clear bacterial infections efficiently, IFNγ is often administered to help these responses. It also enhances ROS production by phagocytes (Cassatella et al., 1990, Schroder et al., 2004).

Macrophages were seeded and differentiated as above. Half the wells were stimulated with 50ng/ml IFNγ in RPMI 1640 +10% v/v HIFCS at 37°C, 5% CO_2 for 18 hours. Half the wells were left in RPMI 1640 + 10% v/v HIFCS without IFNγ. Following this the wells were washed 3 times with 1ml sterile PBS and 500µl fresh RPMI 1640 + 10% v/v HIFCS was added and the macrophages were infected with *S.aureus* Newman for 6 hours as previously outlined. Following lysostaphin addition, some wells were lysed with 1% saponin to determine a starting surviving viable intracellular bacterial burden and the remaining wells were maintained in RPMI 1640 + 10% v/v HIFCS with 2µg/ml lysostaphin for 0.5, 1, 1.5, 2, 3 and 4 hours being lysed at time point with 1% saponin to determine viable, surviving intracellular bacteria.

2.7.2. Trolox infections

Macrophages were incubated with 50µM Trolox for 1 hour prior to the first time point.

2.8. Microscopy

2.8.1. 4'6, diamidino-2-phenylindole and fluorescein isothiocyanate staining

This was done to determine adherent *vs* internalised bacteria. All antibodies used and the concentration is listed in table 2.2. The principle is such that only extracellular and adherent bacteria should stain with the anti-staphylococcal antibody and intracellular bacteria should remain unstained. Macrophages were grown on coverslips and infected at a range of MOI's as previously outlined for up to 5 hours. This was different to other time courses to allow me to measure phagocytosis and adherence at early time points before complete intracellular degradation had occurred. At each time point cells were washed 3 times in 1ml ice cold PBS and fixed for 15 minutes with 2% PF diluted from a 4% stock solution. The cells were left in 1ml sterile PBS following PF treatment. The cells were pre-blocked for 30 minutes in 3% BSA at RT. The cells were washed 3 times with 1ml sterile PBS and then incubated with anti-rabbit IgG primary staphylococcal antibody (Zytomed Biosystems) at RT for 10 minutes. The cells were then washed 3 times with 1ml sterile PBS and incubated with anti-rabbit FITC conjugate (Sigma) at RT in the dark for 10 minutes. The cells were then washed 3 times with 1ml sterile PBS.

A drop of Vectashield mounting medium with DAPI was placed onto a slide and the coverslips from each well removed, inverted and placed on top of the drop. The coverslips were sealed with nail varnish and visualised using the Leica DMRB fluorescent microscope on the blue, green and triple filter for counts at 100x and using the Zeiss confocal microscope at 63x for imaging using the Argon and Chameleon lasers. 100 random macrophages were counted. The total number of DAPI positive bacterial cells was determined, and then those which fluoresced FITC positive were subtracted from the total to give the true number of internalised bacteria *versus* adherent bacteria.

2.8.2. Lysosomal associated membrane protein 1/2 staining

Macrophages were grown and infected as above and fixed as previously outlined. Following removal of the primary anti-staphylococcal antibody, the cells were washed 3 times in 1ml sterile PBS and stained with Alexa Fluor 568 (Invitrogen) at RT in the dark for 10 minutes. Following this the cells were washed 4 times with 1ml sterile PBS and incubated with mouse-anti LAMP-1 (Abcam) or mouse-anti LAMP-2 (Abcam) at 4°C in the dark O/N. The next day the cells were washed 5 times with 1ml sterile PBS and then incubated with Alexa Fluor 488 (Invitrogen) at RT in the dark for 90 minutes. The cells were then washed 5 times with 1ml sterile PBS and mounted and sealed as above. 100 random macrophages were counted.

2.8.3. Microscopic analysis of apoptosis

Macrophages were seeded as above and infected as outlined previously. Following lysostaphin treatment the cells were either fixed in 2% PF for 15 minutes or maintained in low dose lysostaphin at 37°C 5% CO_2. At each time point cultures were washed twice in 1ml sterile PBS and fixed as outlined previously. The cells were stained with DAPI as previously outlined. 300 macrophages/ sample were counted for apoptosis. Apoptosis was determined by fragmented nuclei or nuclei that appeared shrunken with extremely bright DAPI fluorescence. Imaging was performed using the x40 lens on the Zeiss confocal microscope and the x100 lens on the Leica DMRB microscope for counts.

2.8.4. Interferon gamma staining

Macrophages were stimulated as outlined above. They were infected with *S. aureus* Newman at MOI 5 for 1.5-5h. At each time point macrophages were washed 3 times

Antibody	Source	Primary or secondary	Species raised in	Concentration
S.aureus immunoglobulin G (IgG)	Life Sciences	Primary against soluble and structural antigens of the whole bacterium	Rabbit polyclonal (~4mg/ml)	1:1000 in PBS (0.004mg/ml)
Lysosome associated membrane protein 1 (LAMP-1)	Abcam	Primary against LAMP-1	Mouse monoclonal (0.1mg/ml)	1:100 in RPMI 1640 + 10% v/v HIFCS + 0.01% saponin (0.001mg/ml)
Lysosome associated membrane protein 2 (LAMP-2)	Abcam	Primary against CD107b/LAMP-2	Mouse monoclonal (0.1mg/ml)	1:100 in RPMI 1640 + 10% v/v HIFCS + 0.01% saponin (0.001mg/ml)
Fluorescein isothiocyanate (FITC)	Sigma	Secondary	Goat anti rabbit (2mg/ml)	1:500 in PBS + goat serum (0.004mg/ml)
Alexa Fluor 568	Invitrogen	Secondary	Goat anti-rabbit (2mg/ml)	1:250 in PBS + 0.05 Triton X-100 + 1% goat serum (0.008mg/ml)
Alexa Fluor 488	Invitrogen	Secondary	Goat anti-mouse (2mg/ml)	1:250 in PBS + 0.05 Triton X-100 + 1% goat serum (0.008mg/ml)

Table 1.2: List of antibodies used in this study.

with 1ml sterile ice cold PBS and fixed for 15 minutes in 2% PF diluted from a 4% stock. They were then washed once in 1ml sterile PBS and left in 1ml PBS until staining as outlined in 2.6.1. 100 random macrophages were counted/sample.

2.8.5. pH rhodamine staining of intracellular bacteria

pH rhodamine is a fluorescent stain that fluoresces in compartment at about pH 4.5-6. The reaction is reversible and the dye loses fluorescence if in an alkaline or neutral environment. Prior to infection, a vial of bacteria was thawed and incubated with pH rhodamine at a concentration of 10.2μM (diluted from a 10.2mM stock), at 37°C, 5% CO_2 on a shaker for 30 minutes. The bacteria were centrifuged at 9300 *x g* for 1 minute and the pellet resuspended in 1ml sterile PBS. Macrophage infection with pH rhodamine was carried out as described in section 2.4.

2.8.6. Live microscopy

Macrophages were challenged with *S. aureus* Newman~GFP at an MOI of 5 bacteria per cell for 6 hours and extracellular bacteria were killed with lysostaphin. Cultures were maintained with or without lysostaphin, in RPMI-1640 +10% (v/v) HIFCS + HEPES buffer without sodium hydrogen carbonate for up to 72 hours post infection. Imaging was taken from 52 hours to 72 hours post infection, imaging every 10 minutes using the x30 DIC/GFP lasers on the Nikon Ti inverted fluorescence microscope. Images were captured with a Neo camera (Ander) using NIS elements (Nikon). The microscope was enclosed in a temperature and humidity controlled cabinet (OKO Labs) and maintained at 37°C.

In a separate experiment macrophages were challenged with *S. aureus* Newman~GFP at an MOI of 5 bacteria per cell for 1-6 hours imaging every 10 minutes using the x30 DIC/GFP lasers on the Nikon Ti inverted fluorescence microscope. Images were captured as outlined above under the same conditions.

2.9. Bacterial co-infections

2.9.1. Newman and Kanamycin resistant infection

This was done to determine whether having been given one strain of bacteria to phagocytose the macrophages could then phagocytose a second strain. The Kan^R strain

was selected because it is an isogenic mutant and therefore maintains its resistance without antibiotic pressure. Both strains were put onto the macrophages at MOI 5. Macrophages were infected with *S. aureus* Newman for 5 hours, 6 hours or 10 hours and then extracellular bacteria were killed with 50μg/ml kanamycin at 37°C for 30 minutes. The cells were then washed twice with 1ml sterile PBS and some were placed in low dose lysostaphin for up to 7.5 hours and some were incubated *S.aureus* Kan^R for 5-7 hours at 37°C 5% CO_2.

2.10. Flow cytometry

2.10.1. Live and dead flow cytometry

Prior to challenge a vial of *S. aureus* was heat killed at 85°C for 20 minutes and then centrifuged as outlined previously. Macrophages were challenged with either heat killed or live *S. aureus* as outlined above at an MOI of 0.05 or 5 for 5 hours. Following challenge, extracellular bacteria were killed with lysostaphin and then cultures were incubated with 250μl 1% saponin for 12 minutes at 37°C. Following this 750μl sterile PBS was added to the cultures and they were lysed. The supernatants were transferred to sterile 1.5ml eppendorfs and they were centrifuged at 3300 *x g* for 0.5 minutes and then again at 100 *x g* for 8 minutes. The supernatants were transferred to a fresh sterile 1.5ml eppendorf tube and either unstained or stained with 3μM DRAQ7 for 10 minutes on ice and then analysed using the LSRII flow cytometer (BD Biosciences) using the red 660/20 laser capturing 10,000 events. Results were analysed using BD Biosciences software.

2.11. Mathematical modelling using R©

R© is a mathematical programming language allowing users to analyse data and develop new functions. This program was chosen because it is freely accessible and easy to understand and is suitable for the tasks it is being used for. R consists of three main windows, a script window (where the user enters their functions and notes), a console (where you tell R what to solve) and a plot window (where R plots graphically the outcome of the functions you have asked it solve). There is also an important fix function which allows users to check the values of the functions before plotting them, to make sure they make sense.

2.12. Statistics

All statistics are listed in the figure legends.

Chapter 3 Macrophage control of *Staphylococcus aureus* infection

3.1. Introduction

Macrophages have long been recognised as being key cells in the innate immune response to pathogens from the days of Elie Metchnikoff. The role of the macrophage in controlling *Staphylococcus aureus* infection has long been a topic of huge interest, since early work from van de Velde and colleagues showed that virulent *S. aureus* was not readily destroyed by another phagocytic cell, rabbit polymorphonuclear leukocytes whereas avirulent *S. aureus* was (Van de Velde, 1894). Since *S. aureus* seemed capable of retarding the host response of the main recruited phagocyte at sites of infection the capacity of the resident phagocyte, the macrophage, to control infection became an important research question.

The interaction between macrophages and *S. aureus* can be viewed as a triphasic response consisting of a) opsonisation of the bacterium and the migration of monocytes and macrophages towards the site of infection, b) attachment of the bacterium to macrophage phagocytic receptors and internalisation and c) intracellular killing of the bacterium (Verbrugh, 1981). Opsonisation helps the macrophage recognise *S. aureus* and principally involves the C3b component of complement or immunoglobulin G (IgG) antibody (Li and Mudd, 1965). C3b or IgG antibody attaches to the bacterial surface and are then recognised by macrophages. Following opsonisation *S. aureus* are rapidly internalised by phagocytes (Peterson et al., 1977, Verbrugh et al., 1978).

As macrophages mature, their capacity for sustained phagocytosis increases (Baughn and Bonventre, 1975). Research with alveolar macrophages (AMs) showed when the macrophage recognises *S. aureus* it becomes spread out and ingests organisms attached to the cell surface (Lee et al., 1984). Scanning electron microscopy (SEM) by Walters and colleagues in the 1970's revealed that extracellular *S. aureus* attached to receptors on macrophage lamellipodia and then a cup like structure enclosed the organism which was then internalised (Walters et al., 1976).

Macrophages have a range of receptors to bind and internalise *S. aureus* including Fcγ receptors, complement receptors and scavenger receptors, though the role of the latter has been debated (Thomas et al., 2000, DeLoid et al., 2009). Macrophages can

phagocytose both opsonised and unopsonised *S. aureus*, but the phagocytosis of unopsonised *S. aureus* was found to be a lot slower than neutrophils, and, the phagocytosis of opsonised *S. aureus* was found to be similar.

S. aureus can resist phagocytosis. This resistance can be mediated through a range of factors including a polysaccharide capsule which masks key proteins on the bacterial membrane making recognition and phagocytosis of the bacterium more difficult. However, when serum is present to act as an opsonising factor, *S. aureus* are rapidly ingested (Mackaness, 1960). It is now accepted that *S. aureus* phagocytosis is not the rate limiting step in macrophage control of staphylococcal infection (Rogers and Tompsett, 1952, Shayegani and Kapral, 1962, Jonsson et al., 1985).

Macrophage killing of *S. aureus* has been shown to be a lot slower than neutrophil killing and kinetic measurements showed the killing by macrophages to be 7 times slower than neutrophils (Green and Kass, 1964, Devalon et al., 1987). This is in spite of the fact that *S. aureus* can elicit high oxidative burst activation by macrophages early on post infection (Yamada et al., 1987). This has led to the hypothesis that although macrophages are efficient at phagocytosing *S. aureus* they may be less efficient at killing ingested bacteria and maybe need specific activation to optimise killing (Jonsson et al., 1985). Despite this the detailed kinetics of intracellular killing in primary macrophages has not been defined and is the focus of this section of my thesis.

I hypothesised that defects in bacterial clearance were more likely the result of failure to sustain killing in the face of ongoing ingestion of bacteria. I tested this by exploring in detail the kinetics of bacterial killing and its relationship to phagocytosis, carefully examining the effect of time and dose on macrophage killing of intracellular *S. aureus in vitro*. Results were related to the macrophages capacity to control the rate of growth of extracellular bacteria and I explored whether phagocytosis was maintained in the presence of incomplete microbicidal killing. This enabled a more comprehensive understanding of how antimicrobial killing of *S.aureus* in macrophages relates to phagocytosis and where the rate limiting step in bacterial clearance lies. This also provided the data I needed to allow me to start to develop a mathematical model of the interaction of macrophages and *S. aureus*.

3.2. Results

3.2.1. Lysostaphin efficiently reduces extracellular *Staphylococcus aureus*

To measure intracellular bacterial numbers accurately it is necessary to kill all extracellular bacteria efficiently using a technique that has minimal impact on intracellular bacterial numbers. In order for a bacteriolytic agent to be efficient at eliminating adherent and extracellular bacteria *in vitro* it must act rapidly, not be toxic towards the macrophages and not be taken up by the macrophages and allow intracellular killing of bacteria (Easmon et al., 1978, Kumar, 2008). Lysostaphin rapidly kills *S. aureus* and is not cytotoxic (Easmon et al., 1978). Lysostaphin is a metalloendopeptidase produced by *Staphylococcus simulans* first isolated in the 1960's (Schindler and Schuhardt, 1964) and kills *S. aureus* by cleaving the pentaglycine bridge in the peptidoglycan cell wall (Kumar, 2008). Lysostaphin is very effective at killing *S. aureus* because the cell wall contains a lot of pentaglycine bridges. Therefore I decided to investigate how effective lysostaphin was at reducing extracellular *S. aureus* numbers in the extracellular media following macrophage challenge with *S. aureus* in order to determine if lysostaphin would be a suitable bacteriolytic agent to use to kill extracellular and cell surface adherent bacteria in my infection protocols. I also explored the effectiveness of gentamicin as a comparison.

THP-1 differentiated macrophages were challenged with *S. aureus* Newman for 3 or 5 hours at a multiplicity of infection (MOI) of 5 bacteria per macrophage. After challenge cultures were washed and incubated with 5-20μg/ml of lysostaphin for 30 minutes. After incubation the extracellular supernatants were serially diluted and plated onto Columbia blood agar (CBA) plates for extracellular colony forming units (CFU) estimation in order to determine which concentration of lysostaphin to use to eliminate extracellular and cell surface adherent bacteria following exposure to *S. aureus*.

After 3 hours of challenge followed by lysostaphin treatment, extracellular CFU became undetectable at concentrations above 5μg/ml (Figure 3.1A). After 5 hours of challenge followed by lysostaphin treatment, extracellular CFU decreased with increasing lysostaphin concentration (Figure 3.1B). I also confirmed that with this dose I could still detect significant intracellular bacteria (Figure 3.1C) and that macrophage viability was not altered (Figure 3.1D). In comparison I used gentamicin at doses 20-100μg/ml and there were higher numbers of extracellular bacteria and lower numbers of intracellular

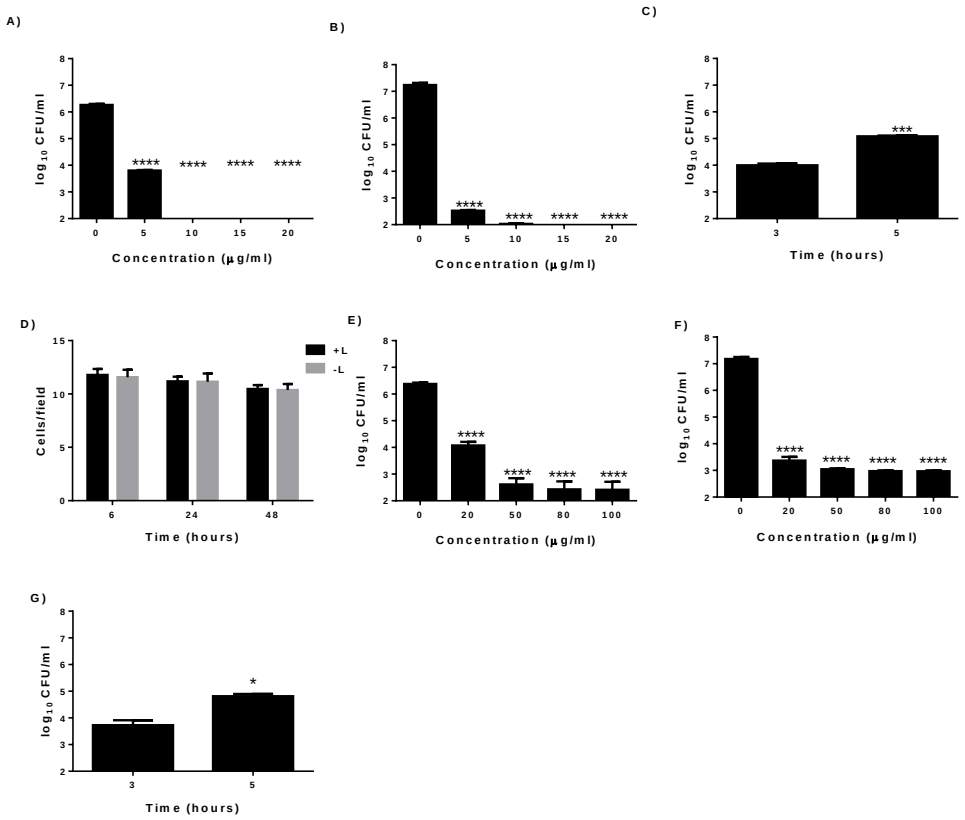

Figure 3.1: <u>Extracellular *S. aureus* colony forming unit's decreases with increasing lysostaphin/gentamicin concentration.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 3 or 5 hours and then treated with various concentrations of lysostaphin or gentamicin. THP-1 differentiated macrophages were also cultured with and without lysostaphin for up to 48 hours. Extracellular supernatants were serially diluted for extracellular colony forming units estimation and cultures were lysed for intracellular colony forming units estimation. **A)** 3 hour **B)** 5 hour challenge. The data shows the error and standard error of the mean; n=3, ****p<0.0001, One Way Anova with Dunnett's Post Test *versus* 5µg/ml. **C)** 3 and 5 hour intracellular colony forming units with lysostaphin. The data shows the error and standard error of the mean; n=3, ***p<0.01 unpaired t test *versus* 3 hours. **D)** 6 hour, 24 hour and 48 hour culture of macrophages with and without lysostaphin. The data shows the error and standard error of the mean; n=3. **E)** 3 hour **F)** 5 hour challenge and treatment with gentamicin. The data shows the error and standard error of the mean; n=3, ****p<0.0001, One Way Anova with Dunnett's Post Test *versus* 20µg/ml. **G)** 3 and 5 hour intracellular colony forming units with gentamicin. The data shows the error and standard error of the mean; n=3, *p<0.05 unpaired t test *versus* 3 hours.

bacteria (Figure 3.1E-G). Based on these results 20μg/ml lysostaphin was chosen to kill cell adherent and extracellular bacteria following macrophage challenge with *S. aureus*.

3.2.2. Dose dependent loss in the control of extracellular staphylococcal infection by macrophages

Since *S. aureus* primarily causes extracellular infections I investigated the macrophages overall ability to control extracellular bacterial replication. I hypothesised that with increasing MOI, the macrophage control of extracellular bacterial replication would become less efficient.

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 0.05, 0.5, 1, 2 and 5 bacteria per macrophage for 2-9 hours. Paraformaldehyde (PF) treated THP-1 differentiated macrophages were used as controls to measure the contribution of macrophage internalisation on regulating extracellular bacterial numbers. Also *S. aureus* was incubated in media without macrophages to determine that the PF treatment of macrophages did not alter extracellular bacterial replication, and to determine how the bacteria would replicate in the absence of macrophages. At each time point the extracellular supernatants from each condition were serially diluted and plated for extracellular CFU estimation. Live and PF treated macrophage cultures were incubated with lysostaphin and then lysed with saponin for intracellular CFU estimation.

Macrophages were able to reduce the replication of extracellular *S. aureus* but for decreasing times with increasing MOI. At an MOI of 0.05 bacteria per cell, macrophages prevented extracellular *S. aureus* replication for at least 7 hours which decreased to 5 hours for an MOI of 0.5 bacteria per cell and 3 hours for an MOI of 1 bacteria per cell (Figure 3.2A-C). With an MOI of 2-5 bacteria per cell the macrophages were overwhelmed and not able to control the extracellular bacterial replication at any time-point (Figure 3.2D-E). These results suggested that macrophages were able to regulate *S. aureus* extracellular replication for varying times at MOI ≤ 1 and above this control became compromised.

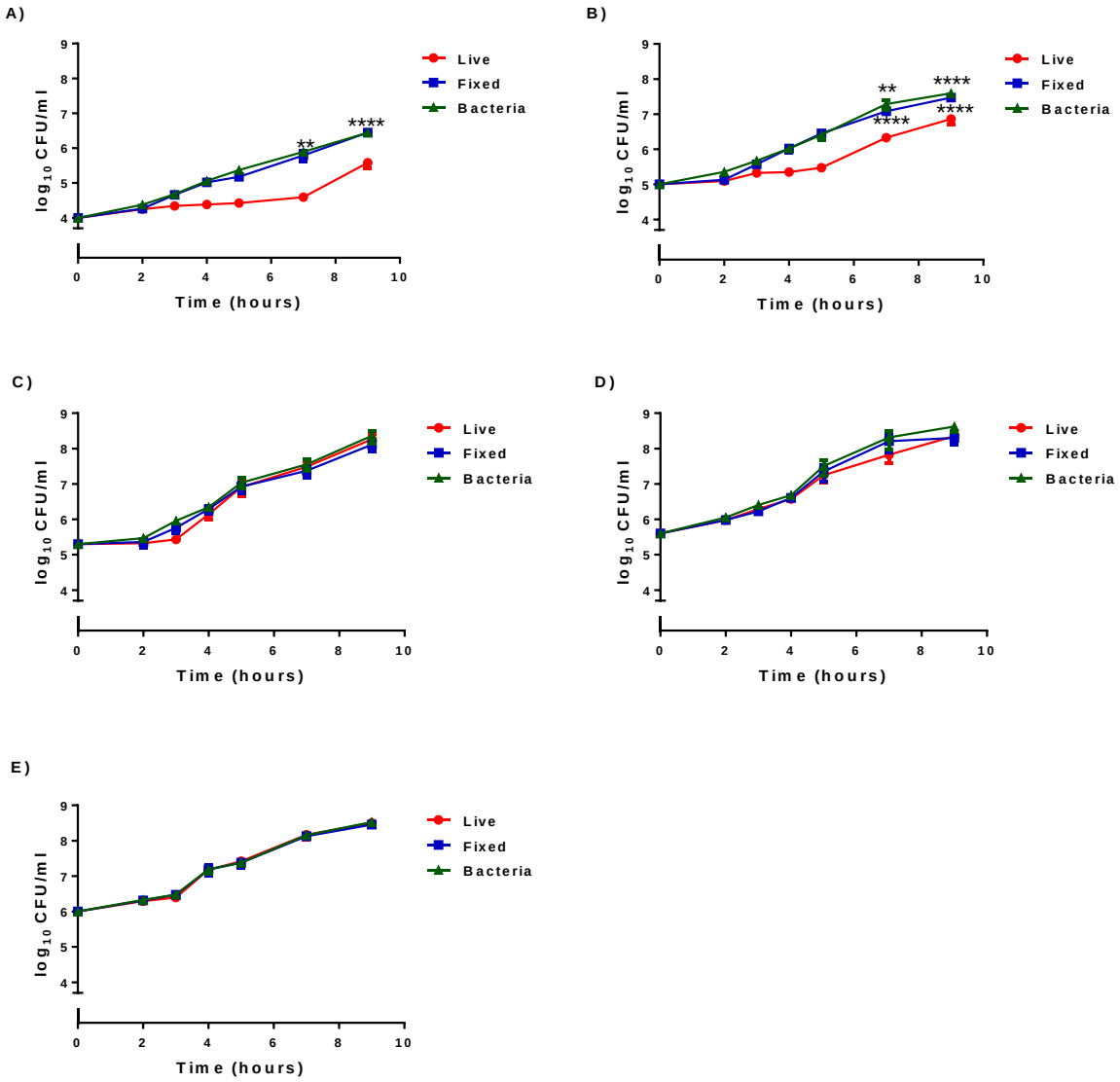

Figure 3.2: <u>Macrophages ability to control extracellular *S. aureus* replication is lost with increasing multiplicity of infection.</u>

Live or fixed THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 0.05-5 bacteria per macrophage or alternatively *S. aureus* was grown in macrophage media at each dose for 2-9 hours and colony forming units estimated in extracellular supernatants **A)** MOI of 0.05, **B)** MOI of 0.5, **C)** MOI of 1, **D)** MOI of 2, **E)** MOI of 5. The data represents the mean and standard error of the mean; n=3, ***p<0.001, ****p<0.0001, Two Way Anova with Dunnett's Post Test of Fixed *versus* Live.

3.2.3. Intracellular *Staphylococcus aureus* accumulate over time in macrophages

I also addressed the changes in intracellular bacterial burden in macrophages in the same set of experiments as I had estimated the changes in extracellular bacterial numbers. Despite the ability of macrophages to control the extracellular bacterial replication at low MOI's, there was accumulation of viable intracellular bacteria over time, even at low MOI and this increased with increasing MOI (Figure 3.3A-E).

Since this analysis was at a population level and examined only viable bacteria I next addressed what the number of adherent extracellular and intracellular bacteria was in individual cells and whether intracellular accumulation was uniformly distributed across the cell population. Since this assay didn't discriminate between viable and non-viable bacteria it had the capacity to be less influenced by killing of intracellular bacteria, as it would only be altered by complete degradation of the bacteria, a process which is predicted to take longer than initial loss of viability. It also had the potential to provide an estimate of the total burden of intracellular bacteria in the population, irrespective of viability, when the results per cell were multiplied by the total number of macrophages.

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI 0.05-25 bacteria per macrophage for 1.5-5 hours, fixed with PF and then analysed by fluorescence microscopy to determine the number of adherent extracellular and intracellular bacteria per macrophage. This protocol exploits the differential staining properties of extracellular and intracellular bacteria such that an anti-staphylococcal antibody and its FITC-conjugated secondary only label extracellular bacteria while intracellular bacteria only stain with a nuclear stain (Gordon et al., 2000).

Following challenge across the range of MOI's I found only a modest increase with time in the numbers of cell surface adherent bacteria per cell which doubled across the range of MOI (Figure 3.4A-F). This suggested the numbers of adherent bacteria is fairly constant either due to a limited and fixed level of cell surface engagement or more rapid internalisation of a greater number of surface bound bacteria as the MOI increases, such that surface numbers of bound bacteria remain at a fairly fixed level and only increase over a limited range. In contrast to adherent cell numbers the number of intracellular bacteria showed a more marked dynamic range with accumulation at the higher MOI over time (Figure 3.4A-F). There was an approximate 5 fold increase in intracellular bacteria as the MOI increased by the 5 hour time point. This suggested there was either an increased capacity to phagocytose bacteria that was offset by a failure to match the

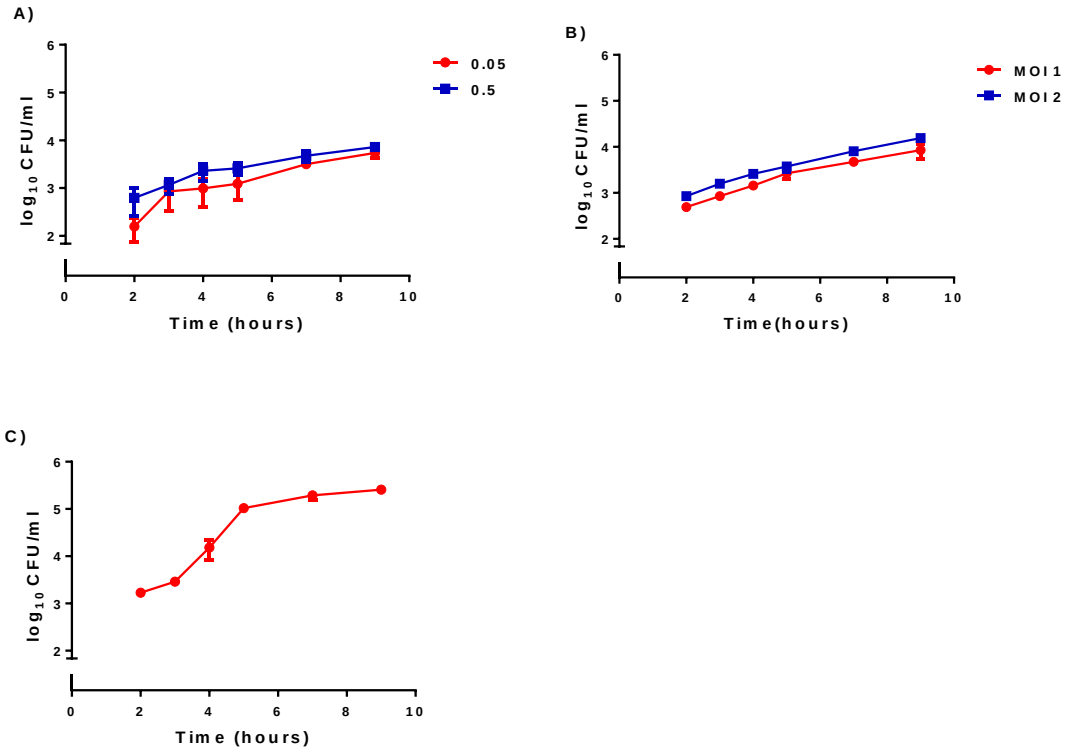

Figure 3.3: <u>Macrophages accumulate intracellular viable *S. aureus* at all multiplicities of infection.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI 0.05-5 bacteria per macrophage for 2-9 hours and then treated with lysostaphin. Cultures were then lysed and intracellular colony forming units estimated **A)** MOI of 0.05 and 0.5, **B)** MOI of 1 and 2, **C)** MOI of 5. The data represents the mean with the standard error of the mean; n=3.

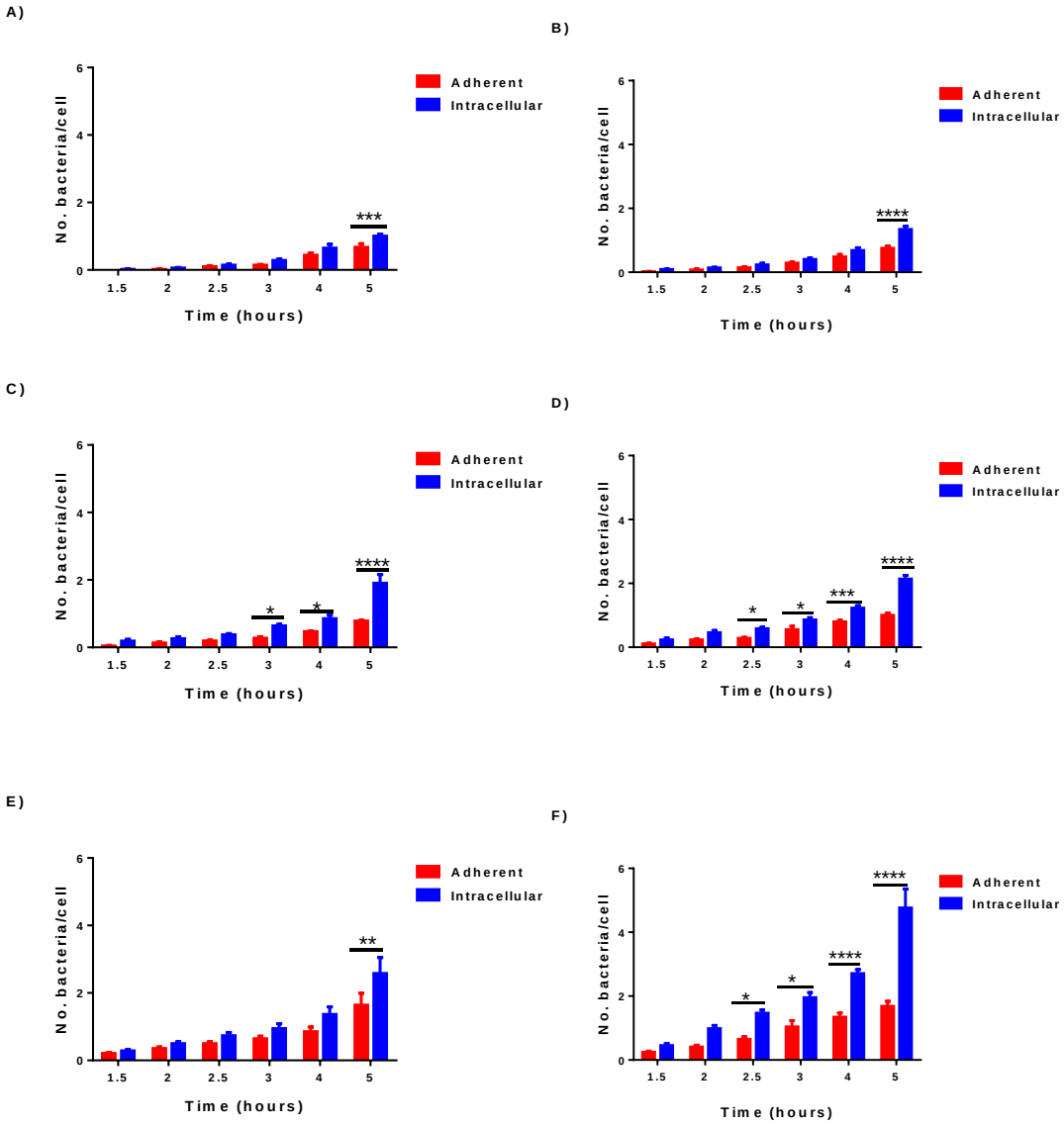

Figure 3.4: <u>The numbers of extracellular adherent bacteria and internalised bacteria with macrophages.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 1.5-5 hours and fixed and stained at each time point. **A)** MOI of 0.05, **B)** MOI of 0.5, **C)** MOI of 1, **D)** MOI of 2, **E)** MOI of 5, **F)** MOI of 25. The data represents the mean with the standard error of the mean; n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Two Way Anova with Bonferonni Post Test, adherent *versus* internalised.

increased ingestion to a comparable increase in the rate of killing or that phagocytosis remained fixed but that intracellular killing capacity decreased. The latter possibility would seem less likely, in particular since the microscopy experiments suggested an increase in the total population intracellular burden at an MOI of 5 as compared to 0.05, and if the difference in viable intracellular bacteria was solely the result of decreased killing with phagocytosis held at a similar level one would have predicted that the numbers calculated by microscopy would have been more similar. Overall these results suggested bacteria were accumulating inside the macrophage due to a failure to match increased ingestion with increased intracellular killing.

Similar to the previous result, the percentage of macrophages that had intracellular bacteria at 5 hours increased approximately 5 fold over the MOI range, suggesting the accumulation of bacteria was the result of more cells accumulating bacteria, rather than a fixed number of cells accumulating more bacteria as the MOI increased (Figure 3.5A-F). It should also be noted that the fold increase (approximately 5 fold) in intracellular CFU and in the percentage of macrophages with bacteria over the MOI range was much less than the increase in the original MOI (100 fold) and shows that the macrophages clearance capacity was failing to keep up with the increased bacterial numbers hence the failure to contain extracellular numbers at higher MOI.

Confocal microscopy images at 5 hours after bacterial challenge at an MOI of 5 bacteria per cell demonstrated there were at least 3 distinct populations of macrophages. The first group clearly showed small numbers of intracellular bacteria and some cell surface adherent bacteria (Figure 3.6A). The second group demonstrated ~ 50 bacteria per macrophage (Figure 3.6B-C) and the final group termed the "super-ingesters" were harbouring large numbers (>50) of intracellular bacteria (Figure 3.6D).

3.2.4. *S. aureus* phagocytosis by macrophages is saturatable

I next investigated whether with very high doses macrophage accumulation of intracellular bacteria would peak. This would suggest that the putative increase in the capacity for phagocytosis of *S. aureus* would become saturated and that the equilibrium between ingestion and intracellular killing would reach a steady state that was unaltered by increased numbers of extracellular bacteria.

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5, 25 or 125 bacteria per macrophage for 1.5-5 hours. At each time point extracellular and cell

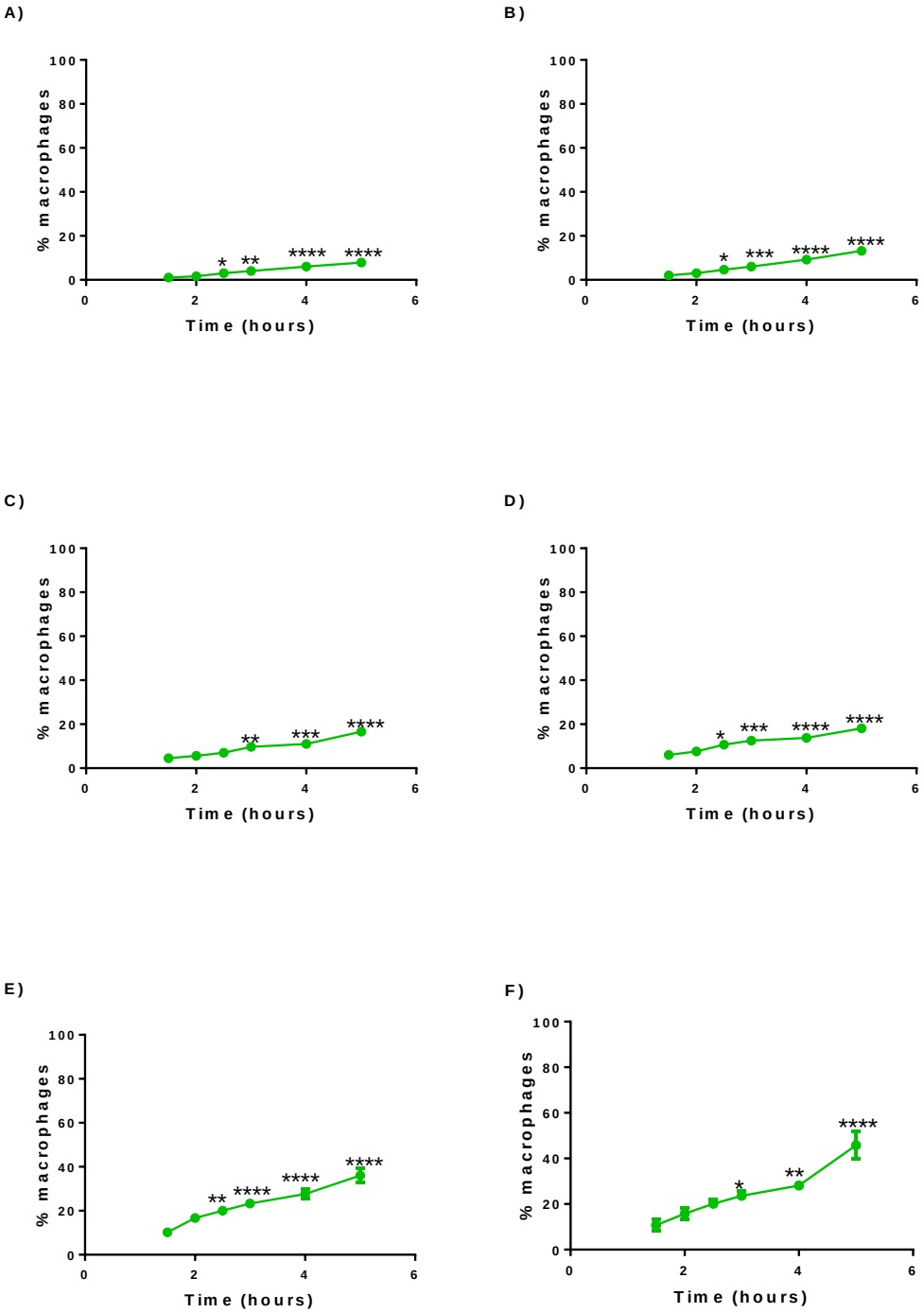

Figure 3.5: <u>The percentage of macrophages ingesting bacteria.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 1.5-5 hours and fixed and stained at each time point. **A)** MOI of 0.05, **B)** MOI of 0.5, **C)** MOI of 1, **D)** MOI of 2, **E)** MOI of 5, **F)** MOI of 25. The data represents the mean with the standard error of the mean; n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, One Way Anova with Dunnett's Post Test *versus* 1.5 hour.

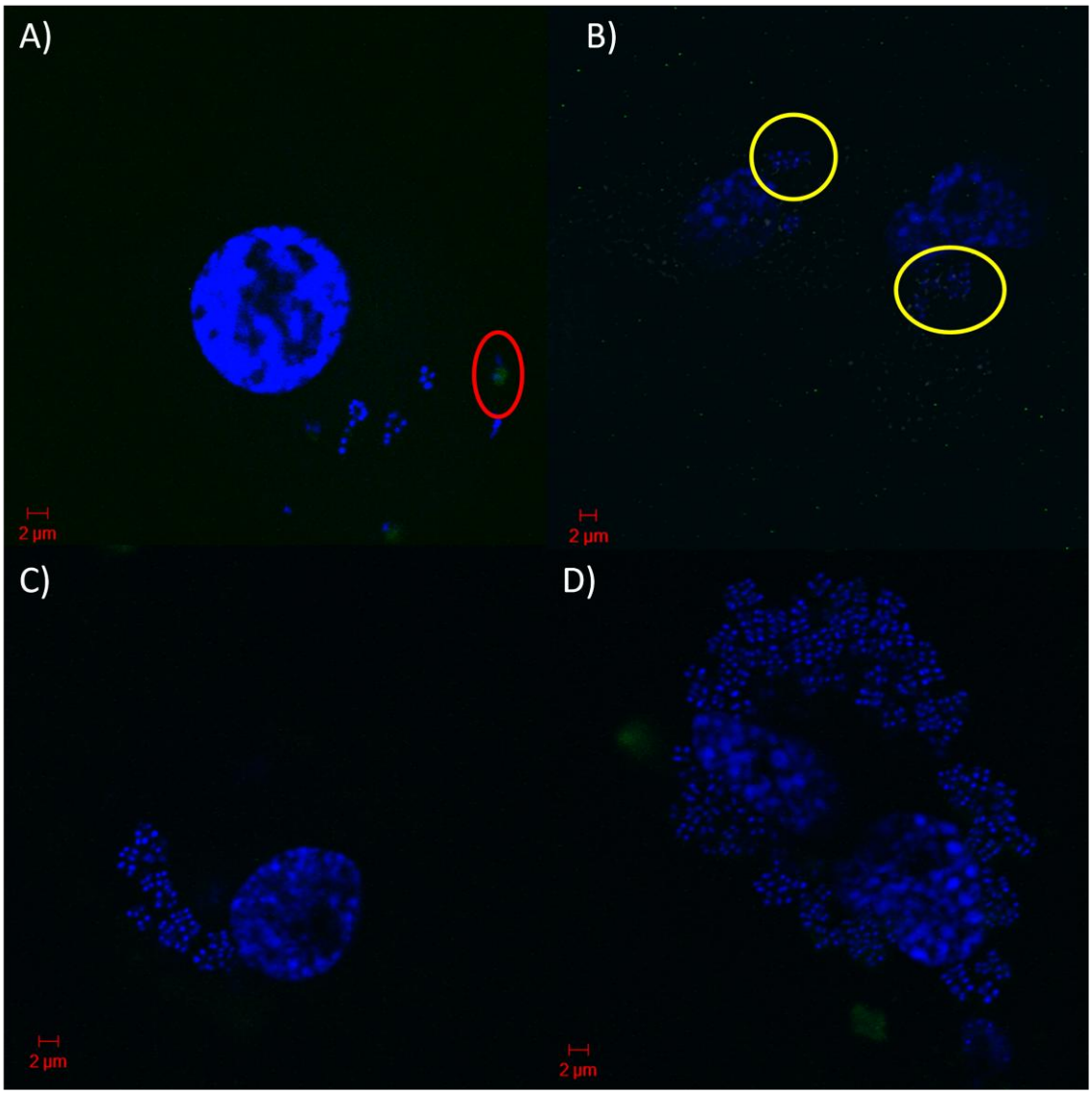

Figure 3.6: <u>Macrophages phagocytose *S. aureus* at varying levels.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 5 hours and fixed and stained. Images were taken using the x63 magnification of the Zeiss laser scanning confocal microscope. The red circle represents adherent bacteria on the surface of a macrophage. The yellow circles indicate internalised bacteria. Macrophages are shown with **A)** Adherent bacteria and internalised bacteria, **B)** Internalised bacteria only, **C)** An intermediate number of internalised bacteria, **D)** a large number of internalised bacteria ("super-ingester.") Scale bar represents 2µm.

surface adherent bacteria were killed with a lysostaphin protection assay, macrophages lysed and intracellular CFU estimated. Fixed macrophages were used as a control to confirm bacteria estimated to be intracellular were not the result of incomplete killing of extracellular bacteria and confirmed CFU in fixed cultures were constantly undetectable.

I found that the numbers of intracellular viable bacteria increased with increasing MOI (Figure 3.7) suggesting the macrophages were continuing to increase the capacity to accumulate *S. aureus*, even at MOI 125. This presumably reflected either continued increases in phagocytosis or further reduction in killing capacity despite maintenance of phagocytosis rate, which as before seemed less likely. However, by an MOI 125 there was little increase in the number of intracellular viable bacteria compared to MOI 25, suggesting accumulation eventually becomes saturated without further capacity to increase phagocytosis or reduce intracellular killing.

3.2.5. Macrophages demonstrate a time and dose dependent loss in the intracellular killing of *S. aureus*

The rate limiting step in infection control by macrophages may be intracellular killing (Jonsson et al., 1985). Having demonstrated in the previous sections that macrophages were efficient at phagocytosing *S. aureus*, and able to increase phagocytic capacity up to a high MOI I next wanted to investigate the proportion of phagocytosed *S. aureus* that were killed over time by macrophages and examine the intracellular fate of phagocytosed bacteria. My data so far have measured numbers of intracellular bacteria, which is a cumulative end-point, influenced by rate of internalisation, intracellular killing and potentially intracellular bacterial replication, though the latter is not believed to be significant for *S. aureus* in macrophages over the time course I have studied (Kubica et al., 2008). To examine this I used an adaptation of the lysostaphin protection assay, which is similar to an adaptation of the gentamicin protection assay and relies on killing extracellular or cell surface adherent bacteria with a higher dose of the antimicrobial and then maintaining cultures in a low dose of the antimicrobial to inhibit extracellular replication and prevent any ongoing bacterial ingestion, without being toxic to the phagocytes or being taken up by the phagocytes to significant levels over extended periods of culture, therefore not reducing intracellular bacterial numbers. Lysostaphin is effective in this assay and was previously described to not be absorbed

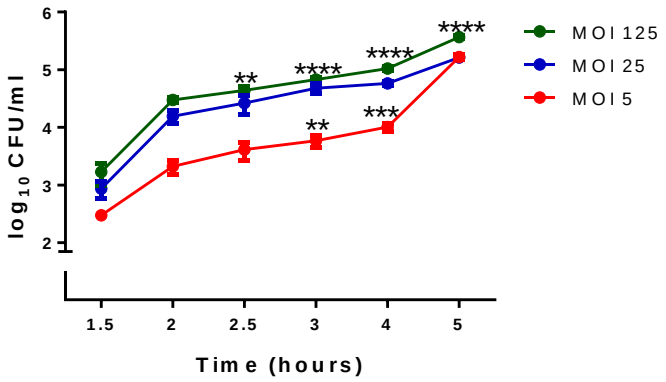

Figure 3.7: <u>Macrophage phagocytosis of *S. aureus* is saturatable.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5, 25 or 125 bacteria per cell for 1.5-5 hours and lysed at each time point to allow intracellular colony forming unit estimation. The data represents the mean with the standard error of the mean; n=3, **p<0.01, ***p<0.001, ****p<0.0001, Two Way Anova with Dunnett's Post Test *versus* 1.5 hour.

by polymorphonuclear leukocytes (Tan et al., 1971). In addition lysostaphin has not been demonstrated to penetrate mammalian plasma membranes over extended time periods e.g. days and weeks (Easmon et al., 1978, Maurin and Raoult, 2001, Kumar, 2008).

To establish the kinetics of intracellular killing I set up a pulse-chase lysostaphin protection assay which involved pulsing differentiated macrophages with *S. aureus* for a defined time period, using lysostaphin to kill extracellular and cell surface adherent bacteria and then maintaining the cultures in lower doses of lysostaphin for defined time periods to ensure there was no ongoing internalisation (chase).

THP-1 differentiated macrophages were challenged with *S. aureus* for 4-6 hours at an MOI of 5, 25 and 125 bacteria per macrophage. Additionally THP-1 differentiated macrophages were challenged with *S. aureus* for 10-16 hours at an MOI of 5 bacteria per macrophage. Following lysostaphin incubation, macrophages were either lysed for intracellular CFU estimation or maintained in culture with the lower dose of lysostaphin for 0.5- 4 hours and lysed at each time point to calculate the intracellular CFU.

In all experiments I found the majority of intracellular killing was occurring within the first 30 minutes following cessation of phagocytosis and then there was a plateau phase with a persistence of intracellular viable bacteria with only very modest killing occurring during this phase. After 4 hours with an MOI of 5, there was ~ 90% killing of intracellular bacteria within the first 30 minutes but the percentage of intracellular bacteria killed within the first 30 minutes decreased with increasing MOI (Figure 3.9). In addition to this, increasing incubation time with 5 and 6 hours led to a decrease in the percentage of killing occurring in the first 30 minutes, as compared to cultures incubated with bacteria for 4 hours, and again, this was dose dependent, decreasing with increasing MOI (Figure 3.8A-C). Increasing the exposure time of the macrophages to *S .aureus* to 10-16 hours further reduced the percentage of intracellular bacteria killed in the first 30 minutes after phagocytosis ceased, the percentage cleared reaching 1% after 16 hours of incubation with bacteria (Figure 3.9A-D). I also performed fluorescence microscopy, and found there was no decrease in intracellular bacteria per macrophage over time and there was no decrease in the percentage of macrophages with intracellular bacteria suggesting intracellular killing was exhausted (Figure 3.10A-B). Interestingly when I compared the absolute numbers of intracellular bacteria killed within the first 30 minutes for each exposure time and compared it to the percentage of intracellular

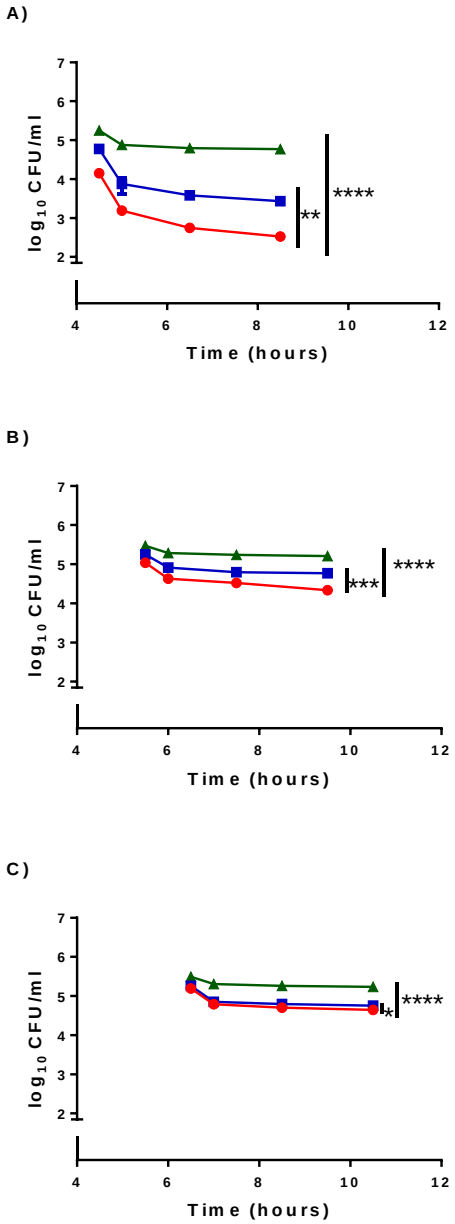

Figure 3.8: <u>Macrophage killing of intracellular *S .aureus* occurs predominantly in the first 30 minutes after ingestion.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5, 25 or 125 bacteria per macrophage for 4-6 hours, and following high dose lysostaphin treatment for 0.5 hours were either lysed or maintained in low dose lysostaphin for 0.5-4 hours and lysed at each time point to calculate intracellular colony forming units **A)** 4 hour **B)** 5 hour, **C)** 6 hour initial bacterial challenge. The data represents the mean with the standard error of the mean; n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Two Way Anova with Dunnett's Post Test *versus* MOI 5.

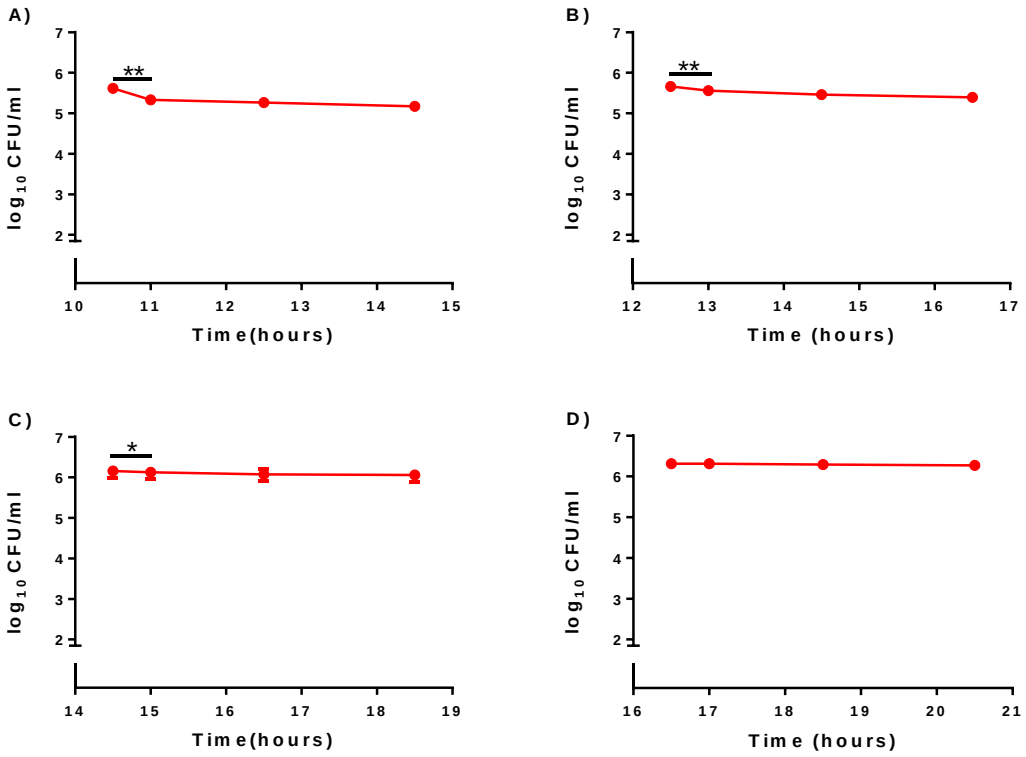

Figure 3.9: <u>Macrophages exhaust their intracellular killing abilities over time.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 10-16 hours, and following high dose lysostaphin treatment for 0.5 hours were either lysed maintained in low dose lysostaphin for 0.5-4 hours and lysed at each time point to allow calculation of intracellular colony forming units **A)** 10 hour, **B)** 12 hour, **C)** 14 hour, **D)** 16 hour initial bacterial challenge. The data represents the mean with the standard error of the mean; n=3, *p<0.05, **p<0.01, Paired t test comparing first two time points.

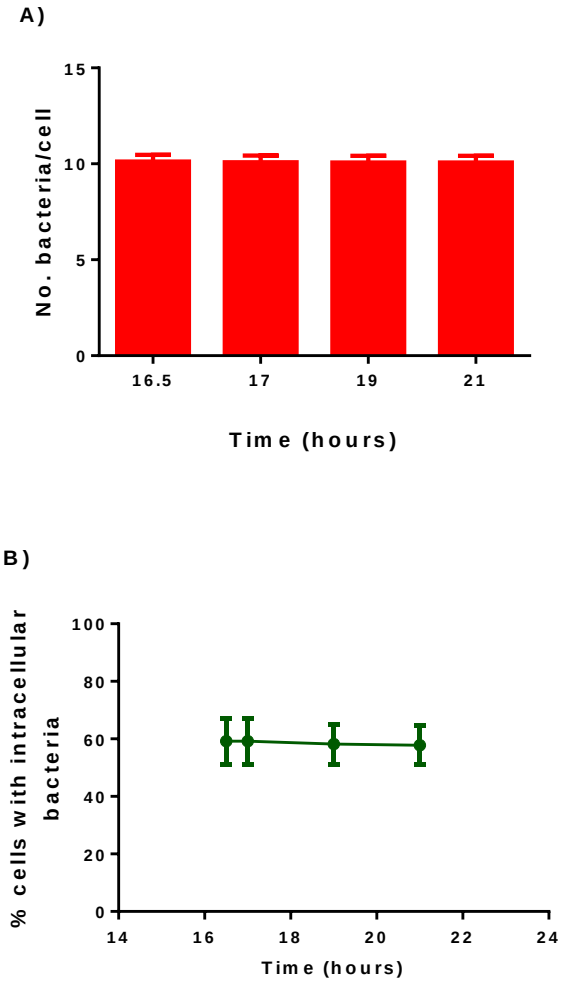

Figure 3.6: <u>Intracellular bacteria are not killed by 16 hours post infection.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 16 hours, and following high dose lysostaphin treatment for 0.5 hours were maintained in low dose lysostaphin for 0.5-4 hours and fixed and stained **A)** Number of intracellular bacteria per cell, **B)** Percentage of macrophages with intracellular bacteria. The data represents the mean with the standard error of the mean; n=3.

bacteria killed within the first 30 minutes, I found the absolute number of intracellular bacteria killed within the first 30 minutes increased for the first few hours of bacterial challenge and then was maintained until approximately 14 hours after which it rapidly decreased, whereas, the percentage of intracellular bacteria killed declined over time (Figure 3.11A-B). This suggested the decreased percentage killing was merely a result of the increasing intracellular bacterial accumulation with time, whereas the absolute level of intracellular killing remained close to maximal for several hours up to 14 hours of exposure to bacteria.

3.2.6. Interferon gamma only modestly enhances intracellular killing of *S. aureus*

Since the intracellular killing of *S. aureus* by macrophages seemed to be occurring at a fairly constant rate despite increasing intracellular accumulation, I next investigated whether stimulation of macrophages would further enhance the intracellular killing capacity. Macrophages can be activated via a range of signals which alter their metabolic and functional capacity (Easmon et al., 1978). Since the initial killing phase of intracellular *S. aureus* in neutrophils is dependent upon reactive oxygen species (ROS) and cationic proteases (Reeves et al., 2002) and the production of these is enhanced by interferon gamma (IFNγ) stimulation (Cassatella et al., 1990, Denis, 1991, Denis et al., 2005, Decker et al., 2005) I investigated the effect of prior IFNγ stimulation of macrophages on their ability to kill phagocytosed intracellular *S. aureus*.

THP-1 differentiated macrophages were stimulated with IFNγ prior to infection. Unstimulated macrophages were used as a control. Unstimulated and stimulated macrophages were then challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 6 hours. Extracellular and cell surface adherent bacteria were killed with lysostaphin and then macrophages were lysed for intracellular CFU estimation or maintained in low dose lysostaphin for 0.5-4 hours and lysed at each time point to calculate intracellular CFU.

I found that IFNγ stimulation only modestly increased intracellular killing (Figure 3.12A), as reflected by a significantly lower baseline level of viable intracellular bacteria, which remained lower as the pulse of bacteria underwent intracellular killing. To eliminate the possibility that the reduction in intracellular counts within stimulated macrophages was due to decreased phagocytosis I challenged stimulated and unstimulated macrophages with *S. aureus* at MOI 5 for 1.5-5 hours and fixed cultures at each time point and stained them as outlined in 3.2.3. I found that both stimulated and

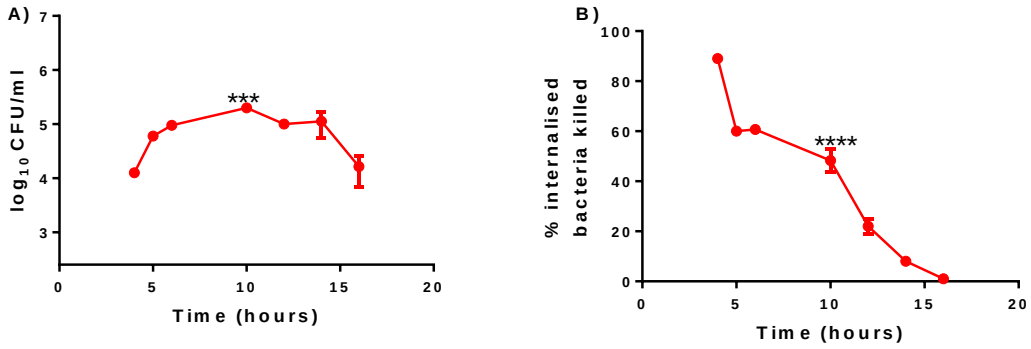

Figure 3.7: <u>Absolute numbers of intracellular bacteria killed and percentage intracellular bacteria killed in the first 30 minutes.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 4-16 hours, and following high dose lysostaphin treatment for 0.5 hours were either lysed or maintained in lysostaphin for 0.5 hours and lysed at each time point to calculate intracellular colony forming units **A)** Absolute intracellular bacteria killed in the first 30 minutes **B)** Percentage intracellular bacteria killed in the first 30 minutes. The data represents the mean with the standard error of the mean; n=3. ***p<0.001, ****p<0.0001, One Way Anova with Dunnett's Post Test *versus* 4 hours.

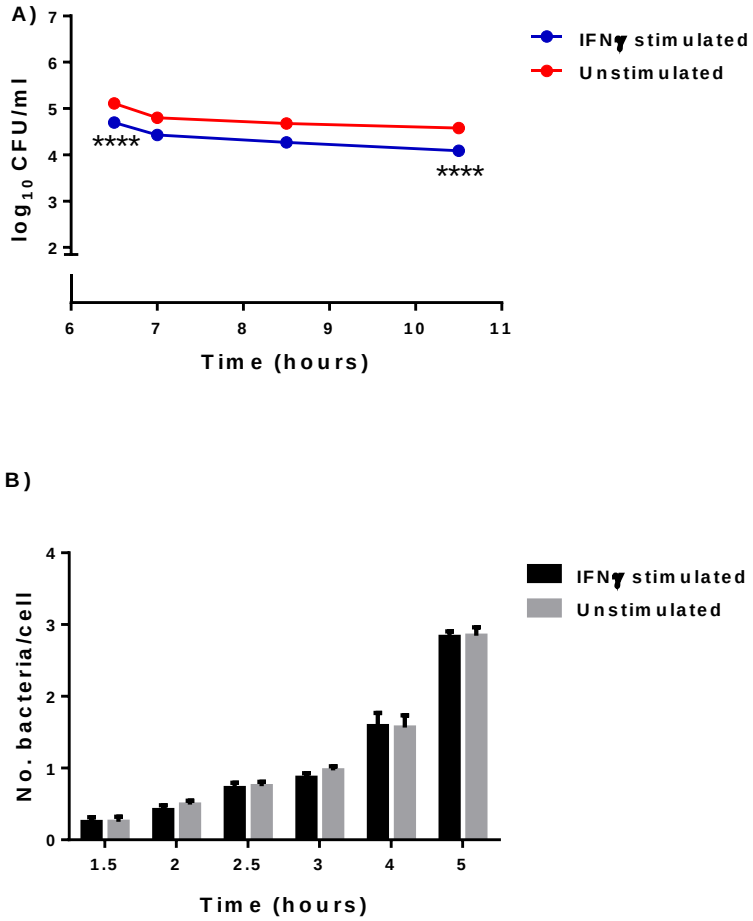

Figure 3.8: <u>Interferon gamma stimulation only modestly enhances intracellular killing of *S. aureus*.</u>

Macrophages were exposed to IFNγ or left unstimulated and then challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 6 hours, and following high dose lysostaphin treatment for 0.5 hours were either lysed or maintained in low dose lysostaphin for 0.5-4 hours and lysed at each time point to allow intracellular colony forming units estimation. Alternatively stimulated or unstimulated macrophages were challenged with *S. aureus* for 1.5-5 hours and fixed and stained at each time point **A)** Intracellular viable colony forming units, **B)** Intracellular bacteria per cell. The data represents the mean with the standard error of the mean; n=3, ****p<0.0001, Two Way Anova with Bonferonni Post Test, IFNγ treated *versus* unstimulated.

unstimulated macrophages demonstrated similar levels of intracellular bacteria over this time course (Figure 3.12B) suggesting they were phagocytosing similar numbers of bacteria.

3.2.7. Macrophages still continue to phagocytose bacteria even after their capacity for intracellular killing becomes exhausted

Having demonstrated that the intracellular killing capacity for *S. aureus* by macrophages became exhausted with time, I next investigated whether macrophages that could no longer kill ingested bacteria could still phagocytose *S. aureus*. To examine this I set up a variation of the pulse chase assay described previously which involved challenging THP-1 differentiated macrophages with wild type (WT) *S. aureus* at an MOI of 5 bacteria per macrophage for 5, 6 or 10 hours. Extracellular and cell surface adherent bacteria were killed with kanamycin, and then cultures were either maintained in low dose lysostaphin for 5-7 hours or exposed to a kanamycin resistant (Kan^R) *S. aureus* at an MOI of 5 bacteria per macrophage for a further 5-7 hours, before extracellular and cell surface adherent bacteria were killed with lysostaphin and macrophages were lysed for estimation of the intracellular CFU. Lysates were plated with and without kanamycin to allow detection of total and Kan^R intracellular CFU, so that the contribution of bacteria ingested at later time points to the total intracellular load could be estimated.

I found that at all time points macrophages were able to phagocytose the Kan^R *S. aureus* (Figure 3.13A-C). Interestingly, the macrophages were still capable of phagocytosing *S. aureus* Kan^R at 16 hours when intracellular killing had decreased and levels of intracellular Kan^R bacteria weren't significantly different after the 5 hour pulse with the resistant bacteria (Figure 3.13C). The second pulse of bacteria did not alter the number of viable intracellular Kan^S resulting from the original bacterial pulse.

3.2.8. Macrophage inability to completely kill intracellular *S. aureus* is not merely the result of exposure to high multiplicity of infection

I next wanted to explore whether the inability of macrophages to clear intracellular *S. aureus* was merely a consequence of exposure to high MOI, as all these experiments had been conducted with an MOI of 5 or greater. THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 0.05 or 1 bacteria per macrophage for 6 and 16 hours. Extracellular and cell surface adherent bacteria were killed with

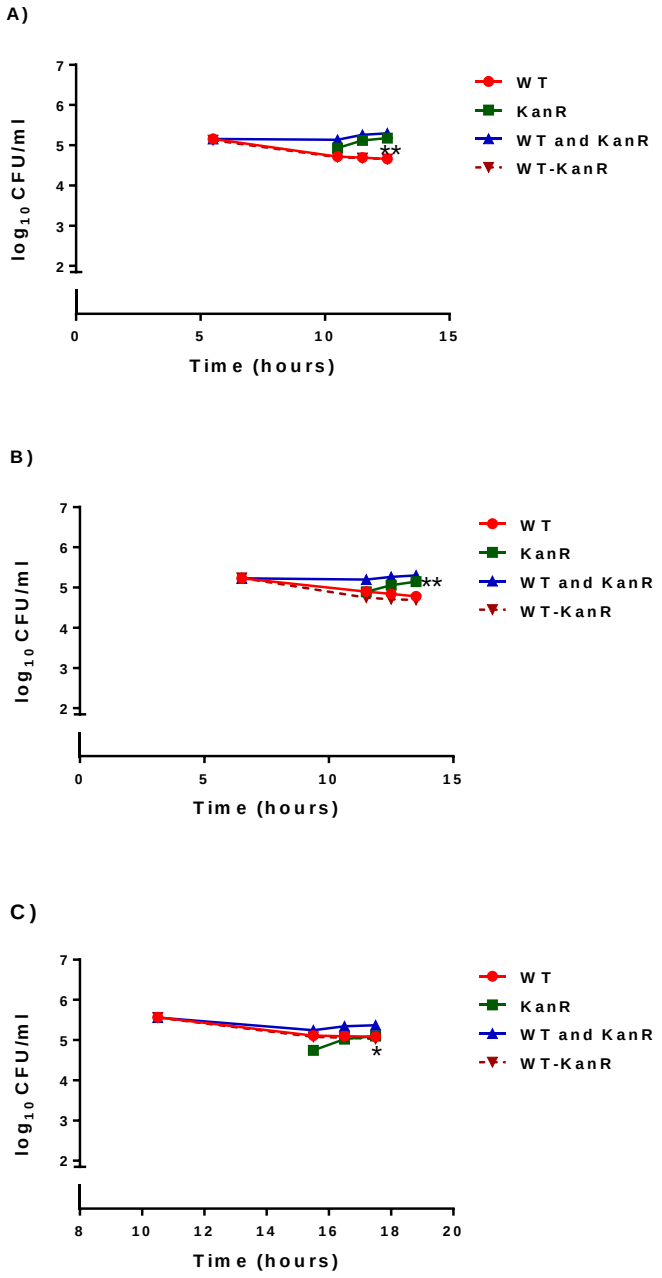

Figure 3.9: <u>Macrophages phagocytose *S. aureus* when intracellular killing is exhausted.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 5, 6 or 10 hours, and following kanamycin treatment for 0.5 hours were either lysed or maintained in lysostaphin for 5.5-7.5 hours or challenged with *S. aureus* Kan^R for 5-7 hours, and following lysostaphin treatment for 0.5 hours were lysed for intracellular colony forming units estimation. **A)** 5 hour, **B)** 6 hour, **C)** 10 hour initial bacterial challenge. The data represents the mean with the standard error of the mean; n=3, * $p< 0.05$, **$p<0.01$, Paired T test 5.5 and 7.5 hours Kan^R.

lysostaphin and followed for varying time periods in the presence of low dose lysostaphin to follow the decay in intracellular CFU estimation.

I found for both MOI's a similar trend was occurring to that observed before for higher MOI, with the majority of intracellular killing occurring within the first 30 minutes after phagocytosis was prevented and then persistence of a small number of viable intracellular bacteria (Figure 3.14A-D). The initial killing phase decreased with increasing dose (~ 90% for MOI 0.05 and ~ 60% for MOI 1) at 6 hours, and was close to 0% by 16 hours post exposure suggesting macrophages had exhausted their intracellular killing capabilities even at lower MOI's.

3.2.9. Macrophage inability to completely kill intracellular *S. aureus* is not cell line specific

I next wanted to explore whether the inability of macrophages to clear intracellular *S. aureus* was merely a consequence of using cell lines, as all these experiments had been conducted with the THP-1 cell line. 14 day differentiated monocyte derived macrophages (MDMs) were challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 4, 6 and 16 hours. The kinetics of viable intracellular bacterial number decay was then measured as before.

I found a similar trend was occurring to that observed before for THP-1 differentiated macrophages, with the majority of intracellular killing occurring within the first 30 minutes after phagocytosis was prevented and then there was persistence of viable intracellular bacteria for up to 24 hours (Figure 3.15A-C). The initial killing phase decreased with increasing time becoming less than 1% by 16 hours post exposure, suggesting macrophage exhaustion of intracellular killing capabilities with *S. aureus* was not merely an artefact of using the THP-1 cell line differentiated macrophages .

3.2.10. Macrophage inability to completely kill intracellular *S. aureus* is not a Newman dependent phenotype

I next wanted to explore whether the inability of macrophages to clear intracellular *S. aureus* was strain specific, as all these experiments had been conducted with the Newman strain. THP-1 differentiated macrophages were challenged with *S. aureus* SH1000 at an MOI of 5 bacteria per macrophage for representative time periods and the decay in intracellular viable bacteria estimated.

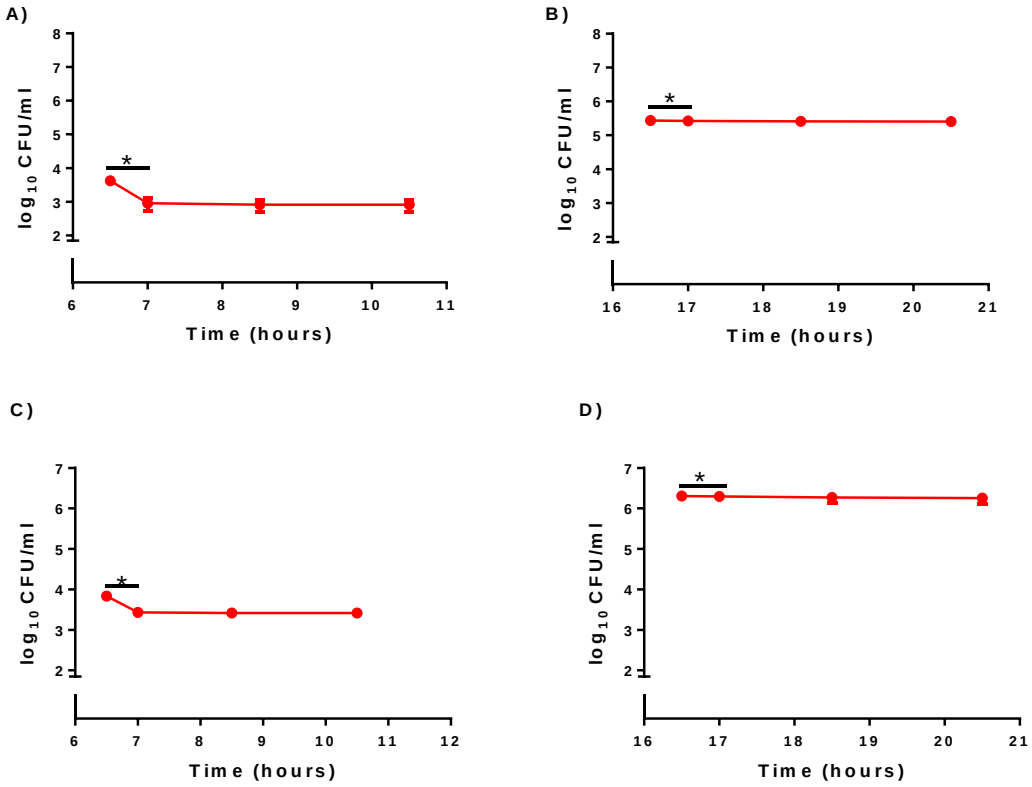

Figure 3.10: <u>Intracellular bacteria persist at low MOIs.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 0.05 or 1 bacteria per macrophage for 6 and 16 hours, and following high dose lysostaphin treatment for 0.5 hours were either lysed or maintained in low dose lysostaphin for 0.5-4 hours and lysed at each time point for intracellular colony forming units estimation **A)** MOI 0.05 6 hour, **B)** MOI 0.05 16 hour, **C)** MOI 1 6 hour, **D)** MOI 1 16 hours bacterial challenge. The data represents the mean with the standard error of the mean; n=3, * p< 0.05, Paired t test comparing first two time points.

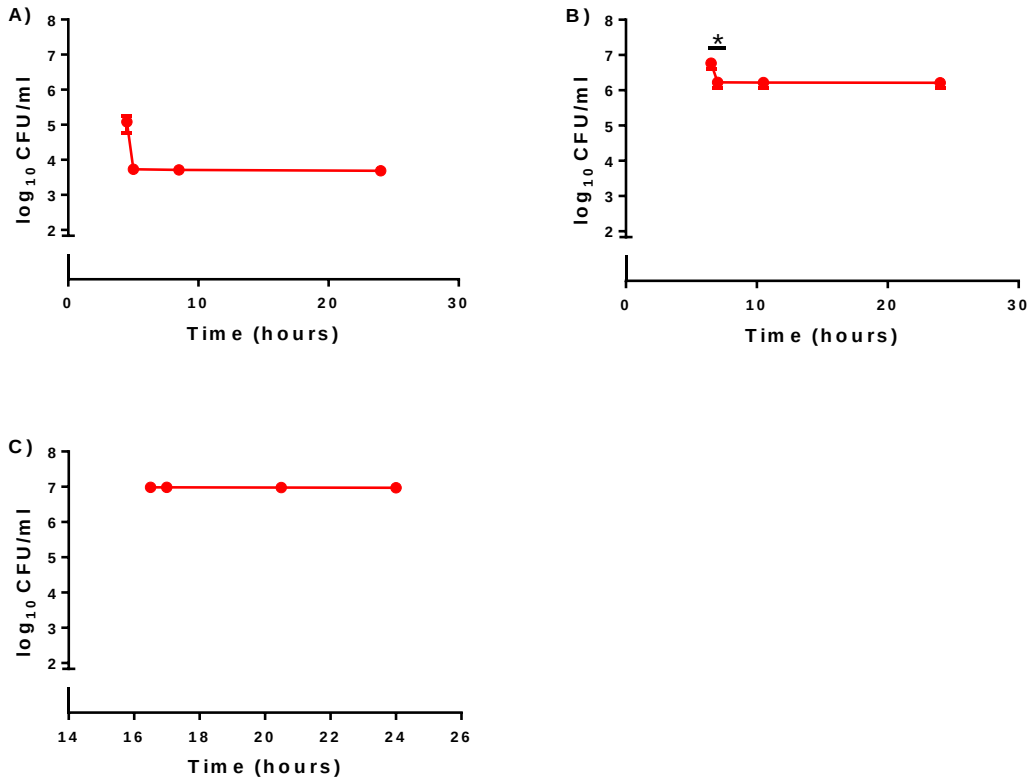

Figure 3.11: <u>Intracellular bacteria persist in MDMs.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 4, 6 and 16 hours, and following high dose lysostaphin treatment for 0.5 hours were either lysed or maintained in low dose lysostaphin for 0.5-4 hours and lysed at each time point for intracellular colony forming units estimation **A)** 4 hours, **B)** 6 hours, **C)** 16 hours challenge. The data represents the mean with the standard error of the mean; n=3, * $p < 0.05$, Paired t test comparing first two time points.

I found a similar trend occurring with SH1000 as was seen with Newman. The majority of intracellular killing was occurring within the first 30 minutes after phagocytosis was prevented and then there was a persistence of viable intracellular bacteria (Figure 3.16A-C). The initial killing phase decreased with increasing time and was less than 1% by 16 hours post exposure suggesting macrophage exhaustion of intracellular killing capabilities with *S. aureus* was not merely a strain specific phenotype.

3.2.11. Intracellular viable bacteria persist in macrophages for up to 40 hours post infection

Having established that the majority of intracellular killing of *S. aureus* by macrophages was occurring early after ingestion and was sustained for only the first 16 hours or so of exposure to bacteria, I next investigated how long intracellular bacteria would persist for. I hypothesised, that there would be no additional second phase of killing occurring at later time points in contrast to what has been described for some other pathogens (Molloy et al., 1994, Dockrell et al., 2003).

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 6 hours. After killing extracellular and cell surface adherent bacteria with high dose lysostaphin, macrophages were maintained in low dose lysostaphin for up to 16-40 hours of culture before calculating the intracellular CFU.

I found that over this time course there was very modest reduction in intracellular viable bacteria, and bacteria persisted intracellularly at similar numbers throughout (Figure 3.17). This suggested that macrophages had become overwhelmed and exhausted their intracellular killing capabilities but also that there was no late phase killing being initiated and therefore intracellular bacteria persisted within macrophages for at least 40 hours. Since bacterial numbers were fairly constant it also did not suggest significant intracellular replication was occurring.

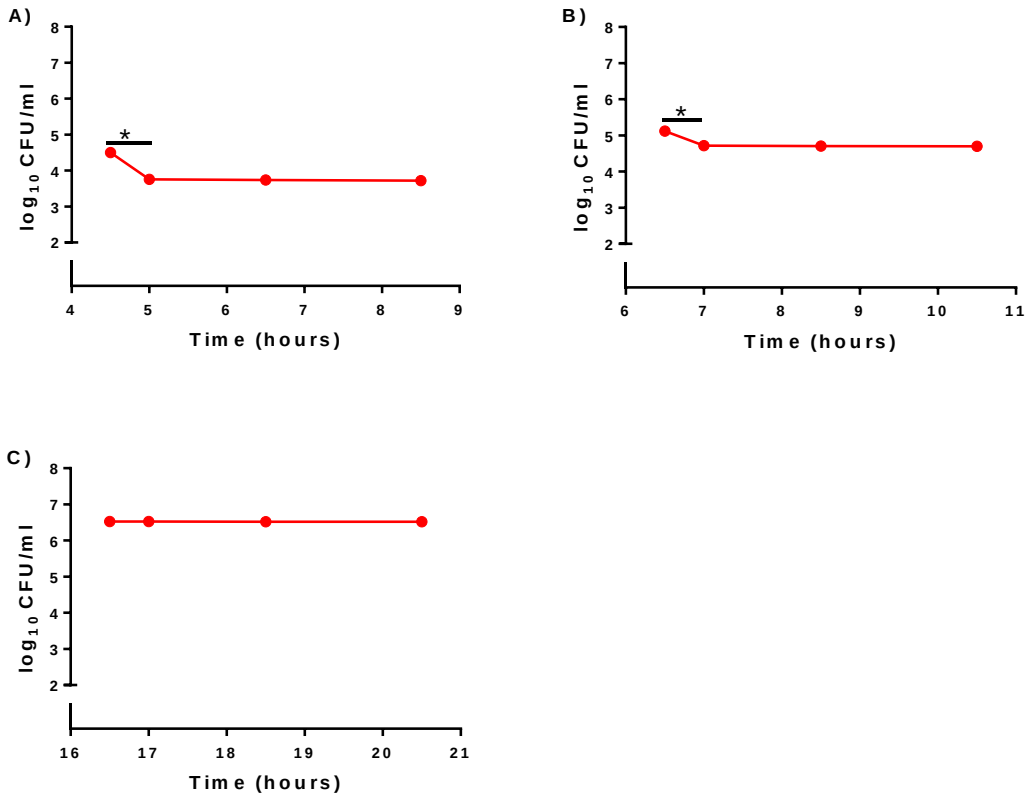

Figure 3.12: <u>SH1000 persists intracellularly.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* SH1000 at an MOI of 5 bacteria per macrophage for 4, 6 and 16 hours, and following high dose lysostaphin treatment for 0.5 hours were either lysed or maintained in low dose lysostaphin for 0.5-4 hours and lysed at each time point for intracellular colony forming units estimation **A)** 4 hours, **B)** 6 hours, **C)** 16 hours challenge. The data represents the mean with the standard error of the mean; n=3, * $p < 0.05$, Paired t test comparing first two time points.

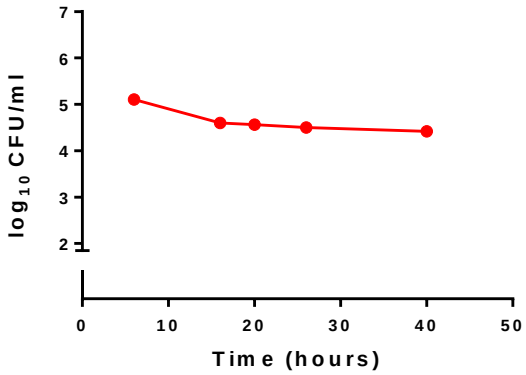

Figure 3.13: <u>Intracellular bacteria persist for up to 40 hours post infection.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per macrophage for 6 hours, and following high dose lysostaphin treatment for 0.5 hours were either lysed or maintained in low dose lysostaphin for 16- 40 hours after bacterial challenge and lysed at each time point for intracellular colony forming units estimation. The data represents the mean with the standard error of the mean; n=3.

3.3. Discussion

The persistence of *S. aureus* within professional and non-professional phagocytes has been previously documented, though there is very little data specifically concerning survival in differentiated macrophages (Kapral and Shayegani, 1959, Melly et al., 1960, Gresham et al., 2000, Hess et al., 2003). There is some evidence for the failure of macrophages to kill intracellular *S. aureus* leading to intracellular persistence (Kim et al., 1976, Galbenu et al., 1979). Galbenu *et al.*, reported that pulmonary macrophages were unable to kill intracellular *S. aureus* but they used macrophages from patients with pulmonary cancer (Galbenu et al., 1979) which may have affected the results. Likewise, Kubica *et al.*, have suggested that MDMs fail to kill *S. aureus* completely and instead *S. aureus* can indirectly kill the macrophage by an uncharacterised mechanism (Kubica et al., 2008). The reasons for incomplete intracellular killing in macrophages are unknown and the molecular mechanisms that account for the failure of the macrophage to control *S. aureus* following phagocytosis are uncharacterised. Another limitation is that there is little known about the kinetics of this process, which prompted me to investigate in detail how time and dose affect the intracellular killing kinetics following *S. aureus* phagocytosis by macrophages.

Since differentiated tissue macrophages are crucial to the innate immune response to *S. aureus* (Martin et al., 2011) and are the resident phagocytes that first encountered the bacterium within host tissues, I investigated the interaction between differentiated macrophages and *S. aureus*. I investigated the rates of intracellular accumulation, if the rates of intracellular bacterial killing of *S. aureus* were rate limiting and how long intracellular *S. aureus* persisted.

In this section I have demonstrated that macrophages contain extracellular *S. aureus* but this capacity is overwhelmed at increasing MOI. Lysing the macrophages showed that for all doses there was an accumulation of viable intracellular bacteria over time, even at low doses of infection when extracellular bacteria were being contained. This suggests that even when controlling bacteria macrophage host responses might be occurring at the expense of complete intracellular control and would allow some intracellular persistence. At higher doses, extracellular *S. aureus* replication exceeds the rate at which macrophages can clear bacteria and extracellular bacterial numbers are not controlled. However, as the macrophage response is pushed to the point at which it is overwhelmed, phagocytosis is maintained at near maximal rates. This results in the

macrophages becoming overwhelmed with more intracellular bacteria than they can kill, resulting in accumulation of intracellular viable bacteria.

I found broadly similar levels of adherent bacteria at all doses, with only a modest increase in dynamic range, suggesting that macrophage surface receptors were optimally engaged and had the capacity to ensure a steady supply of surface bound bacteria for internalisation without allowing accumulation of bacteria on the surface. Since only a sub-population of macrophages had intracellular bacteria it is possible that macrophage heterogeneity results in differential activation so that only some cells are activated to phagocytose or alternatively only a subset of macrophages may be able to ingest bacteria irrespective of activation state. An alternative but less likely explanation is a subset of those macrophages that have phagocytosed bacteria are differentially activated to enhance killing so that bacteria are more rapidly killed and are not present on microscopy, although as many of these experiments were conducted in the first few hours after ingestion it would seem less likely that significant numbers of macrophages had completely killed and degraded all internalised bacteria, thus this possibility seems less plausible.

Although not performed in my thesis there are other methods I could have used to more directly analyse phagocytosis and differentiate between adherent, internalised and killed bacteria. The reason for not carrying out these methods was because my results were designed to be descriptive in order to feed into my mathematical model. One of these methods involves manipulating protein A present on *S. aureus*. I could fuse gold particles to protein A and using electron microscopy to determine internalised and killed bacteria. The internalised bacteria would be present intracellularly attached to the gold particles and killed bacteria would be represented by areas where there is just gold. Another method is flow cytometry using fluorescently labelled bacteria. Using the labelled bacteria, I could follow internalisation directly by blocking phagocytosis and looking at the fluorescent signal to provide a read-out of adherent bacteria and compare this to macrophages without blocking to get a measurement of the internalisation. A final method is time lapse imaging using labelled bacteria. By doing this, I could follow individual macrophages and look at their behaviour over a number of hours to determine the adherence *versus* internalisation rates. These methods would provide a more direct measurement of phagocytosis compared to DAPI staining and viability counts.

I have demonstrated that for a range of bacterial doses macrophages efficiently phagocytose *S. aureus.* They continue to increase internalisation of bacteria up to a high MOI of 125, though by this level there was minimal enhancement over the levels seen at an MOI of 25 suggesting phagocytosis was maximal and there was no capacity to further enhance phagocytosis.

Phagocytosis of *S. aureus* is known to occur rapidly and both opsonised and non-opsonised *S. aureus* is ingested by macrophages (Jonsson et al., 1985). Detailed studies of phagocytosis with other bacteria have been undertaken. *Streptococcus pneumoniae* is known to be inherently resistant to phagocytosis because it possesses a polysaccharide capsule which masks bacterial ligands (Jonsson et al., 1985, Gordon et al., 2000). In order for *S. pneumoniae* to be phagocytosed efficiently by macrophages requires opsonisation by IgG and complement present in serum (Gordon et al., 2000). Despite this opsonisation however, *S. pneumoniae* is ingested at a lower level than *S. aureus* and macrophages exhibit lower levels of intracellular bacteria (Jonsson et al., 1985). A similar pattern has been observed with *Pseudomonas aeruginosa* which requires IgG mediated opsonisation for efficient phagocytosis although IgA has also been shown to be important but the mechanism remains elusive (Reynolds et al., 1975). Following opsonisation, *P. aeruginosa* is ingested via Fc receptors present on macrophages (Reynolds et al., 1975). *Escherichia coli* is ingested at a similar rate to *S. aureus* by macrophages, and opsonisation shows a slight enhancement of this rate (Hoidal et al., 1981, Jonsson et al., 1985). Early literature seemed to suggest with *E. coli* there was a saturation effect which limited the efficiency of phagocytosis which might explain why macrophages showed lower intracellular *E. coli* numbers compared to *S. aureus* (Hoidal et al., 1981). Non-typeable *Haemophilus influenzae* (NTHi) was also shown to be rapidly ingested by AMs and to a similar extent as *S. aureus* (Jonsson et al., 1985). Electron microscopy revealed that NTHi and *S. aureus* were intracellular and not adherent to the macrophage and present in tight vacuoles; the key difference between the bacteria were NTHi appeared to be undergoing degradation whereas *S. aureus* was not (Jonsson et al., 1985). There are similarities between these ingestion processes but the requirements for opsonisation for example differ between different bacteria, which can be a determining factor in the level of internalisation achieved. The key differences are in the receptors utilised; *S. pneumoniae* uses complement and Fc receptors for internalisation following opsonisation for example (Gordon et al., 2000), whereas *S. aureus* primarily utilises scavenger receptors (Thomas et al., 2000).

I found that at all times and doses the majority of intracellular killing was occurring early on, in the first 30 minutes after phagocytosis, and then there was a subsequent plateau phase with very modest intracellular killing, which became less pronounced with increasing dose and time. This lead to the persistence of intracellular bacteria which occurred for at least 40 hours after first exposure to bacteria. As bacterial dose and time increased, the percentage of intracellular killing occurring in the first 30 minutes decreased in spite of the fact that the actual numbers of intracellular bacteria killed in the first 30 minutes remained fairly similar from 5-14 hours of bacterial exposure. By 16 hours of incubation there was very little reduction in intracellular bacterial numbers suggesting macrophages had exhausted their intracellular killing capacity and were becoming overwhelmed with intracellular bacteria. I found this response was not dose dependent and even at physiologically relevant lower doses, such as MOI of 0.05 and 1, there was still persistence of intracellular viable bacteria. As well as this, the response was not cell line dependent or strain dependent and I found MDMs controlled intracellular *S. aureus* in a similar manner to THP-1 differentiated macrophages. *S. aureus* SH1000 also persisted intracellularly in a similar fashion to *S. aureus* Newman. Interestingly, stimulating macrophages with IFNγ prior to infection only modestly enhanced the intracellular killing capacity. This suggests that the reason for exhaustion of killing of intracellular bacteria by the macrophages was not merely because the macrophages were sub-optimally stimulated to kill intracellular bacteria in culture but instead reflected a fixed capacity of intracellular killing, which was overwhelmed by dose and time.

There are many potential reasons why there is an initial killing period and then a plateau phase. ROS production via the NADPH oxidase system has previously been shown to be activated by phagocytosis and the act of phagocytosis seems to be an important stimulus for the recruitment and activation of the various components that form the NADPH oxidase complex in phagocytes (DeLeo et al., 1999). It is possible that there is a threshold level of bacteria required for the macrophages to become fully activated and achieve maximal phagocytosis and intracellular killing of bacteria. Once maximal there is no capacity to further enhance killing. One reason is that intracellular killing requires adenosine triphosphate (ATP) generation and the increasing demands of phagocytosis and killing could result in failure to ensure adequate ATP levels to support ongoing killing. Alternatively the capacity to generate ROS, to acidify the phagolysosome or the supply of lysosomes with proteases to engage with the phagosome could become

limited, leading to intracellular persistence. The fact that the intracellular killing became exhausted before phagocytosis ceased suggests the reasons are most likely related to aspects specific to the killing process.

Alternatively, it is also possible that a subset of *S. aureus* is resisting the macrophages killing response; *S. aureus* has been demonstrated to resist ROS (Karavolos et al., 2003, Das and Bishayi, 2009) and NO (Richardson et al., 2008). *S. aureus* can also resist degradation; peptidoglycan is relatively resistant to degradation by enzymes such as lysozyme (Bera et al., 2005, Kaplan et al., 2012). Since killing mechanisms require activation and intracellular recognition systems respond to antimicrobial products of digested bacteria to enhance activation this could allow some of the intracellular bacteria to persist in a 'silent' fashion if the necessary killing mechanisms are not completely engaged. Thus the model of resistance to degradation could modify further killing (Wolf et al., 2011). As a result, the macrophages might not be eliciting further responses to these intracellular bacteria allowing them to survive for extended periods of time. Finally it could be that the remaining intracellular bacteria traffic to a different compartment intracellularly where they cannot be killed or are in a phagolysosome that is not sufficiently bactericidal and therefore cannot kill the intracellular bacteria and they persist. This could also result from a bacterial subversion response that alters lysosome trafficking to and fusion with phagosomes as they mature through stages into a mature phagolysosome. In a related mechanism it is possible the intracellular bacteria traffic elsewhere intracellularly over time and avoid the macrophages killing response.

An interesting finding from this section was that macrophages were still able to phagocytose *S. aureus* despite the fact they were no longer killing the bacteria. This would seem to further predispose macrophages to accumulation of viable bacteria. I found that at 16 hours of incubation when macrophages had exhausted their intracellular killing capacity and become overwhelmed with intracellular bacteria, they were still able to phagocytose further extracellular bacteria. This suggests macrophages are unable to match phagocytosis to the capacity to clear ingested bacteria. In the initial period of the macrophage's response to bacteria, phagocytosis and killing are inter-related since phagocytosis stimulates ROS dependent killing (DeLeo et al., 1999), but at later times phagocytosis not only fails to induce killing but becomes an uncoupled process that is unable to sense that there is no longer a capacity to kill ingested bacteria.

A key function of the macrophage is antigen presentation to T cells. There is a lot of evidence in the literature demonstrating that macrophages can present antigen to $CD4^+$ primed T cells and $CD8^+$ primed T cells (Kovacsovics-Bankowski and Rock, 1994, Nair et al., 1995). Initially it was thought that DCs were the potent antigen presenting cell (APC) that presented antigens to the T cells and primed the immune response. In summary the DC recognises antigens present on the bacterium and presents these to the T cells. The T cells can then proliferate increasing the number of T cells present to respond to the antigen. This response can then go on to stimulate B cell responses leading to the development of protective antibodies and then the DC can undergo apoptosis to turn off the response (Folcik et al., 2007). Recent research has shown that macrophages can also prime the immune response. Therefore in my research, culturing macrophages with naive T cells could enhance intracellular killing by macrophages. This could occur through the macrophages presenting antigens to the T cells stimulating them to proliferate, enhancing their effector functions and stimulating them to mature into memory T cells which can respond faster on another exposure to the bacterium (Pozzi et al., 2005). This would be important because macrophages are abundant at the site of infection and if they are potent at presenting antigens and can therefore prime T cells it could lead to enhanced clearance of intracellular bacteria because now there are two immune cells interacting together to contribute to overall pathogen control (Pozzi et al., 2005).

It is possible that some of the findings presented in this section of my thesis could be related to how activated the THP-1 cells are at the start of the experiment. The activation of THP-1 differentiated macrophages at the start of experiments can influence some of the results presented in this thesis. Unlike MDMs, the THP-1 differentiated macrophages are known to be of a more M1 polarisation state (Daigneault et al., 2010), in comparison to MDMs which are more of an M2 polarisation state. This enhanced activation could mean the THP-1 differentiated macrophages are more stimulated initially to start phagocytosing and killing bacteria in comparison to MDMs, which would become M1 on exposure to bacteria. It could also mean, that when analysing the effect of additional pro-inflammatory stimulation on the ability of THP-1 differentiated macrophages to kill intracellular bacteria, further stimulation might have modest effects, especially if the THP-1 differentiated macrophages are already maximally activated. Despite this, the actual polarisation profile of THP-1 macrophages has not been studied in extensive detail, but it was noted that similar to MDMs, THP-1 differentiated

macrophages demonstrated the ability to become similar in phenotype to MDMs following challenged with heat killed bacteria (Daigneault et al., 2010). In addition, despite the additional, suggested M1 polarisation of THP-1 macrophages, the behaviour towards *S. aureus* is similar between THP-1 differentiated macrophages, MDMs and bone marrow derived macrophages (BMDMs), suggesting that the initial interaction between the cells and bacteria might differ and lead to for example increased adherence and internalisation, but the later processes and intracellular killing are broadly similar.

A lot of the work presented in this section of my thesis used THP-1 differentiated macrophages which have advantages and disadvantages as models of differentiated tissue macrophages. One of the major advantages is unlike MDMs the differentiation protocol is a lot shorter and allows more data to be collected in a shorter space of time. As well as this, despite some heterogeneity between the cells, there is generally a lot less variation than with MDMs, owing to the lack of donor variation. The main advantage of THP-1 differentiated macrophages is they are extremely similar to MDMs and therefore are good models of tissue macrophages but the functional consequences requires further characterisation and to study more specific macrophage subsets requires an altered differentiation protocol. Despite these obvious advantages there are some disadvantages with using the THP-1 model. During differentiation, unlike MDMs, there was a greater proportion of lysosomes within THP-1 differentiated macrophages which could impact on their degradative capacity (Daigneault et al., 2010). Also in respect to surface receptors, there could be differences in responses to certain pathogens e.g. because of different expression of TLRs (Daigneault et al., 2010). The differentiation protocol pushes the THP-1 cells towards differentiated tissue macrophages and therefore does not account for the environmental factors accounting for the arisal of specific macrophage subsets.

The findings presented in this section of my thesis demonstrate that macrophages efficiently phagocytose *S. aureus* but have a finite capacity to kill intracellular bacteria. Intracellular killing is not complete and even at low dose exposures some bacteria remain viable in the intracellular environment but the accumulation of these viable bacteria becomes accentuated once killing is exhausted. The level of surviving viable intracellular bacteria increased with both time and dose, but, when the macrophages could no longer kill they were still able to phagocytose. Stimulation of macrophages only modestly enhanced their intracellular killing capacity. It was not clear whether this intracellular persistence was primarily the result of intrinsic limitations on the

macrophages ability to kill intracellular bacteria or mediated by specific bacterial factors. However, this data provided the basis on which to develop a mathematical model to explain the interaction between *S. aureus* and macrophages, which is the focus of the next section of this thesis.

Chapter 4 A mathematical model to describe the interaction of *Staphylococcus aureus* with the macrophage

4.1. Introduction

Over recent years, mathematical models of biological systems have increasingly proved a useful tool in helping to inform our understanding of host: pathogen interactions. The development of new mathematical models to describe a range of biological systems has become very attractive because they can offer important insights, are quick to generate and allow predictions to be made. The ability to use these models to make predictions and inform experimental design is at the heart of what makes mathematical models extremely useful. Based on this, the development of a mathematical model to describe the interaction of macrophages with *S. aureus* will prove important for future research.

Mathematical modelling is a particularly powerful research tool in disease biology, including at a microbiology and immunology scale. As well as being able to complement the study of biological interactions, it can offer new insights into host: pathogen behaviour (Kirschner and Marino, 2005). Mathematical models, which can be simple or complex may be able to successfully match experimental observations, make a range of hypotheses about the system under investigation and offer ideas for experiments necessary to extend or inform the model (Kirschner and Marino, 2005, Callard and Yates, 2005). A lot of infectious disease models model a single target cell and a single pathogen to maintain tractability, but complex models with multiple and heterogeneous populations are common in biology (Dixit and Perelson, 2005, Pienaar and Lerm, 2014).

Over the years there have been a number of mathematical models of host: pathogen interactions at the pathogen-cell scale developed e.g. models of TB dynamics (Warrender et al., 2006), Human Immunodeficiency Virus (HIV-1) (De Boer et al., 2010) and Hepatitis (Ciupe et al., 2007). These models have offered important insights into the behaviour of the immune response following exposure to these infectious agents, and, how they are able to cause the diseases they do. Also they have allowed us to analyse how treatment can be used in the control of these diseases and when it is best to administer treatment (Kepler and Perelson, 1998, Di Mascio et al., 2004). However,

there has been no detailed mathematical analysis of the interaction between macrophages and *S. aureus*.

One of the most modelled bacterial pathogens is TB and mathematical models of TB infection have examined the roles played by both macrophages and T cells. It has been demonstrated that if macrophages are the only responding cell to a mycobacterial infection, then the bacteria are able to grow unrestricted (Warrender et al., 2006). With T cell influx the growth is controlled (Warrender et al., 2006). Another study demonstrated using an agent based model (ABM) that resting macrophages ingested extracellular bacteria leading to bacterial clearance (Segovia-Juarez et al., 2004). On the other had activated macrophages were necessary to ingest and kill mycobacteria. Too many resting macrophages could provide a perfect bacterial niche preventing the necessary interactions for pathogen clearance, whereas, too many activated macrophages could cause severe tissue damage (Segovia-Juarez et al., 2004). An interesting study from 2010 found that although macrophages were important in the response to mycobacteria, the phase of growth of the bacilli influenced the differentiation state of the macrophages (Bru and Cardona, 2010). A recent study found that if macrophage recruitment was too low then mycobacteria would grow unrestricted (Pienaar and Lerm, 2014). Increasing macrophage recruitment offered more control to the infection and even a moderate rate of recruitment could eliminate the pathogen through the generation of inflammatory mediators (Pienaar and Lerm, 2014). The model suggested there was a constant interplay between the host and the bacterium, whereby periods of inactive macrophage response would allow the bacteria to disseminate to a new location (Pienaar and Lerm, 2014).

The interaction of *S. aureus* with macrophages is a very underdeveloped field. There have been various reports about the ability of *S. aureus* to persist in macrophages but the papers are conflicting in their nature (Melly et al., 1960, Kubica et al., 2008). It is well established that *S. aureus* can avoid a range of innate immune defences from recognition to intracellular killing (Rooijakkers et al., 2006, Richardson et al., 2008) and this allows it to successfully establish an infection. However, there has never been an extensive study exploring the kinetics of phagocytosis or intracellular killing of *S. aureus* by macrophages which formed the basis of my thesis. Although there are mathematical models describing the quorum sensing system of *S. aureus* (Jabbari et al., 2012a, Jabbari et al., 2012b) there hasn't been a current model describing the interaction with macrophages.

While there are a number of mathematical models addressing the dynamics of TB infections there is not currently a mathematical model describing the macrophage response to *S. aureus*. I therefore decided to use my *in vitro* results to inform a novel mathematical model. I developed a model to describe the extracellular and intracellular phases of *S. aureus* infection by using my *in vitro* results to estimate parameters for the model. These results provided important insights into how macrophages respond to *S. aureus* and provided suggestions as to why macrophage control of extracellular and intracellular *S. aureus* was very different.

4.2. The Model

4.2.1. Model structure

In order to develop my mathematical model I first developed a schematic diagram outlining the key biological processes occurring within my system and also the relevant cellular and bacterial populations present (Figure 4.1). I described four populations (or variables) namely unexposed (or 'free') macrophages (Mf) and actively ingesting (or 'active') macrophages (Ma) and extracellular (Se) and intracellular (Si) bacteria. Initially, I did also have an additional macrophage population, regarded as 'chronically infected' macrophages (see below), but as the model developed, this population was combined with the actively ingesting macrophage population. Using some of my initial experiments I then decided on the key events that could give rise to each population and how each population behaved.

4.2.2. Macrophage and bacterial populations

Initially, a population of Mf macrophages would be present in culture at a fixed density. Over the time course I am focussing on I assume that these macrophages do not decay and that no additional cells are recruited. They would respond to Se bacteria and actively internalise them making them Ma macrophages with Si bacteria. Ma macrophages kill Si bacteria at a fixed rate and in theory if they clear their entire load could become Mf macrophages once more. With increasing bacterial dose, macrophages ingest more Se bacteria, but because killing occurs at a fixed rate, over time their relative killing rate decreases leading to intracellular bacterial persistence.

Se bacteria have the capacity to replicate in the extracellular medium until it reaches its critical population density ('carrying capacity'), with the growth rate slowing as the carrying capacity is approached ('logistic growth'). They can be ingested by Mf or Ma macrophages to become Si bacteria. Si bacteria are assumed not to replicate, at least at early time points (as suggested by my experimental data) but can be killed by Ma macrophages at a fixed rate. These events are summarised in figure 4.1.

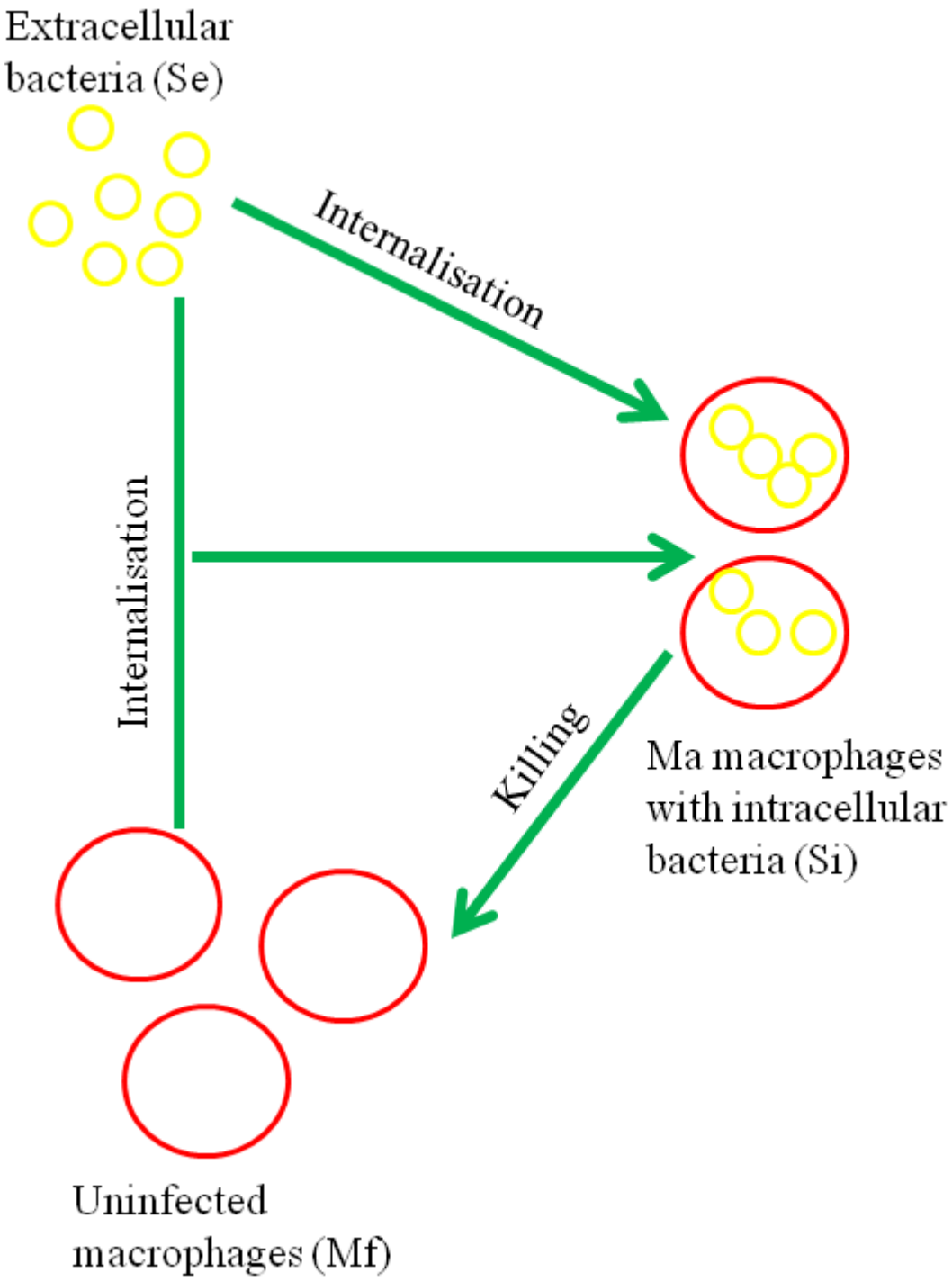

Figure 4.1: <u>Summary of the interaction of macrophages with *S.aureus* during an extracellular infection.</u>

Se bacteria can be internalised by Mf or Ma macrophages to become Si bacteria. Ma macrophages can kill intracellular bacteria and become Mf macrophages if they clear their entire load. Se bacteria can replicate to a maximum population density.

4.2.3. Extracellular model equations and assumptions

Having formed the representative diagram shown in figure 4.1, I translated it into an ordinary differential equation (ODE) model. This relies on forming differential equations for each population (variable) which contains various processes (parameters) within them to describe in detail how the population changes with time. In coming up with my equations I had two key assumptions in my model:

- A subset of Mf macrophages were capable of phagocytosing Se bacteria.
- Each sub-population of macrophages, those with and without the potential to become Ma, was homogeneous.

I produced four equations to describe the dynamics of the populations shown in figure 4.1 over time. I assigned parameters for each of the processes namely: internalisation of Se by Mf macrophages (γ); replication of Se (r); the maximum population density for Se (k); internalisation of Se by Ma macrophages (β) and; killing of intracellular bacteria by Ma macrophages (μ).

For internalisation of extracellular bacteria by Mf the equation was written as γ*Mf*Se. This takes into account that the ability to internalise Se bacteria is a property of an interaction of an Mf macrophage with a Se bacteria that would lead to it being ingested. The ingestion term is a density dependent rate (i.e. the rate of ingestion by a macrophage is proportional to the density of Se bacteria). This is similar for internalisation by Ma macrophages which for this model is assumed to occur at the same rate as for Mf macrophages (i.e. $\gamma=\beta$). This assumption was partly to make the model less complicated. Although internalisation rates might be anticipated to increase after a period of bacterial exposure, as a result of activation of macrophages, my experimental data did not suggest that this represented a major effect. Moreover the assumption was pragmatic because there was no easy way to accurately determine the Mf and Ma populations over time, there was no simple method to accurately split up the two internalisation rates. The killing of intracellular bacteria is written as μ*Ma, to account for the property that intracellular killing is a feature of the Ma population with each macrophage killing at some fixed rate.

The Se equation also has an additional term to account for the replication of bacteria. I have written this as (r*Se*(1-Se/k)). This shows that the Se population can replicate but by engineering in the k, the bacterial population cannot grow indefinitely but instead

has ‘logistic’ growth where, in the absence of macrophages, it will grow to reach its carrying capacity, k. The Si equation contains both the internalisation and killing terms.

4.2.4. Macrophage equations

The Mf equation shows that Mf macrophages can internalise Se (-γ) and become Ma macrophages (Equation 4.1 (1)). As well as this if a Ma macrophage killed its Si bacteria it would once again form part of the Mf macrophage population (+μ) (Equation 4.1 (1)). In the model, it is assumed that above a certain Si level, Ma macrophages can no longer clear their entire intracellular bacterial load over time despite intracellular killing still occurring at a fixed rate. As there was not a specific term for this in the original model, I engineered functions that would allow killing to lead to clearance over a certain range of Si numbers and to prevent clearance above a threshold value. These terms were the piecewise functions represented at the end of the Mf and Ma equations i.e. the (Si/Ma<1) (Equations 4.1). These terms allow the clearance term to be discontinuous as intracellular numbers are varied (i.e. it behaves differently over different values of Si). They are designed to allow Ma macrophages to clear Si bacteria if the Si number is less than 1 bacterium per cell. In the second half of the function, if the Si number is greater than 1 bacterium per cell, the function prevents intracellular clearance of Si bacteria by Ma macrophages, leading to intracellular killing but Ma macrophages do not become Mf macrophages.

Ma macrophages arise when Mf macrophages internalise Se (+γ) (Equation 4.1 (2)). Secondly if a Ma macrophage clears its intracellular bacterial burden it becomes an Mf macrophage again (–μ) (Equation 4.1 (2)).

4.2.5. Bacterial equations

Se bacteria could replicate (r) to a maximum population density (k) (Equation 4.1 (3)). As well as this, Se could be internalised by Mf macrophages (–γ), or internalised by Ma macrophages (–β) (Equation 4.1 (3)). These internalisation processes would give rise to Si bacteria (Equation 4.1 (4)). Also Si bacteria could be killed by Ma macrophages; hence there is a –μ in the Si equation (Equation 4.1 (4)).

$$(1)\ \frac{dM_f}{dt} = \begin{cases} (-\gamma M_f S_e) + (\mu M_a)\ if\ \ S_i/M_a < 1 \\ 0\ if\ \ S_i/M_a \geq 1 \end{cases}$$

$$(2)\ \frac{dM_a}{dt} = \begin{cases} (\gamma M_f S_e) + (\mu M_a)\ if\ \ S_i/M_a < 1 \\ 0\ if\ \ S_i/M_a \geq 1 \end{cases}$$

$$(3)\ \frac{dSe}{dt} = \left(rS_f\left(1 - \frac{S_e}{k}\right)\right) - \ (\beta M_a S_e) - \ \left(\gamma M_f S_e\right)$$

$$(4)\ \frac{dSi}{dt} = \left(\gamma M_f S_e\right) + \ (\beta M_a S_e) - (\mu M_a)$$

Equations 4.1: <u>Equations describing the extracellular model.</u>

Equations were formed based on the diagram in figure 4.1. There are 4 cellular populations and each of these is assigned parameters based on how each population arises. **(1)** Mf equation. **(2)** Ma equation. **(3)** Se equation. **(4)** Si equation. The – and + signs designate how each parameter causes a population to shift from one to the other. Key: γ= internalisation rate of Se by Mf macrophages, μ=killing rate of Si by Ma macrophages, r=replication rate of Sf, k=carrying capacity of Sf, β=internalisation rate of Sf by Ma macrophages.

4.2.6. The initial interaction of *S. aureus* with macrophages

Se replication was estimated from *S. aureus* replication experiments in media over 0-9 hours. *S. aureus* at an MOI of 5 was incubated in macrophage media for 1 hour on ice and then transferred to 37°C for up to 9 hours. Importantly, there were no macrophages present in this experiment. At each time point I took a measurement of the extracellular colony forming unit (CFU). I input these results into R® and used a linear model (lm) which fitted a straight line through my logged data points. When doing an lm model, R comes up with a set of values called residuals which is the difference between the observed values and the predicted values. It then tests this for homoscedascity, which is the distribution of these residual values around the mean of the data. It plots the fitted line *versus* the residuals and checks the residual values lie close to the line. Secondly, it checks the residuals are normally distributed. If these criteria are met, which is checked by making R® plot the two graphs, you can be assured that the lm model has produced the best fit through the data.

The internalisation rate of Se by Mf macrophages was estimated from experiments where I had allowed macrophages to internalise *S. aureus* for 0-9 hours. Macrophages were challenged with *S. aureus* at an MOI of 5 for 0-9 hours (1 hour on ice and 8 hours at 37°C). At each time point I took a measurement of extracellular CFU. I input the data into R® and tried fitting an lm through my logged data. However, trying to employ the above method to calculate the internalisation rate of Se by Mf was not simple. Using the lm function in R® produced fitted *versus* residuals plot with points scarcely close to the fitted line and therefore this model in R® could not be trusted to have produced the most accurate estimation of the data. Therefore I used another method, using the function 'fminsearch' in the mathematical programming software Matlab®, which fits the model output to the experimental data and optimises by minimising the sum of squares error between the data and model. This method, which is similar to how R® calculates the best fit using lm function allowed a more accurate estimation to be achieved and produced a value for γ that was 0.00000011. For simplicity in the model I assumed that $\gamma=\beta$ and therefore β was also set at 0.00000011.

To calculate the killing rate of Si by Ma macrophages, I used my results from experiments where I had challenged macrophages with *S. aureus* at an MOI of 5 for 6 hours. At each time point extracellular bacteria were removed and the experiment followed without internalisation for defined time periods. I took a measurement at 30

minutes post phagocytosis ceasing and used this to calculate μ in a similar fashion to what has been described. When I had an estimate for μ over 30 minutes the value was doubled to estimate killing/hour (μ=0.16). This value implies that on average an intracellular bacterium can survive for about 6 hours.

I set the Mf macrophages at 80,000 because my experimental results suggested only a subset of macrophages were capable of phagocytosis, and I usually observe no more than 40% of macrophages with Si bacteria at 6 hours at an MOI of 5 in my experimental results. This would suggest that either 40% of macrophages are capable of ingesting or some have ingested, cleared Si bacteria and then not re-ingested. For simplicity, I assume the first statement and set the Mf starting at 40% of my Mf seeding density. I initially ran the model over 6 hours because this was the time course over which most of my experiments were conducted. The code I used for R® for the model is shown in appendix 1.

4.3. Results

Having estimated the parameters I input them into my model and ran the model at an MOI of 0.05 (Figure 4.2A-C) and an MOI of 5 (Figure 4.2D-F) over 6 hours. To calculate my parameter estimations I had used data recorded for an MOI of 5. I found this produced a good correspondence to my *in vitro* data at this MOI. I also used these parameters to plot the trends for a lower MOI of 0.05.

The model showed for an MOI of 0.05 Ma macrophages slowly increased in number and by 6 hours post infection about ~9.4% of the total Mf macrophage starting population (80,000) had become Ma (Figure 4.2A). For an MOI of 5 there was a sharper increase in Ma macrophages and by 6h ~99% of the total Mf macrophage starting population had become Ma macrophages (Figure 4.2D). For both MOI's the Se line shows a similar increase in the gradient because the model assumes relative bacterial growth rather than absolute bacterial growth (Figure 4.2B, E). Over time at both MOI's there is a gradual accumulation of Si bacteria within Ma macrophages as a result of ongoing internalisation (Figure 4.2C, F). Plotting the *in vitro* Se and Si data alongside the model outputs shows a good correspondence at both MOIs (Figure 4.3B, C, E, F). These results suggest that as the MOI increases more Mf macrophages become activated to control the extracellular infection leading to increased accumulation of intracellular bacteria.

Figure 4.3 shows Se and Ma dynamics for three further bacterial MOI's (MOI 0.5, 1 and 10). Figure 4.3 suggests that macrophages control extracellular bacterial replication for longer at lower bacterial doses compared to higher bacterial doses (Figure 4.3B, D and F). Figure 4.3 also shows that as MOI increases, the number of Ma macrophages recruited to help control extracellular bacterial replication increases from 60% of the starting Mf macrophage population at an MOI of 0.5 to ~100% of the starting Mf macrophage starting population at an MOI of 10 (Figure 4.3A, C and E). These results suggest that macrophages have the capacity to control small infections over a number of hours, even though there will eventually be exponential growth of Se bacteria. This control may have an important role in allowing time for the recruitment of other immune cells to the site of infection. At higher bacterial doses, macrophages have to work harder to control the large infection, and the influx of other immune cells is probably more crucial in determining the outcome of the infection.

4.3.1. The effect of varying the extracellular bacterial replication rate

Having shown the extracellular model produced reasonable dynamics at low and high bacterial MOI, I next examined the effect of varying the replication rate of extracellular bacteria on the population of Se and Si bacteria. To do this, I used the standard error from my lm model for bacterial replication, which was 0.058. I varied the replication rate using the standard error to make r=0.758 and r=0.642. I input these values into my model and re-ran it at an MOI of 0.05 and 5 over 6 hours.

At both MOI's for both a higher r value (Figure 4.4A, C) and lower r value (Figure 4.4A, C), there is similar behaviour in Se bacteria. As the bacteria replicate faster or slower, they take less time or longer respectively to reach k. As the bacteria replicate faster, more macrophages in the Mf population become Ma and accumulate Si bacteria.

4.3.2. Modelling later events in the macrophage: *S. aureus* interaction

Having modelled the initial phase of infection, I next modelled what was happening to intracellular bacteria over time following an initial pulse of *S. aureus* i.e. events post lysostaphin treatment. In experiments involving macrophages and bacteria this protocol is standard. The reason behind adding lysostaphin at 6 hours is in order to kill extracellular bacteria that have not been ingested. This is important because in order to follow intracellular bacteria and intracellular kinetics it is important to make sure that extracellular bacteria and therefore ongoing ingestion are not contributing. In my

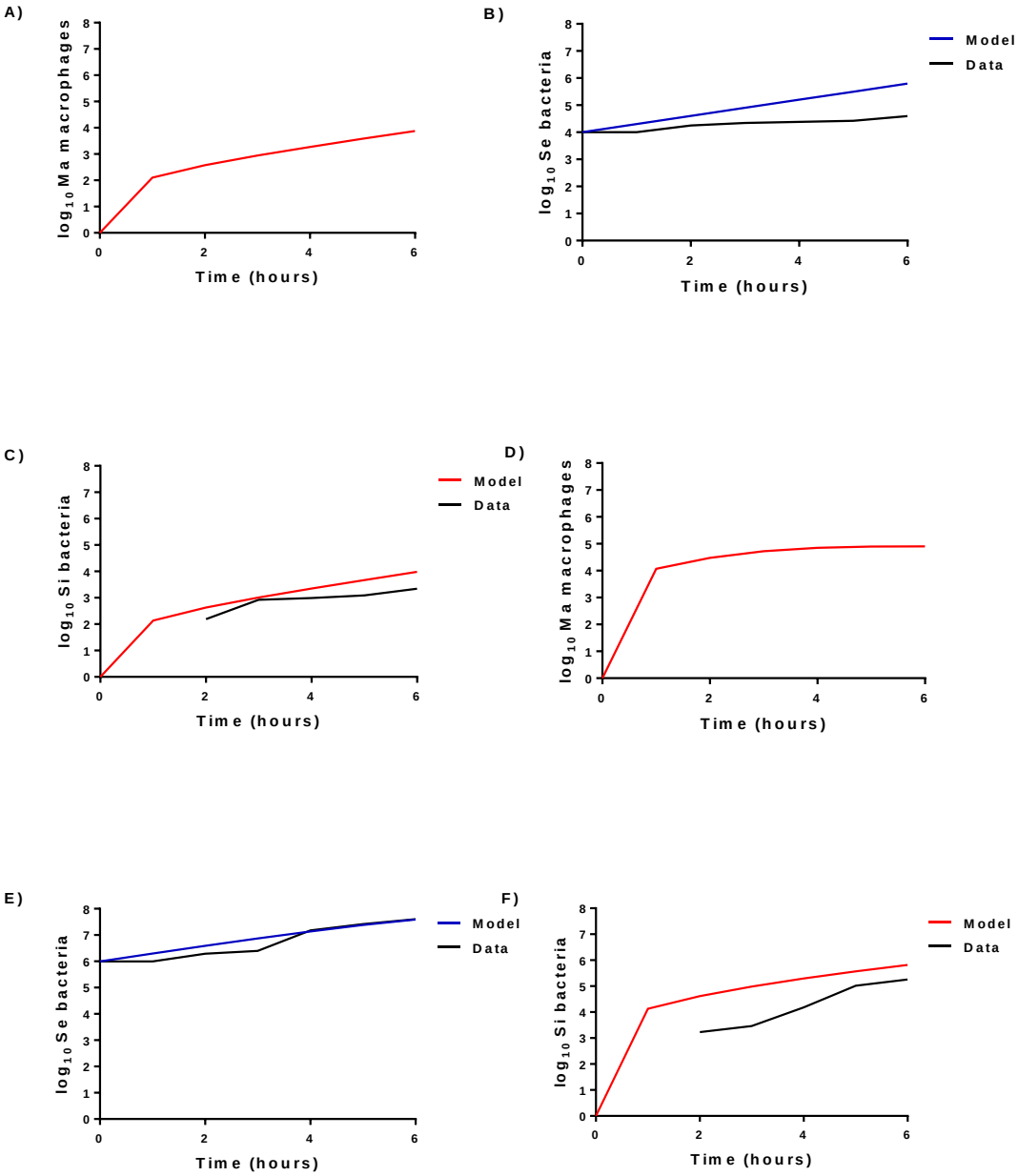

Figure 4.2: <u>Extracellular model outputs.</u>

Output from the model showing the dynamics of **A)** Ma macrophages, MOI of 0.05, **B)** Se bacteria, MOI of 0.05, **C)** Si bacteria, MOI of 0.05 and the *in vitro* results, **D)** Ma macrophages, MOI of 5, **E)** Se bacteria, MOI of 5, **F)** Si bacteria, MOI of 5 and the *in vitro* results. Experimental data is also plotted in B, C, E and F. Parameter values were estimated from experimental data as: (r=0.7, k=10^8, γ=1.1 x 10^7, μ=0.16).

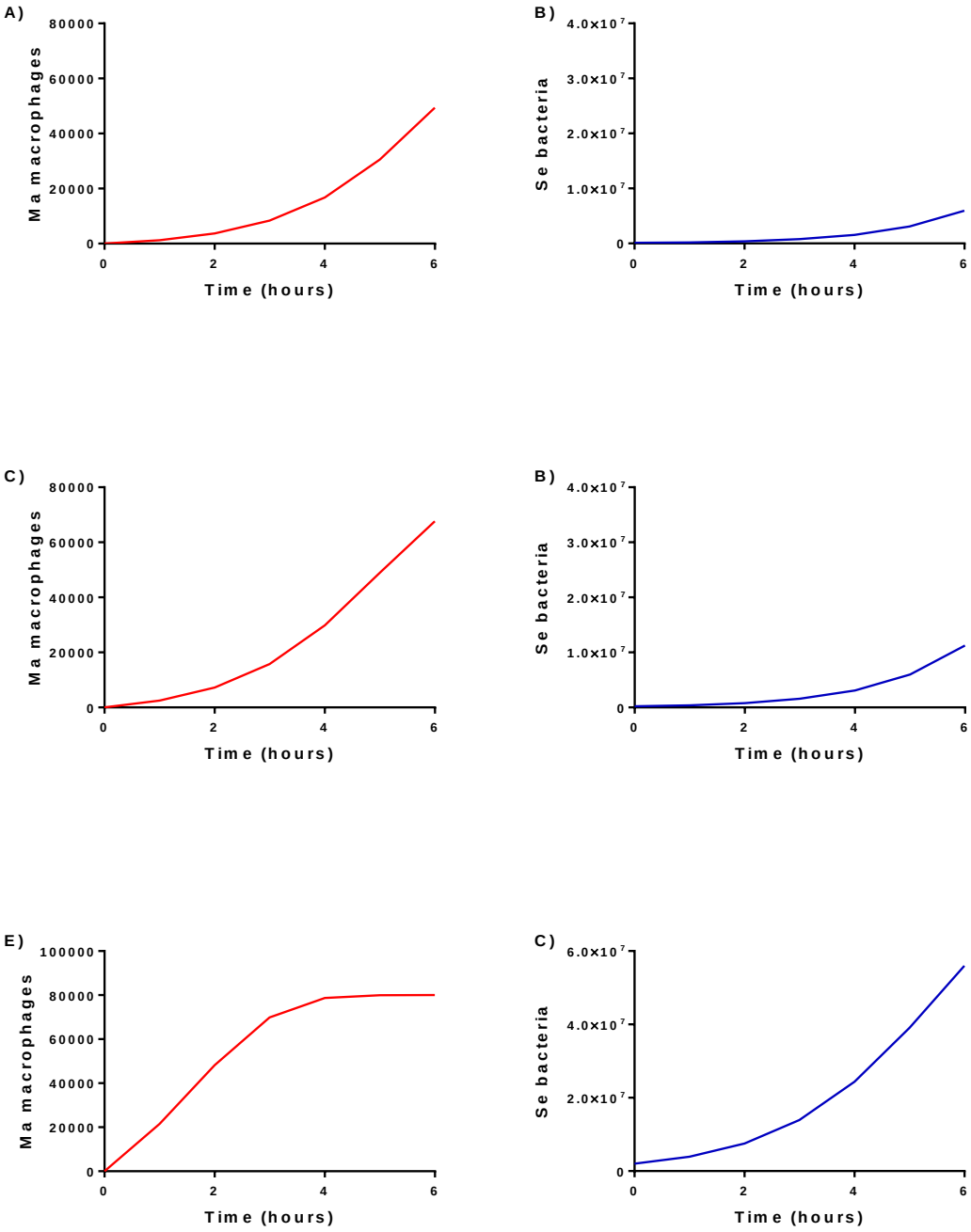

Figure 4.3: <u>Increasing bacterial dose compromises macrophage control of bacterial replication.</u>

Using the parameters calculated above, the model was run at an MOI of 0.5, 1 and 10 over 6 hours. **A)** Ma macrophages, MOI of 0.5, **B)** Se bacteria, MOI of 0.5, **C)** Ma macrophages, MOI of 1, **D)** Se bacteria, MOI of 1, **E)** Ma macrophages, MOI of 10, **F)** Se bacteria, MOI of 10. Parameter values were estimated from experimental data as: (r=0.7, k=10^8, γ=1.1 x 10^7, μ=0.16).

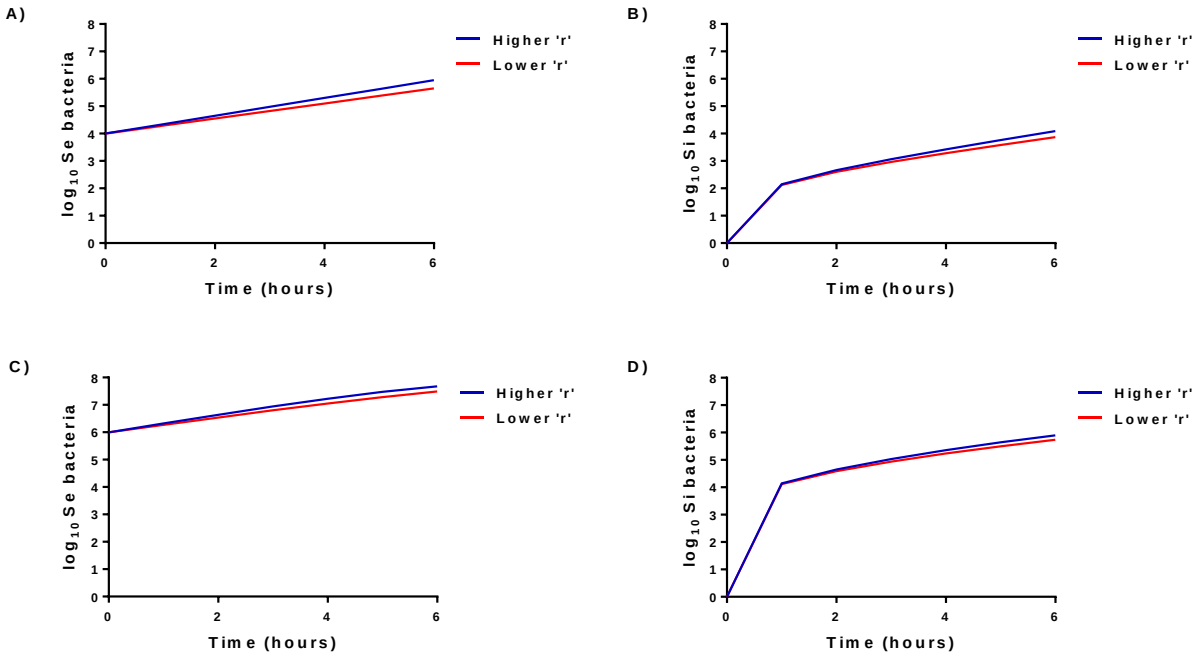

Figure 4.4: <u>The effect of varying 'r' on Se and Si bacteria.</u>

Using the parameters calculated above, and altering r to 0.758 or 0.642, the model was run at an MOI of 0.05, and 5 over 6 hours. **A)** Se bacteria MOI of 0.05, **B)** Si bacteria, MOI of 0.05, **C)** Se bacteria, MOI of 5, **D)** Si bacteria, MOI of 5. Parameter values were estimated from the lm model for r and experimental data for all other parameters: (r=0.758 (Higher 'r') or r=0.642 (Lower 'r'), k=10^8, γ=1.1 x 10^7, μ=0.16).

experiments, I always record post lysostaphin treatment that extracellular bacterial numbers are undetectable and therefore in the model I assume that the treatment is efficient and hence Se numbers become zero preventing ongoing phagocytosis and Se replication.

I modified my equations to take account of the result of adding lysostaphin to my *in vitro* cultures following the initial infection pulse. As well as this I had one assumption for this phase of the model:

1) Above 1 bacterium per cell, the clearance of intracellular bacteria by Ma macrophages ceases.

I modified three equations to describe the populations (Mf and Ma macrophages and Si bacteria) present post lysostaphin treatment. My initial Mf equation had a term regulating internalisation of Se bacteria by Mf macrophages and a term for the killing of Si bacteria by Ma macrophages. In the intracellular model, the term for internalisation of Se bacteria has been removed because lysostaphin treatment removes Se bacteria and therefore internalisation is no longer a parameter needed within the model (Equations 4.2). The Ma macrophage equation has been modified in a similar fashion and now only contains a term for intracellular killing of bacteria. My Se equation has been removed from this part of the model. The Si equation now only holds a term for the intracellular killing of Si bacteria by Ma macrophages. Having modified my equations appropriately, I assigned a mathematical code for the parameters within each equation. The parameters were killing of intracellular bacteria by Ma macrophages (μ) and the average number of Si bacteria per cell (K). This K term was added into the Mf and Ma equations to account for the fact that there was a certain threshold of Si bacteria when macrophages would be able to clear intracellular bacteria and above this threshold clearance would not occur.

In my model I assume that intracellular killing of Si bacteria by Ma macrophages occurs at a constant rate. Depending on the Si number, intracellular killing can lead to Ma macrophages clearing Si bacteria where a Ma macrophage once again becomes an Mf macrophage. The Mf equation shows that Mf macrophages can arise when Ma macrophages clear Si bacteria ($+\mu$) (Equation 4.2 (5)). Ma macrophages can clear Si bacteria ($-\mu$) (Equation 4.2 (6)). Finally the Si equation has been modified to account for the fact that Si can be cleared by Ma macrophages ($-\mu$) (Equation 4.2 (7)).

4.3.3. Modelling the intracellular phase of infection

Having edited the initial equations to take into account what could happen to intracellular bacteria in the absence of bacterial internalisation, I next substituted in the parameter values for each term which were assumed to be the same as the original model, but with the addition of the clearance process.

Prior to running the model, I re-analysed my killing data and it became evident that there were two phases of intracellular killing occurring by macrophages. I have termed these phase α to represent the initial fast killing that occurs when phagocytosis ceases and phase β to represent the more gradual killing that occurs later (Figure 4.5). My intracellular model presented in this chapter of my thesis models a single rate of killing for analytical ease but further extensions to the model should look to include both phases α and β.

Having substituted my estimated parameter values into the model I ran the model at an MOI of 0.05 (Figure 4.6A-B) and an MOI of 5 (Figure 4.6C-D). Again in estimating my parameters I used the values from my MOI of 5 experiments and was able to then use these to estimate what would happen at an MOI of 0.05. For the purposes of this model, the starting densities of Mf and Ma macrophages and Si bacteria were set at the values from 6 hours based on the previous extracellular model for each bacterial MOI. I ran the model up to 16 hours because this was one of the longer time courses I had performed in my experiments. The script for this part of the model is shown in appendix 2.

I found that using my calculated values I was able to show the phase β behaviour with a good degree of accuracy. I found for an MOI of 0.05 Ma macrophages slowly decreased in number and by 16 hours post infection almost the entire Ma population had once again become Mf macrophages, but there was still a small population of Ma macrophages with Si bacteria (Figure 4.6A). For an MOI of 5 there was no decrease in Ma macrophages, indicative of the fact that intracellular killing had become exhausted and these Ma macrophages were now harbouring more than 1 bacterium per cell (Figure 4.6C). At both doses, there was a gradual decline in Si bacteria over time (Figure 4.6B, D). However, the single rate of killing used here does not account for the initial fast phase of killing after lysostaphin is added.

These results suggest that at low bacterial doses almost every killing event leads to intracellular bacterial clearance seen by the decrease in Si numbers almost paralleling

$$(5)\ \frac{dM_f}{dt} = \begin{cases} (\mu M_a)\ if\ \ S_i/M_a < 1 \\ 0\ if\ \ S_i/M_a \geq 1 \end{cases}$$

$$(6)\ \frac{dM_a}{dt} = \begin{cases} (-\mu M_a)\ if\ \ S_i/M_a < 1 \\ 0\ if\ \ S_i/M_a \geq 1 \end{cases}$$

$$(7)\ \frac{dSi}{dt} = (-\mu M_a)$$

Equations 4.2: <u>Intracellular model equations.</u>

The equations presented in figure 4.2 were modified to account for the effect of lysostaphin on Se bacteria. There are 3 cellular populations and each of these is assigned parameters based on how they behave post internalisation. **(5)** Mf equation. **(6)** Ma equation. **(7)** Si equation. Key: μ=killing rate of Si by Ma macrophages, K=average number of Si bacteria per cell. The functions at the end of the Mf and Ma equation indicate that above a certain value, killing becomes exhausted.

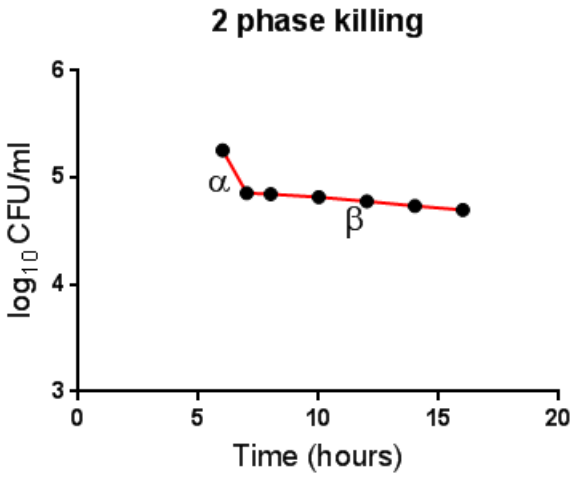

Figure 4.5: <u>Two phase intracellular killing dynamics.</u>

Results from *in vitro* data shows there are 2 phases of intracellular killing. Phase α represents a fast killing step occurring within the first 30 minutes post phagocytosis whereas phase β represents a more gradual killing phase.

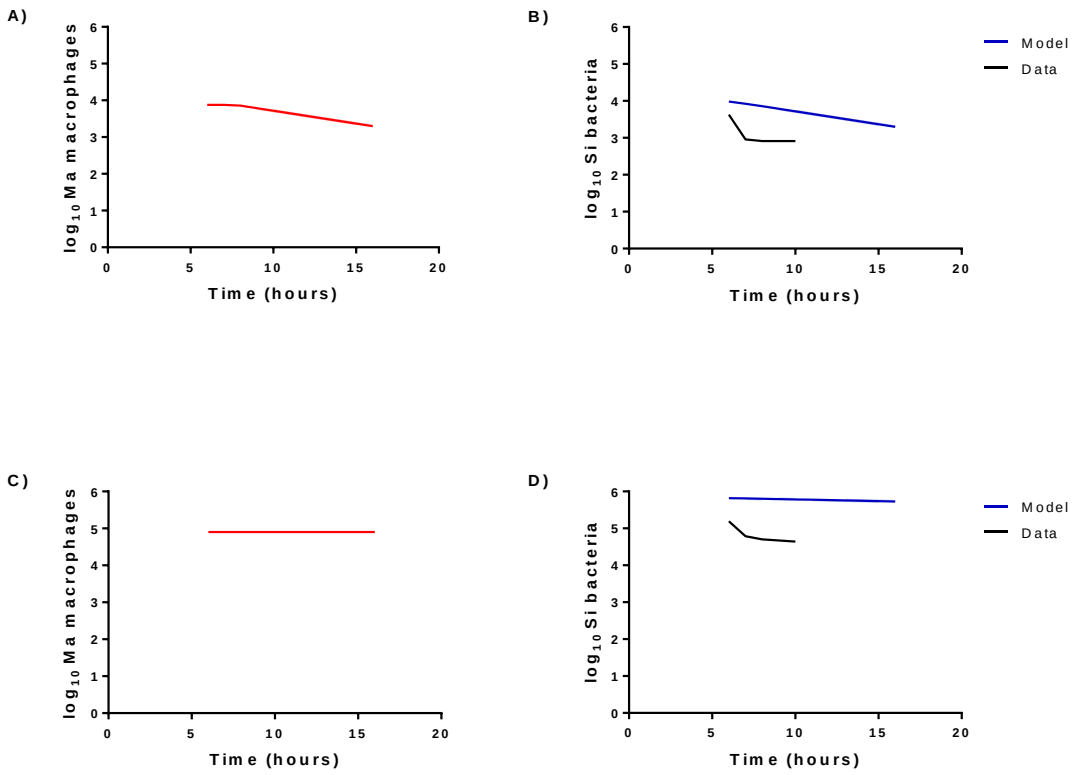

Figure 4.6: <u>Intracellular model outputs.</u>

Parameters were calculated from the biological results. These altered parameters for intracellular killing of bacteria by Ma macrophages, μ (0.16) and the average number of bacteria per cell, K (1) were then inputted into the model and the model was run at an MOI of 0.05 and an MOI of 5 over 6-16 hours. **A)** Ma macrophages, MOI of 0.05, **B)** Si bacteria, MOI of 0.05, **C)** Ma macrophages, MOI of 5, **D)** Si bacteria, MOI of 5.

the decrease in Ma macrophages. In contrast at higher bacterial doses, there is a gradual decrease in Si, but killing does not lead to clearance of intracellular bacteria and hence the Ma macrophages line stays flat over the 10 hour time course. Finally, the model demonstrates that intracellular killing is most likely the rate limiting step in the macrophages control of *S. aureus* and hence over time there is a small amount of Si that persist for an extended period of time. This suggests that even at low bacterial doses, and with killing of Se with an antimicrobial agent, some Si bacteria can persist intracellularly for an extended period of time.

4.4. Discussion

I have developed and analysed a mathematical model to explore the dynamics of the interaction between *S. aureus* and macrophages. My key results are that: (i) macrophages are unlikely to fully control an *S. aureus* infection over a long time period as bacterial replication is always faster than phagocytosis, but; (ii) at low MOI's macrophages may control the infection for as long as 6 hours, potentially allowing time for further immune cells to be recruited; (iii) intracellular bacterial numbers will gradually accumulate over the course of an infection, assuming persistent extracellular bacteria, as phagocytosis is proportional to extracellular bacterial numbers but killing is fixed; (iv) treatment that clears all extracellular bacteria may allow full clearance over time at low doses. Overall, my model highlights that macrophages alone can never fully clear *S. aureus* infection, but at low bacterial MOI's they can delay bacterial replication for longer time periods compared with high bacterial MOI's. My model also demonstrates, that removing the Se bacterial stimulus leads to a gradual decrease in Si bacteria over time, at both low and high bacterial doses, but small numbers of Si bacteria persist intracellularly for extended periods of time. Finally my model highlights how Si bacterial numbers influence the macrophages ability to clear intracellular bacteria, and when Si numbers are sufficiently high enough, macrophages can gradually reduce Si numbers but never clear Si bacteria and return to an Mf state again.

The results from my model have shown that, with realistic parameter values estimated from data, the macrophage population will never fully control *S. aureus* infection. The reason is simply that bacterial replication will always outstrip phagocytosis meaning extracellular numbers are always increasing. However, at low MOIs I found that extracellular numbers could be kept at a low level for long time periods. This is important because during human infection it is unlikely to see infection with *S. aureus*

at such high MOI's. The ability of tissue macrophages to be able to delay extracellular *S. aureus* replication for extended periods at low doses would allow tissue macrophages to elaborate chemotactic cytokines and recruit additional immune cells to the site of infection such as neutrophils. This interplay allows the macrophage to begin responding to and controlling *S. aureus* infection and then as time progresses the combination of a range of innate immune cells ultimately contributes to the successful elimination of the pathogen.

It has been shown in neutrophils that phagocytosis can stimulate oxidative killing (DeLeo et al., 1999). The movement between Mf ('free') and Ma ('active') macrophages is dependent on both the extracellular bacterial concentration and the intracellular bacterial concentration. However, there are also inherent feedbacks in the model. For example, higher Se numbers would cause higher Si numbers and over time less transition from Ma to Mf. The schematic diagram I created at the start of the model development suggested to me that the overall outcome of the system i.e. the control of bacterial infection was a dynamic interplay between bacterial and host factors. This mirrors a typical initial phase of *S. aureus* infection, whereby the ability of the infection be localised or systemic depends upon an interplay between the host immune system and bacterial virulence factors (Lowy, 1998).

As the model demonstrates as the bacterial dose increases, macrophages begin to lose control of the extracellular phase of *S. aureus* infection. Potentially, macrophages require additional help from other immune cells such as neutrophils in order to efficiently manage *S. aureus* infection (Lowy, 1998). This has been demonstrated for TB where early T cell influx combined with the macrophage response reduced bacterial growth (Warrender et al., 2006). The earlier peak in Ma macrophages might reflect Ma macrophages elaborating cytokines to activate nearby Mf macrophages to respond to infection. However, these models might suggest that the timing of macrophage activation is important, although this is not something I have addressed in my experiments or my model. If they respond to too great an extent, the control of infection might be compromised if activated macrophages release factors that damage tissues and enable bacteria to escape to other environmental niches (Segovia-Juarez et al., 2004).

The effect of ongoing activation and internalisation by macrophages is the accumulation of Si bacteria. My results show that intracellular numbers continue to increase over the course of an infection while internalisation increases proportionately with extracellular

numbers, and killing by macrophages is fixed. Increased intracellular bacterial load may have important implications to the infection and could affect cell viability as I will investigate in the next section.

For low bacterial MOI there is a close correlation between Ma and Si numbers after the addition of lysostaphin. Over time there is a decrease in Ma macrophages which closely mirrors a decrease in Si bacteria. This suggests that the Ma population are still able to actively kill Si bacteria and return to an Mf state. For higher bacterial MOI's there is no decrease in Ma macrophages over time suggesting intracellular killing has become limited relative to the total intracellular bacterial burden. However, there is still a gradual decrease in Si numbers. This may suggest that treatments that can clear extracellular bacteria may help the immune system to fully control infection, if given whilst the MOI is still low. Despite this, at both a low and a high MOI, Ma killing of Si bacteria appears to be rate limiting. Even though at low MOI's it appears there are Ma macrophages able to clear Si bacteria, over time, there is a subset of Ma macrophages where Si bacteria persist for extended periods of time that could lead to relapse or recrudescence of infection.

The reason why macrophages show a decreased ability to completely kill intracellular bacteria above certain thresholds could be two fold. Firstly, over time an essential resource required to maintain rapid intracellular killing (phase α) is depleted reducing intracellular killing leading only to the less effective killing of phase β. In addition, following initial killing there could be energy limitation leading to a more gradual reduction in Si number and ultimately the persistence of small numbers of Si bacteria.

However, the response to intracellular bacteria could also be influenced by bacterial factors. The initial rapid phase of killing at low doses could represent intracellular *S. aureus* that are sensitive to the macrophage defence and therefore are rapidly killed. This means that there could be a subset of intracellular bacteria that are sufficiently degraded within phagolysosomes to release bacterial ligands to activate macrophages to initiate a signalling cascade resulting in sustained intracellular killing of bacteria (Ip et al., 2010). This is important as it has been demonstrated that for macrophages to sufficiently mount a response to *S. aureus* requires firstly engagement of bacteria by cell surface receptors and secondly intracellular recognition of bacteria by receptors (Wolf et al., 2011) recruited to phagosomes (Underhill et al., 1999). In contrast, it is possible, that in the subset of cells in which bacteria persist there is incomplete

engagement of this activation process since intracellular bacteria are preventing their recognition by the macrophage and persisting in a quiet, non-active state (Tuchscherr et al., 2010, Tuchscherr et al., 2011). Regardless of this at higher doses the killing mechanisms would become overwhelmed and even appropriately activated cells will ingest more bacteria than they can kill.

This model assumed each population was homogeneous. This assumption can be criticised because *in vitro* the population would not be homogeneous, but for example, there would be macrophages at different levels of differentiation or activation. In order to determine how this affects results, it would be necessary to differentiate cells in different ways then mix them and challenge them with *S. aureus*. These results could then be incorporated into a future model with new equations to describe the new populations and also modifications to existing parameters. By doing this, I would expect to see the model possibly showing a more oscillatory nature with different groups of macrophages responding in different times.

Another assumption from this system was that extracellular bacteria activated the Mf macrophage population. This is a useful assumption for the model, but *in vivo* there might be subsets of cells that could be activated quicker than others and might contribute to earlier responses. Stimulating macrophages with different activation protocols prior to bacterial challenge and then comparing internalisation and killing between stimulated and unstimulated macrophages would allow this to be explored. These results could then be incorporated into the model.

From my *in vitro* observations it is clear that macrophage killing of intracellular *S. aureus* appears to follow two distinct phases (Figure 4.5). There is an initial phase of rapid killing which reduces intracellular bacterial numbers over a short time frame (phase α) and then a more gradual phase which reduces bacterial numbers more slowly and 'keeps the intracellular population in check' (phase β). To improve the model, better characterisation of the phases of intracellular killing are required. It will be necessary to work out the exact timing of the killing occurring in phase α to determine if the rapid reduction occurs early or within the 30 minutes. This would then lead to a calculation to work out better the rate of intracellular killing within this first 30 minutes which could be incorporated into a more complex model that accounts for the two phases. This would produce a more accurate model which has a killing rate based on phase α and phase β separately rather than one overall killing rate.

Mathematical models of infectious disease dynamics are very useful in forming hypotheses about biological processes and in confirming observed phenomena (Kirschner and Marino, 2005). The reason behind the lack of mathematical models observing the interaction of *S. aureus* with macrophages and other immune cells could be because *S. aureus* has always been observed to be an extracellular pathogen (Archer, 1998) and it is only recently coming to light as surviving in both an extra and intracellular environment. However, a lot of the early HIV mathematical models also did not examine intracellular kinetics in great detail (Perelson et al., 1993). Therefore it is possible that there are many processes affecting the intracellular dynamics and kinetics that make these models more difficult to generate. However, the amount of useful data and insights gained from generating simple or complex intracellular models far outweighs the time and difficulty in generating them. The lack of a good mathematical model to describe the interaction of *S. aureus* with macrophages prompted me to carry out a range of *in vitro* experiments with the central aim of using the results to develop and inform a new mathematical model.

The model presented in this section of my thesis used an ODE system to generate it. ODE models are examples of population based models and determine how the population changes with time (Miao et al., 2011). As such, they are very useful for simple models where the populations are assumed to be homogenous. For the model presented in this section of my thesis, an ODE system was excellent at generating a simple model which can now be extended.

As models become more complicated there are other modelling systems which would be more appropriate. One type of model which is very popular is agent based models (An et al., 2009). These models are very useful because they allow the modeller to follow individual cells that behave differently and model how they change with time. Essentially the model consists of an environment where the different cells reside and this can be altered to account for the behaviour of the different cells (Bauer et al., 2009). As the model becomes more complicated, agent based models are useful at incorporating elements such as cytokine secretion, cell movement etc and to develop a further understanding of how different components interact, as has been shown for TB (Segovia-Juarez et al., 2004). ABMs are also useful because they allow the overall contribution of individual cell types to for example, infection control to be studied, in a way that ODE models just follow the whole population of cells. However, the drawback

with ABMs is they are time consuming and require multiple simulations to produce accurate results, and as such, are selected only for very complicated models.

Another type of model which is only starting to prove popular is known as the CyCells system (Warrender et al., 2006). This type of model is similar to an ODE model and follows how populations change with time. The one key difference here is that there are already cellular populations present in an 'environment' and the influx of new cells is controlled by the cells already present (Warrender et al., 2006). Like other types of models, in the CyCells system, the cells can respond and interact with each other and respond to the environment. For more complicated these models are useful because they allow signalling pathways to be activated and can look at threshold levels of activation of the cells which are much harder to do by other modelling techniques (Warrender et al., 2006).

The findings presented in this section of my thesis outline a novel mathematical model describing the interaction between *S. aureus* and macrophages. I have formed a model that accounts for at least four populations within my *in vitro* system, and that Mf macrophages are capable of internalising Se bacteria at a range of bacterial doses to become Ma macrophages. I have also shown that the response of Mf macrophages to Se bacteria determines and influences the outcome of the infection i.e. full control of Se replication or minimal control. I have extended the model to describe how intracellular bacteria change over time and have been able to demonstrate that as bacterial dose increases macrophages ability for sustained intracellular killing is overwhelmed and is most likely the reason for bacterial persistence seen *in vitro*.

Chapter 5 Exploring the persistence of *Staphylococcus aureus* in macrophages

5.1. Introduction

It has become increasingly apparent that *S. aureus* inhibits aspects of the host response (Melly et al., 1960, Gresham et al., 2000, Hess et al., 2003, Kubica et al., 2008). As a result of these findings, the biological mechanisms allowing *S.aureus* to survive intracellularly in macrophages, within what can be considered a rather hostile environment, became a very important and engaging research question.

Once *S. aureus* has been recognised and phagocytosed by macrophages the bacteria become encased in the phagosome (Gruenberg and van der Goot, 2006). The phagosome matures into a phagolysosome (Desjardins et al., 1994) and a final drop in pH mediated by proton movement across the membrane acidifies the phagolysosome (Pitt et al., 1992). Acidification is energy dependent with energy coming from vacuolar ATPases (V-ATPase) and hydrogen pumps required to activate proteases within the phagolysosome (Pitt et al., 1992, Hackam et al., 1997).

The phagolysosome destroys invading microorganisms using oxidative and non-oxidative mechanisms. These include the generation of reactive oxygen species (ROS) through the NADPH oxidase system, and the mitochondrial electron transport chain (ETC) during oxidative phosphorylation, and the generation of NO. Non oxidative mechanisms include the production of various proteases e.g. lysozyme matrix metalloproteinases and cathepsins as well as pore forming peptides such as β defensins.

ROS and NO attack various components of microorganisms (Nathan and Shiloh, 2000, Fang, 2004). Hydrogen peroxide causes DNA damage to microorganisms (Imlay and Linn, 1986, Imlay and Linn, 1988) and attacks DNA bases leading to deoxyribose changes which culminate in DNA strand breaks (Fang, 2004). NO inhibits DNA replication in bacteria (Schapiro et al., 2003). NO blocks bacterial respiration (Pacelli et al., 1995, Stevanin et al., 2000) and interacts with tyrosyl residues inhibiting ribonucleotide reductase which puts a blockade on the availability of the precursors for bacterial DNA synthesis (Lepoivre et al., 1991). Lysozyme rapidly inactivates bacteria (Biggar and Sturgess, 1977). Cathepsin D restricts the growth of microorganisms and it

is thought that as the phagosome matures cathepsin D gets released and is activated by the low phagosomal pH and cleaves bacterial proteins such as the toxin listeriolysin preventing their cytosolic escape and subsequent replication (del Cerro-Vadillo et al., 2006). Macrophages produce elastase (matrix metalloproteinase 12 or MMP12) that gets transported to the phagolysosome (Houghton et al., 2009). Within the phagolysosome a new antimicrobial peptide, formed from the C terminal domain (CTD) portion of the protein disrupts bacterial cell walls (Houghton et al., 2009). Macrophages deficient in MMP12 have higher intracellular viable bacterial burdens compared to WT macrophages (Houghton et al., 2009). It has also been shown that AMs produce defensins (Patterson-Delafield et al., 1980) which inactivate bacteria by binding to the cell membrane and permeabilising it leading to the efflux of intracellular solutes (Lehrer et al., 1993).

S. aureus can evade oxidative defences using a variety of mechanisms e.g. staphyloxanthin (Clauditz et al., 2006, Olivier et al., 2009), and catalase (Das and Bishayi, 2009). *S. aureus* also possesses two lactate dehydrogenase genes (Ldh) and a haem metalloprotease enabling it to survive intracellularly with NO (Richardson et al., 2006, Richardson et al., 2008). *S. aureus* overcomes non-oxidative defences by producing staphylokinase for example which can inactivate defensins (Jin et al., 2004). *S. aureus* is hugely resistant to lysozyme through the synthesis of O-acetyltransferase A (OatA) (Bera et al., 2005). This resistance to intracellular killing leads to *S. aureus* persistence.

As mentioned previously, *S. aureus* persistence has been seen in a range of professional and non-professional phagocytes. The molecular mechanisms behind this persistence are only just starting to become understood. There are some reports that intracellular *S. aureus* has the ability to persist, even for example in human MDMs, and eventually the MDMs succumb to the infection and release intracellular bacteria into the extracellular media, by an unknown mechanism, although these reports have not been consistent in the literature (Kubica et al., 2008). Added to this it has recently been shown that intracellular *S. aureus* has the capabilities to lyse the phagosome and replicate in the cytoplasm (Grosz et al., 2013). This is not just a feature of *S. aureus* infection of macrophages and has been demonstrated for other cell types as well (Giese et al., 2011). Therefore it is becoming apparent that *S. aureus* may manipulate host defences, leading to intracellular persistence, bacterial survival and dissemination of infection.

Evasion of intracellular killing by bacteria can be the result of pathogen induced macrophage apoptosis, which was first demonstrated for *Shigella flexineri* and allows the destruction of macrophages before they can kill bacteria (Zychlinsky et al., 1992). Alternatively it may be the result of preventing a programme of host-mediated macrophage apoptosis, which enables killing of intracellular bacteria. (Dockrell et al., 2003). *S. aureus* has been demonstrated to prevent macrophage apoptosis through the upregulation of Mcl-1, which may facilitate intracellular persistence and might prevent this form of apoptosis-associated bacterial killing (Koziel et al., 2009, Koziel et al., 2013) though this remains to be firmly established.

I hypothesised that *S. aureus* was capable of intracellular persistence in differentiated macrophages and that this was the result of intracellular bacteria residing in a phagolysosomal compartment, which was unable to mediate efficient intracellular killing. I explored the features of the phagolysosome in which intracellular bacteria resided determining phagolysosome markers and whether the phagolysosome was sufficiently acidified. I also explored whether key steps required to sensitise macrophages for host mediated apoptosis during bacterial infection were engaged. These results gave insights into the reasons as to why macrophage antimicrobial killing of *S. aureus* was limited and provided an understanding of potential mechanisms by which *S. aureus* survives intracellularly in macrophages and could subvert host defence.

5.2. Results

5.2.1. Macrophages phagocytose *Staphylococcus aureus* into lysosome associated membrane protein 1 positive organelles

Having demonstrated in chapter 3 that macrophages phagocytosed *S. aureus* and there was a residual burden of intracellular bacteria I wanted to investigate where the intracellular bacteria were localised. To address this I stained macrophages with the late endosomal protein lysosome associated membrane protein -1 (LAMP-1) (Gordon et al., 2000).

Following challenge of THP-1 differentiated macrophages with *S. aureus* at an MOI of 5 bacteria per cell for 1.5-5 hours, cells were fixed. The cultures were then stained with the same anti-staphylococcal antibody previously used for fluorescence microscopy and a conjugated Alexa Fluor 568 red secondary antibody to label cell surface adherent bacteria. The cultures were then incubated with an unconjugated anti-LAMP-1 primary antibody in the presence of a low concentration of saponin to permeabilise the phagolysosome and then with a conjugated Alexa Fluor 488 green secondary antibody. All nuclei were counterstained with DAPI.

I found the number of intracellular bacteria (defined as DAPI positive but anti staphylococcal antibody negative) colocalising with LAMP-1 to steadily increase over time (Figure 5.1A) and by 5 hours of bacterial challenge approximately 85% of all intracellular bacteria colocalised with LAMP-1 (Figure 5.1B). This suggested macrophages were phagocytosing *S. aureus* predominantly into a LAMP-1 positive organelle. Confocal microscopy revealed that the intracellular bacteria colocalising with LAMP-1 had very tight LAMP-1 protein rings formed around them (Figure 5.2). Interestingly, there was the appearance of some bacteria not colocalising with LAMP-1 but still in a cluster formation (Figure 5.2).

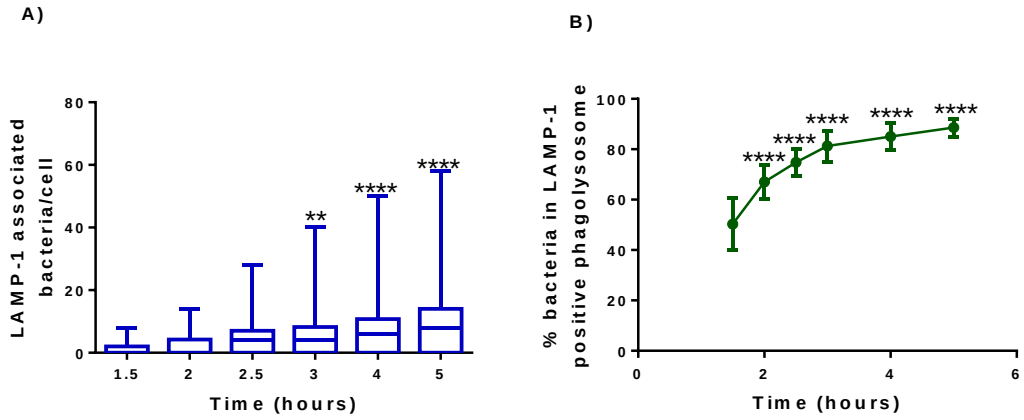

Figure 5.1: <u>The majority of intracellular bacteria colocalise with LAMP-1.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 1.5-5 hours and fixed and stained at each time point **A)** The number of intracellular bacteria per macrophage colocalising with LAMP-1 (box and whisker plot showing minimum to maximum), **B)** The percentage of intracellular bacteria per cell colocalising with LAMP-1. The data represents the mean with the standard error of the mean; n=4, **p<0.01, ****p<0.0001, One Way Anova with Dunnett's Post Test *vs* 1.5 hour.

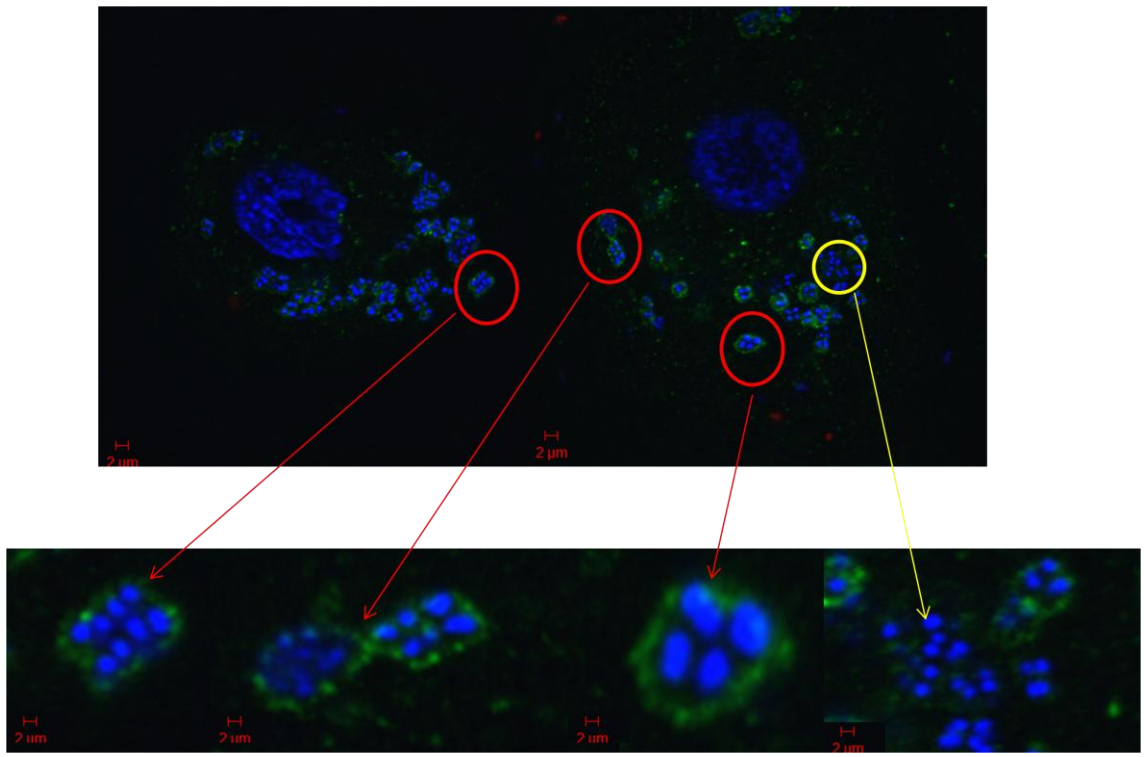

Figure 5.2: <u>Macrophages phagocytose *S.aureus* into LAMP-1 positive organelles.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 5 hours, then were fixed and stained. Images were taken using the x63 magnification of the Zeiss laser scanning confocal microscope. The red circle represents intracellular bacteria colocalising with LAMP-1. The yellow circle indicates intracellular bacteria not colocalising with LAMP-1. Scale bar represents 2µm.

5.2.2. Intracellular *Staphylococcus aureus* persist within lysosome associated membrane protein 1 and 2 organelles

Having demonstrated previously that macrophages always had a residual burden of intracellular bacteria I next questioned where this residual burden was persisting within the LAMP-1 or 2 positive organelle. To address this I stained macrophages with LAMP-1 or LAMP-2.

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per cell for 6 hours and extracellular bacteria were killed with lysostaphin. Cultures were then maintained in low dose lysostaphin for up to 48 hours post infection and fixed at each time point. The cultures were then stained as outlined in 5.2.1 but using LAMP-2 instead of LAMP-1 as appropriate in certain experiments.

I found the number of intracellular bacteria colocalising with LAMP-1 to decrease within the first 30 minutes post phagocytosis and then there was a plateau, with the number of intracellular bacteria colocalising with LAMP-1 remaining fairly static. (Figure 5.3A) but by 48 hours approximately 70% of all intracellular bacteria still remained colocalised with LAMP-1 (Figure 5.3B). For LAMP-2, I found the number of intracellular bacteria colocalising with LAMP-2 again decreased (although in contrast to LAMP-1 the percentage increased) within the first 30 minutes post phagocytosis and then the number of intracellular bacteria colocalising with LAMP-2 gradually increased over time (Figure 5.4A). The percentage of intracellular bacteria colocalising with LAMP-2 gradually increased from 50% at 6.5 hours to about 70% by 48 hours post exposure (Figure 5.4B). This suggested intracellular *S. aureus* persisted within a LAMP-1 or LAMP-2 positive phagolysosome possibly because they were resisting macrophage intracellular killing mechanisms or the phagolysosomal killing was exhausted.

Having determined the above I decided to dual stain my macrophages with both LAMP-1 and LAMP-2 together. I found a similar pattern was occurring. Following bacterial exposure there was a marked decrease in the number of intracellular bacteria colocalising with LAMP-1 or LAMP-2 within the first 30 minutes post phagocytosis (Figure 5.5A).

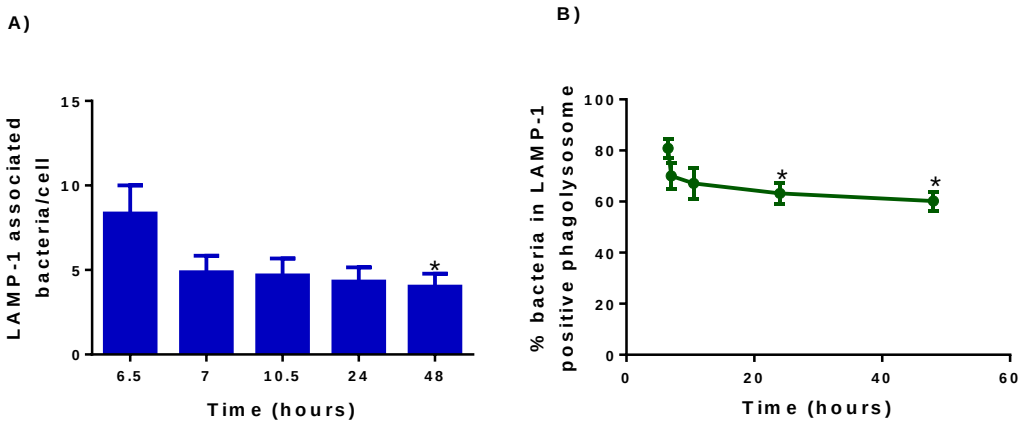

Figure 5.3: <u>The majority of intracellular bacteria persist in a LAMP-1 positive compartment.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 6 hours and extracellular bacteria were killed with lysostaphin for 0.5 hours and macrophages were fixed and stained or maintained in lysostaphin for up to 48 hours post infection **A)** The number of intracellular bacteria per macrophage colocalising with LAMP-1, **B)** The percentage of intracellular bacteria per cell colocalising with LAMP-1. The data represents the mean with the standard error of the mean; n=3, *$p<0.05$, One Way Anova with Dunnett's Post Test *versus* 6.5 hour.

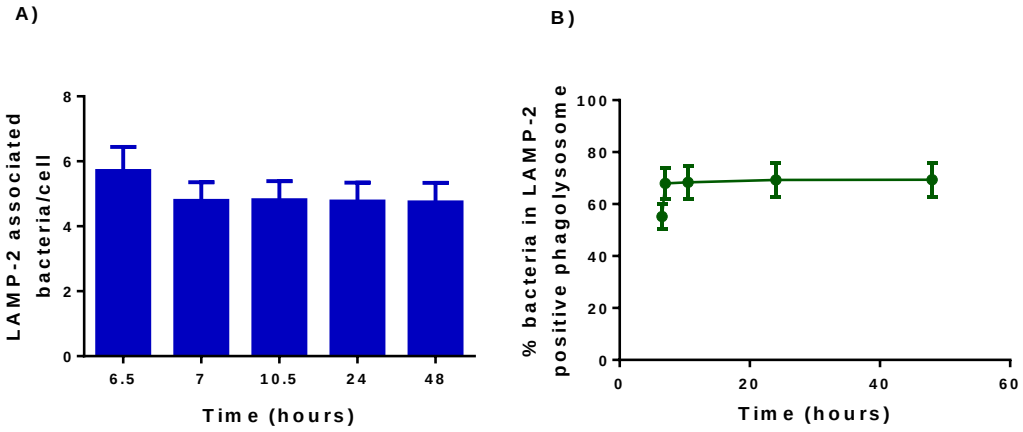

Figure 5.4: <u>The majority of intracellular bacteria persist in a LAMP-2 positive compartment.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 6 hours and extracellular bacteria were killed with lysostaphin for 0.5 hours and macrophages were fixed and stained or maintained in lysostaphin for up to 48 hours post infection **A)** The number of intracellular bacteria per macrophage colocalising with LAMP-2, **B)** The percentage of intracellular bacteria per cell colocalising with LAMP-2. The data represents the mean with the standard error of the mean; n=3.

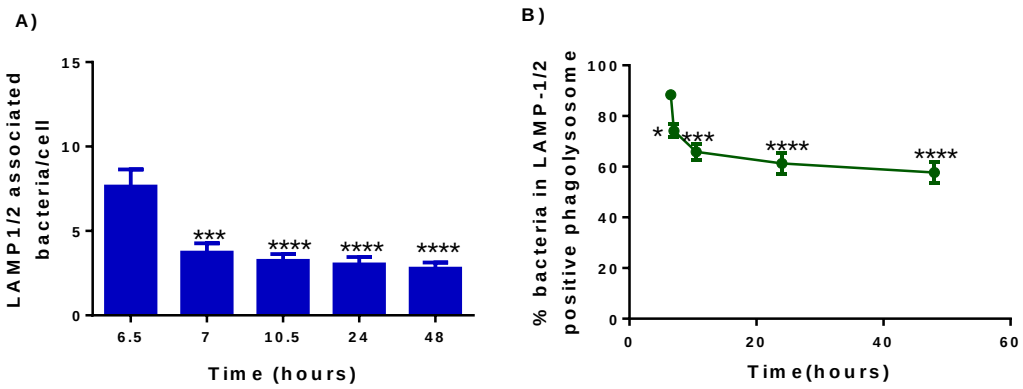

Figure 5.5: <u>The majority of intracellular bacteria persist in a LAMP-1/2 positive compartment.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 6 hours and extracellular bacteria were killed with lysostaphin for 0.5 hours and macrophages were fixed and stained or maintained in lysostaphin for up to 48 hours post infection **A)** The number of intracellular bacteria per macrophage colocalising with LAMP-1 or LAMP-2, **B)** The percentage of intracellular bacteria per cell colocalising with LAMP-1 or LAMP-2. The data represents the mean with the standard error of the mean; n=3, *p<0.05, ***p<0.001, ****p<0.0001, One Way Anova with Dunnett's Post Test *versus* 6.5 hour.

This was followed by a plateau phase with the number of intracellular bacteria colocalising with LAMP-1 or 2 remaining fairly constant (Figure 5.5B). This suggested that the majority of intracellular bacteria persist within a LAMP-1 and/or 2 positive phagolysosome also that there are some bacteria persisting in a LAMP-1/2 negative compartment.

5.2.3. The majority of intracellular *S. aureus* do not traffick to acidic compartments

Having determined that the majority of intracellular *S. aureus* trafficked to LAMP-1 and 2 positive compartments, I next investigated whether persistence of intracellular bacteria was associated with any failure to acidify the phagolysosome. To do this I decided to examine whether the intracellular bacteria were in a compartment of low pH. To do this I used the pH sensitive dye pH-rhodamine, which fluoresces bright red in pH 4.5-6, and is non-fluorescent in a pH > 6.

S. aureus was labelled with pH rhodamine for 30 minutes at 37°C and then THP-1 differentiated macrophages were challenged with the labelled *S. aureus* at an MOI of 5 bacteria per cell for 4-6 hours and extracellular bacteria were killed with lysostaphin. In a parallel experiment, THP-1 differentiated macrophages were challenged with labelled *S. aureus* at an MOI of 0.05 bacteria per cell for 6 hours.

Cultures were either fixed and stained as outlined previously or maintained for 0.5-48 hours post infection and then fixed and stained at each time point. The percentage of intracellular bacteria that were rhodamine positive was quantified in 100 random macrophages. Alternatively macrophages were exposed to labelled *S. aureus* at an MOI of 5 bacteria per cell for 1.5-6 hours and fixed and stained as outlined previously to determine adherent *versus* intracellular bacteria to determine the percentage of intracellular bacteria colocalising with pH rhodamine in 100 random macrophages. As a side line, I verified the labelling procedure by placing the bacteria in a range of different pH from 4-8 and confirmed fluorescence at pH 4-6 which decreased as pH increased (Figure 5.6).

I found challenging macrophages with labelled *S.aureus* at MOI of 5 for 4-6 hours only led to 30% of intracellular bacteria being contained in a phagolysosome with pH of 6 or below (Figure 5.7A-D). Using an MOI of 0.05 I still found that only 30% of

intracellular bacteria were in an acidified organelle with pH 6 or below (Figure 5.7E-F). This suggested that the majority of intracellular bacteria were in a compartment that was not appropriately acidified. I investigated whether the peak in acidification occurred earlier than 6 hours. Challenging the macrophages to *S. aureus* for 1.5-6 hours demonstrated there was a gradual increase in fluorescence of pH rhodamine from 20% to 30% by 4 hours which remained constant for the next 2 hours (Figure 5.7G-H). This suggested that not only were intracellular bacteria in a compartment that was not sufficiently acidified, but also that there might be a block on acidification of the phagolysosome.

5.2.4. The failure to traffick to an intracellular compartment of high pH is *S. aureus* specific

Having determined that only a minority of intracellular *S. aureus* trafficked to a compartment of low pH, I next investigated whether this phenotype was specific to *S. aureus*. To do this I decided to examine whether two other bacteria, *Escherichia coli* and *Streptococcus pneumoniae* trafficked to intracellular compartments of low pH, by staining with pH rhodamine as outlined previously.

S. pneumoniae was first opsonised in immune serum for 30 minutes at 37°C to enhance internalisation and then labelled with pH rhodamine for 30 minutes as outlined above. THP-1 differentiated macrophages were challenged with the labelled *S. pneumoniae* at an MOI of 5 bacteria per cell for 4 hours and extracellular bacteria were killed with gentamicin. Cultures were maintained for up to 24 hours post infection and fixed at each time point. *E. coli* was labelled with pH rhodamine for 30 minutes at 37°C and then THP-1 differentiated macrophages were challenged with the labelled *E. coli* at an MOI of 5 bacteria per cell for 4 hours and extracellular bacteria were killed with gentamicin. Cultures were either fixed as outlined previously or maintained for 0.5-24 hours post infection and then fixed at each time point. The percentage of intracellular bacteria that were pH rhodamine positive was quantified in 100 random macrophages.

I found challenging macrophages with labelled *S. pneumoniae* or *E. coli* at a high MOI for 4 hours led to ~ 60% of intracellular bacteria fluorescing indicative of their presence in an endosome of reduced pH (Figure 5.8A-D). Over time there was a gradual decrease, for both organisms that were in an acidic compartment and by 24 hours post infection there were very few intracellular bacteria in an acidic endosomal compartment (Figure 5.8A-D).

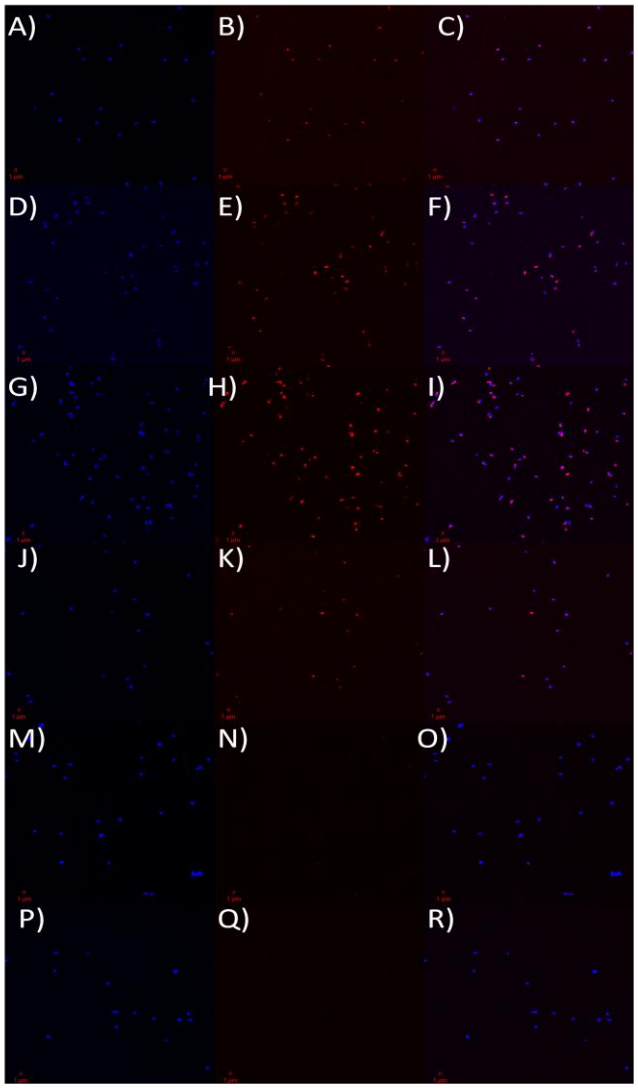

Figure 5.6: <u>pH rhodamine labelled *S. aureus* fluoresces maximally at pH of 6 or below.</u>

S. aureus were labelled with pH rhodamine and fixed in paraformaldehyde. Labelled bacteria were then incubated in solutions of varying pH and images taken using the confocal multiphoton microscope. **A)** DAPI image of *S. aureus* following staining. **B)** pH rhodamine image of *S. aureus* following staining. **C)** Merged image of *S. aureus* following staining. **D)** DAPI image of *S. aureus* at pH 4. **E)** pH rhodamine image of *S. aureus* at pH 4. **F)** Merged image of *S. aureus* at pH 4. **G)** DAPI image of *S. aureus* at pH 5. **H)** pH rhodamine image of *S. aureus* at pH 5. **I)** Merged image of *S. aureus* at pH 5. **J)** DAPI image of *S. aureus* at pH 6. **K)** pH rhodamine image of *S. aureus* at pH 6. **L)** Merged image of *S. aureus* at pH 6. **M)** DAPI image of *S. aureus* at pH 7. **N)** pH rhodamine image of *S. aureus* at pH 7. **O)** Merged image of *S. aureus* at pH 7. **P)** DAPI image of *S. aureus* at pH 8. **Q)** pH rhodamine image of *S. aureus* at pH 8. **R)** Merged image of *S. aureus* at pH 8.

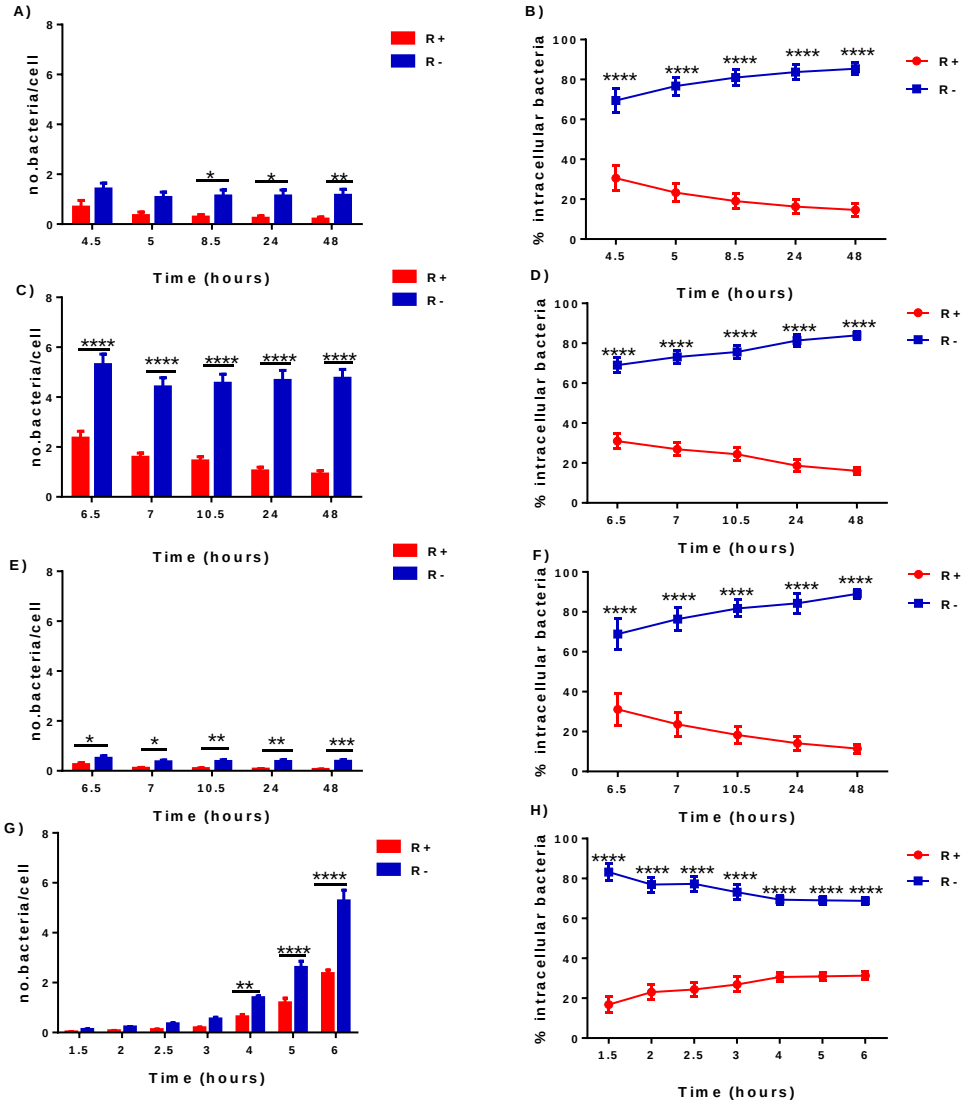

Figure 5.7: <u>The majority of *S. aureus* do not localise in a compartment of low pH.</u>

A-B) THP-1 differentiated macrophages were challenged with pH rhodamine labelled *S. aureus* at an MOI of 5 for 4 hours or **C-D)** for 6 hours and extracellular bacteria were killed with lysostaphin for 0.5 hours and macrophages were fixed for microscopy. Cultures were also maintained in low dose lysostaphin and fixed at the indicated time points. Alternatively THP-1 differentiated macrophages were challenged with pH rhodamine labelled *S. aureus* at **E-F)** an MOI of 0.05 for 6 hours or **G-H)** an MOI of 5 for 1.5-6 hours and fixed at each time point for microscopy. **A, C, E, G)** Number of intracellular bacteria per cell showing pH rhodamine fluorescence, **B, D, F, H)** Percentage of intracellular bacteria showing pH rhodamine fluorescence. The data represents the mean with the standard error of the mean; n=3 *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Two Way Anova with Bonferonni Post Test *versus* rhodo negative.

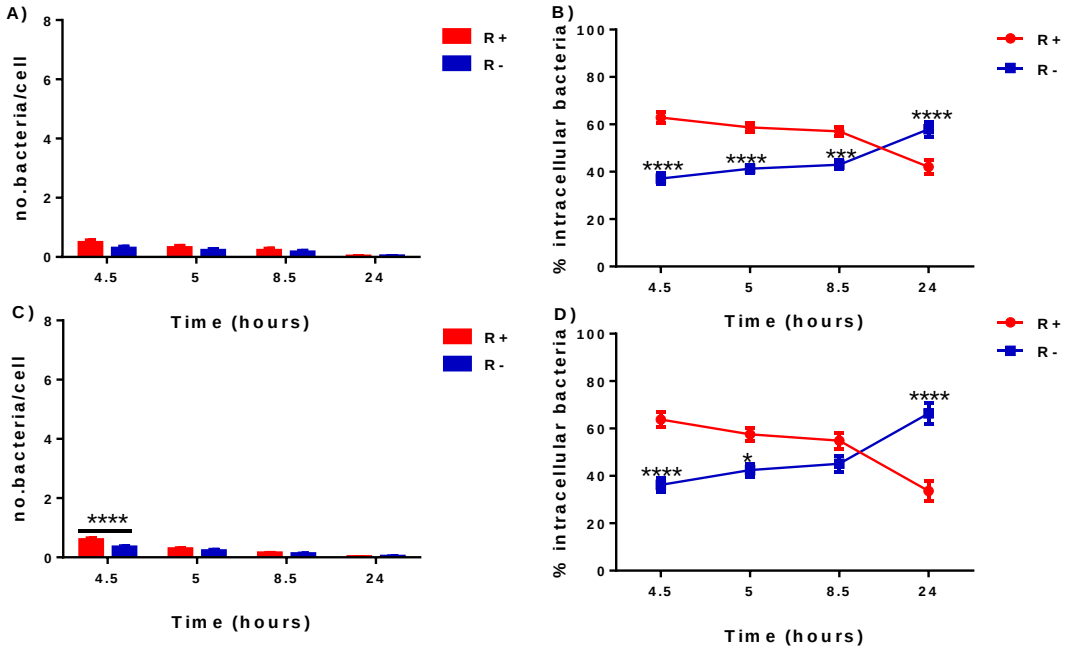

Figure 5.8: <u>Failure to localise in a compartment of low pH is specific to *S. aureus*.</u>

THP-1 differentiated macrophages were challenged with pH rhodamine labelled *S. pneumoniae* or *E. coli* at an MOI of 5 for 4 hours and extracellular bacteria were killed with gentamicin for 0.5 hours and macrophages were fixed for microscopy. Cultures were also maintained in low dose gentamicin and fixed at the indicated time points. **A)** Number of intracellular *S. pneumoniae* per cell fluorescing at 4.5-24 hours, **B)** Percentage of intracellular *S. pneumoniae* fluorescing at 4.5-24 hours, **C)** Number of intracellular *E. coli* per cell fluorescing at 4.5-24 hours, **D)** Percentage of intracellular *E. coli* fluorescing at 4.5-24 hours. The data represents the mean with the standard error of the mean; n=3 ***p<0.001, ****p<0.0001, Two Way Anova with Bonferonni Post Test *versus* rhodo negative.

5.2.5. Host mediated macrophage apoptosis is not engaged during *S. aureus* infection

Host-mediated macrophage apoptosis has been shown to be an additional mechanism by which the macrophage can clear intracellular bacteria, and has been demonstrated for bacteria as diverse as *S. pneumoniae* infection (Dockrell et al., 2001) and mycobacterial species (Keane et al., 1997). I sought to investigate whether this additional element of killing was engaged during *S.aureus* infection of macrophages.

THP-1 differentiated macrophages were challenged with *S. aureus* Newman at an MOI of 5 or 25 bacteria per cell or mock-infected (MI) for 6 hours and extracellular bacteria were killed with lysostaphin. Alternatively macrophages were challenged with *S. aureus* SH1000 at an MOI of 5 bacteria per cell for 6 hours and extracellular bacteria were killed with lysostaphin. Cultures were then maintained in low dose lysostaphin for up to 40 hours post infection and fixed at each time point. The cultures were then stained with DAPI to stain nuclei and assessed for apoptosis by fluorescence microscopy, counting the number of cells per field to determine cellular viability and also scoring apoptosis based on nuclear features such as nuclear fragmentation.

I found for both *S. aureus* Newman at an MOI of 5 and 25 bacteria per cell over 40 hours there was no significant decrease in macrophage numbers compared to MI macrophages (Figure 5.9A-B). As well as this macrophage apoptosis was not engaged at a high level compared to MI macrophages (Figure 5.9C-D) unlike what is reported for *S. pneumoniae* infection for example. These results suggest the maintenance of macrophage cell numbers per field and the non-engagement of apoptosis is not dose dependent. I also found this response was not strain dependent and using *S. aureus* SH1000, macrophage viability was again maintained for at least 40 hours *versus* MI macrophages (Figure 5.10A) and there was only a small percentage of macrophage apoptosis evident *versus* MI macrophages (Figure 5.10B). As a positive control, already performed by our research group, macrophages were UV irradiated and this induced nuclear fragmentation (Marriott et al., 2005). Representative images of *S. aureus* Newman infection *versus* MI for 6, 20 and 26h are shown in figure 5.11.

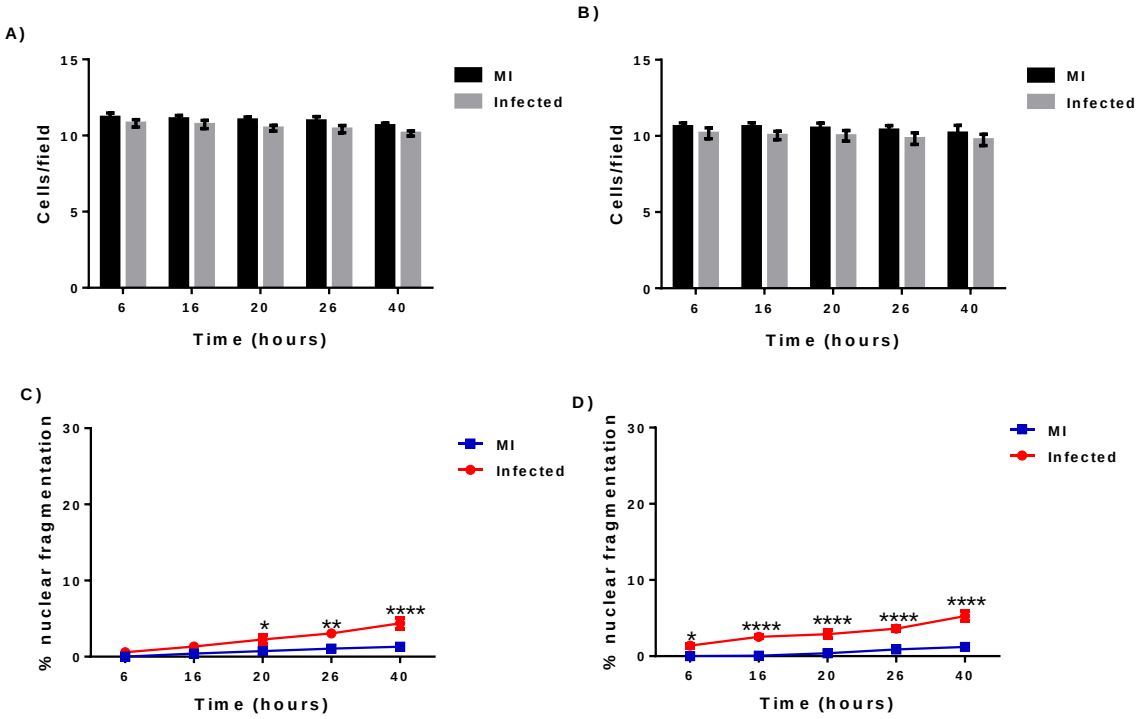

Figure 5.9: <u>Macrophage associated apoptosis is not engaged during *S.aureus* infection.</u>

THP-1 differentiated macrophages were mock infected or challenged with *S. aureus* (Newman strain) for 6 hours and extracellular bacteria were killed with lysostaphin for 0.5 hours and macrophages were fixed and stained or maintained in lysostaphin for up to 40 hours post infection **A)** Macrophage viability at an MOI of 5, **B)** Macrophage viability at an MOI of 25, **C)** Macrophage apoptosis at an MOI of 5, **D)** Macrophage apoptosis at an MOI of 25. The data represents the mean with the standard error of the mean; n=5 (MOI of 5) and n=3 (MOI of 25), *p<0.05, **p<0.01, ****p<0.0001, Two Way Anova with Bonferonni Post Test comparing mock infected to infected.

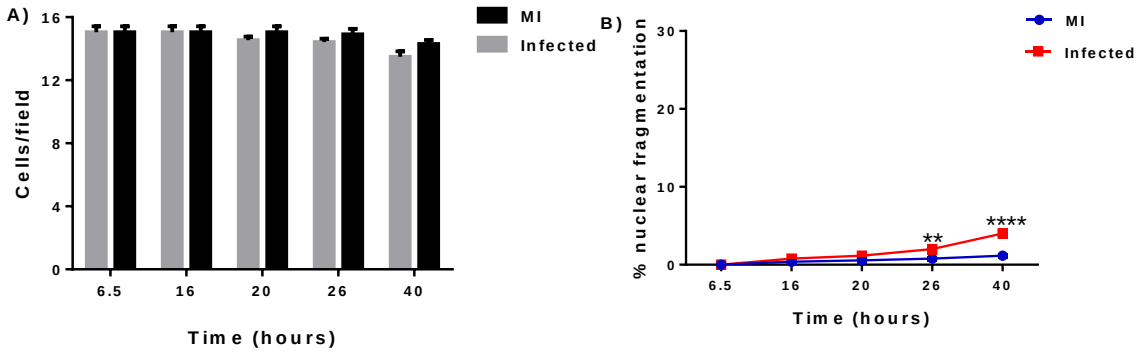

Figure 5.10: <u>Failure of macrophages to undergo apoptosis is not strain dependent.</u>

THP-1 differentiated macrophages were mock infected or challenged with *S. aureus* SH1000 for 6 hours and extracellular bacteria were killed with lysostaphin for 0.5 hours and macrophages were fixed and stained or maintained in lysostaphin for up to 40 hours post infection **A)** Macrophage viability at an MOI of 5, **B)** Macrophage apoptosis at an MOI of 5. The data represents the mean with the standard error of the mean; n=3, **p<0.01, ****p<0.0001, Two Way Anova with Bonferonni Post Test comparing mock infected to infected.

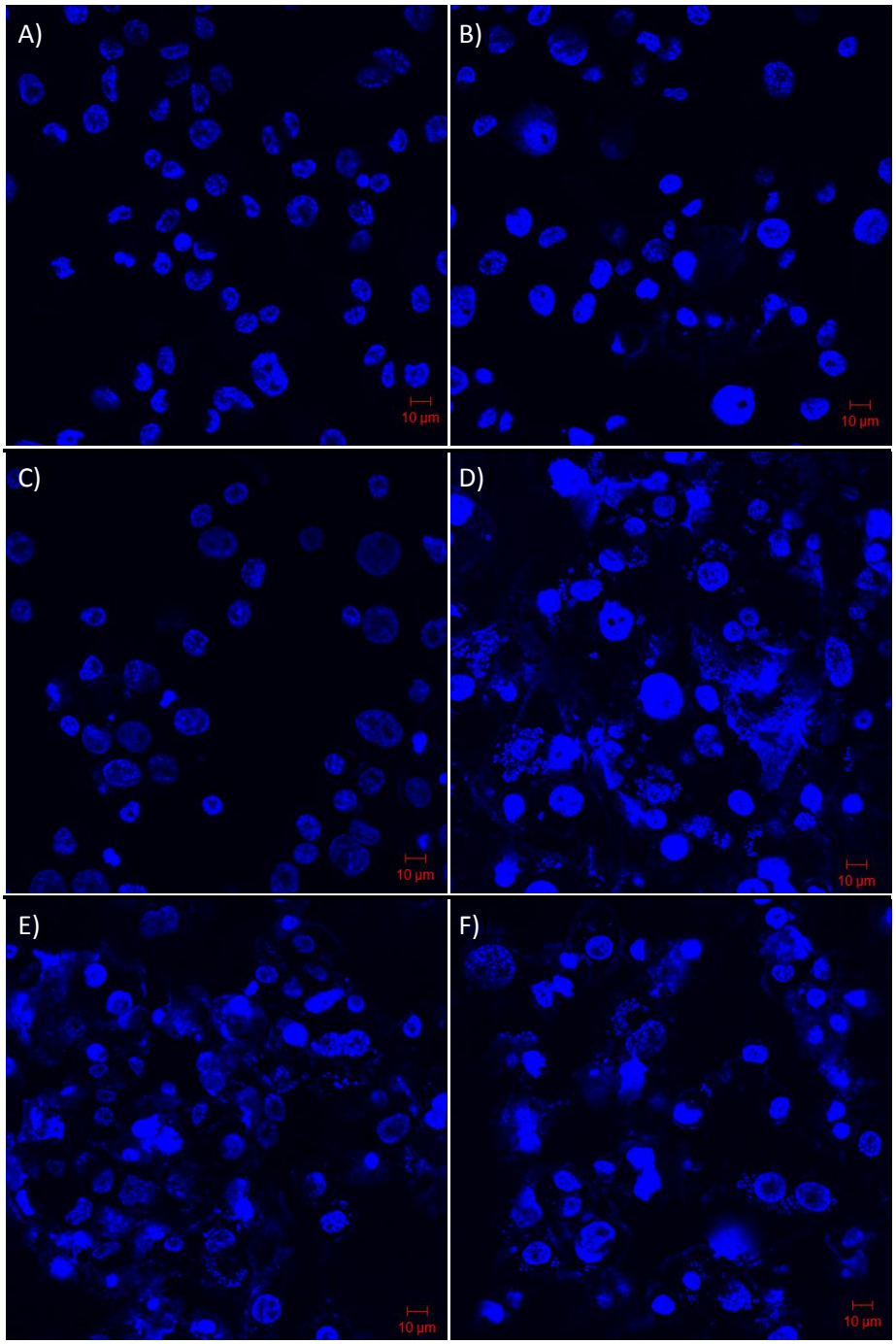

Figure 5.11: <u>Representative images of macrophage apoptosis with *S. aureus* MOI 5.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* Newman or mock infected for 6 hours and extracellular bacteria were killed with lysostaphin for 0.5 hours and macrophages were fixed and stained or maintained in lysostaphin for up to 20 and 26 hours post infection. Images were taken using the x40 magnification of the Zeiss laser scanning confocal microscope. **A)** Mock infected 6 hours, **B)** *S. aureus* infection 6

hours, **C)** Mock infected 20 hours, **D)** *S. aureus* infection 20 hours, **E)** Mock infected 26 hours and **F)** *S. aureus* infection 26 hours. Scale bar represents 10μm.

5.2.6. Macrophages retain viability for extended periods with intracellular *S. aureus*

Having demonstrated that macrophage cell numbers were maintained for up to 40 hours with intracellular *S. aureus* at a high MOI, I next determined if this phenotype would also occur at a lower MOI over an extended period of culture. I hypothesised that if the bacteria produced factors that inhibited host-mediated apoptosis that at a lower dose might be reengaged. I also reasoned that if this did not occur macrophages would continue to ingest bacteria and I wanted to know what the ultimate fate of these cells and the bacteria they contained was. I reasoned that if macrophages remained viable over an extended period of time, there would be a point when the macrophages succumbed to the infection and intracellular bacteria would be released from the macrophages intracellular environment.

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 0.05 bacteria per cell or MI for 6 hours and extracellular bacteria were killed with lysostaphin. Cultures were then either maintained in low dose lysostaphin for up to 14 days post infection or maintained in low dose lysostaphin for up to 2 days post infection and then cultured in lysostaphin free media. At each time point, cultures were either lysed for an estimation of intracellular CFU (extracellular CFU were also quantified) or fixed and stained as outlined previously for viability, apoptosis and LAMP-1 staining.

I found as outlined in chapter 3, that over the first 24 hours, there was an initial decline in intracellular bacterial numbers over the first 30 minutes post internalisation and then a persistence of intracellular bacteria (Figure 5.12A). Extracellular CFU were consistently 0 over these time points. In cultures without lysostaphin at 3 days post infection there was a burst of extracellular bacterial replication which increased over the next 4 days and then plateaued between days 7 and 14 post infection. For cultures maintained in lysostaphin there were no detectable extracellular bacteria over the same time course. In accordance with these results for cultures maintained without lysostaphin there was an increase in intracellular viable bacteria from 3 days post infection (Figure 5.12A), whereas in cultures maintained with lysostaphin the intracellular viable bacteria numbers gradually declined and became undetectable by 14 days post infection (Figure 5.12A). I verified these results by microscopy and showed

for cultures maintained without lysostaphin there was increase in the number of intracellular bacteria per cell from 3 days post infection (Figure 5.12B). Cultures

maintained with lysostaphin showed a decrease in intracellular bacteria per cell becoming 0 by day 14 (Figure 5.12B). This was matched by an increase in the number of macrophages with internalised bacteria from 3 days p/i for cultures without lysostaphin compared to a gradual decrease for cultures with lysostaphin (Figure 5.12C). Staining for LAMP-1 association revealed that in cultures maintained without lysostaphin the number of intracellular bacteria trafficking to LAMP-1 positive compartments increased from 3 days post infection (Figure 5.12D). In those cultures maintained in lysostaphin the opposite occurred and the number of intracellular bacteria colocalising with LAMP-1 became 0 by day 14 (Figure 5.12D). Despite this, the percentage of intracellular bacteria colocalising with LAMP-1 was fairly constant for cultures maintained without lysostaphin and decreased for cultures without lysostaphin (Figure 5.12E).

When I looked at macrophage viability, I found this to be fairly similar for infected *versus* MI cultures for both experimental setups for the first 2 days post infection and then there was a gradual decline in both setups by 3 days post infection (Figure 5.13A-B). However, the decrease in macrophage numbers continued for cultures without lysostaphin reaching about a 60% decrease by day 14 *versus* mock infected, whereas for cultures maintained in lysostaphin there was an initial 20% decrease by day 3 and then no further decrease in macrophage numbers (Figure 5.13A-B). Despite this, the percentage of apoptosis noted for both experimental conditions was markedly low (Figure 5.13C-D).

Taken together these results suggest that there is a point during *S. aureus* infection when macrophages become overwhelmed by intracellular bacteria and succumb to the infection by a cytolytic death process. These results suggests that sometime between 48 and 72 hours, there is a release of intracellular bacteria from the macrophages which can then replicate extracellularly and be further ingested by other macrophages. This release and further ingestion is predicted to be ongoing leading to the gradual decline in overall macrophage numbers by day 14 for cultures without lysostaphin. In the case of cultures with lysostaphin, these results suggest the same phenomenon with cytolytic cell death might be occurring initially but any released bacteria are killed by the extracellular lysostaphin minimising any further detectable internalisation and further waves of

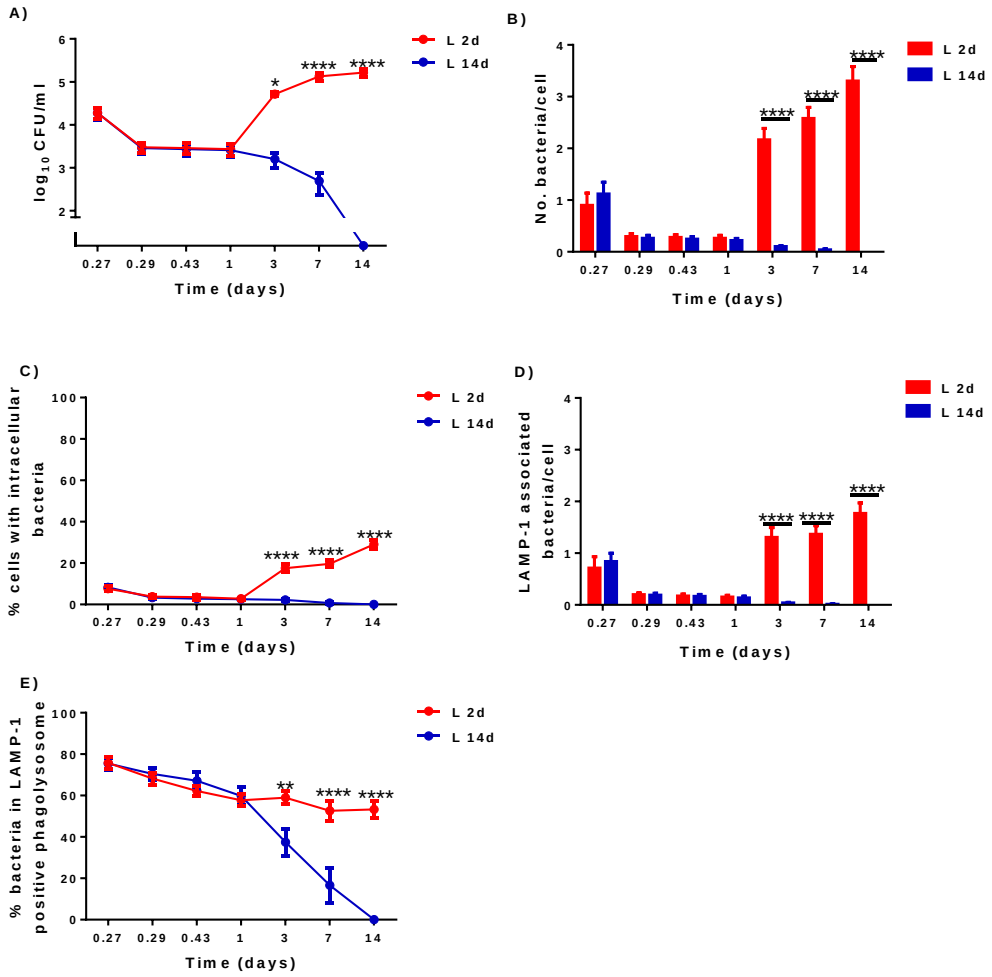

Figure 5.12: <u>Without inhibition of extracellular replication, intracellular bacterial numbers increase.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 6 hours at an MOI of 0.05 and extracellular bacteria were killed with lysostaphin and macrophages were maintained in lysostaphin for up to 48 hours post infection. Some cultures were then maintained without lysostaphin for up to 14 days post infection. **A)** The intracellular logarithmic colony forming units value of viable bacteria in macrophages treated with or without lysostaphin, **B)** Number of intracellular bacteria per macrophage in cultures with or without lysostaphin. **C)** The percentage of macrophages with intracellular bacteria in cultures with or without lysostaphin. **D)** Number of intracellular bacteria in a LAMP-1 positive compartment in macrophage cultures with or without lysostaphin. **E)** The percentage of intracellular bacteria in a LAMP-1 positive compartment in macrophage cultures with or without lysostaphin. The data represents the mean with the standard error of the mean; n=3, *p<0.05, **p<0.01, ****p<0.0001, Two Way Anova with Dunnett's Post Test comparing with and without lysostaphin.

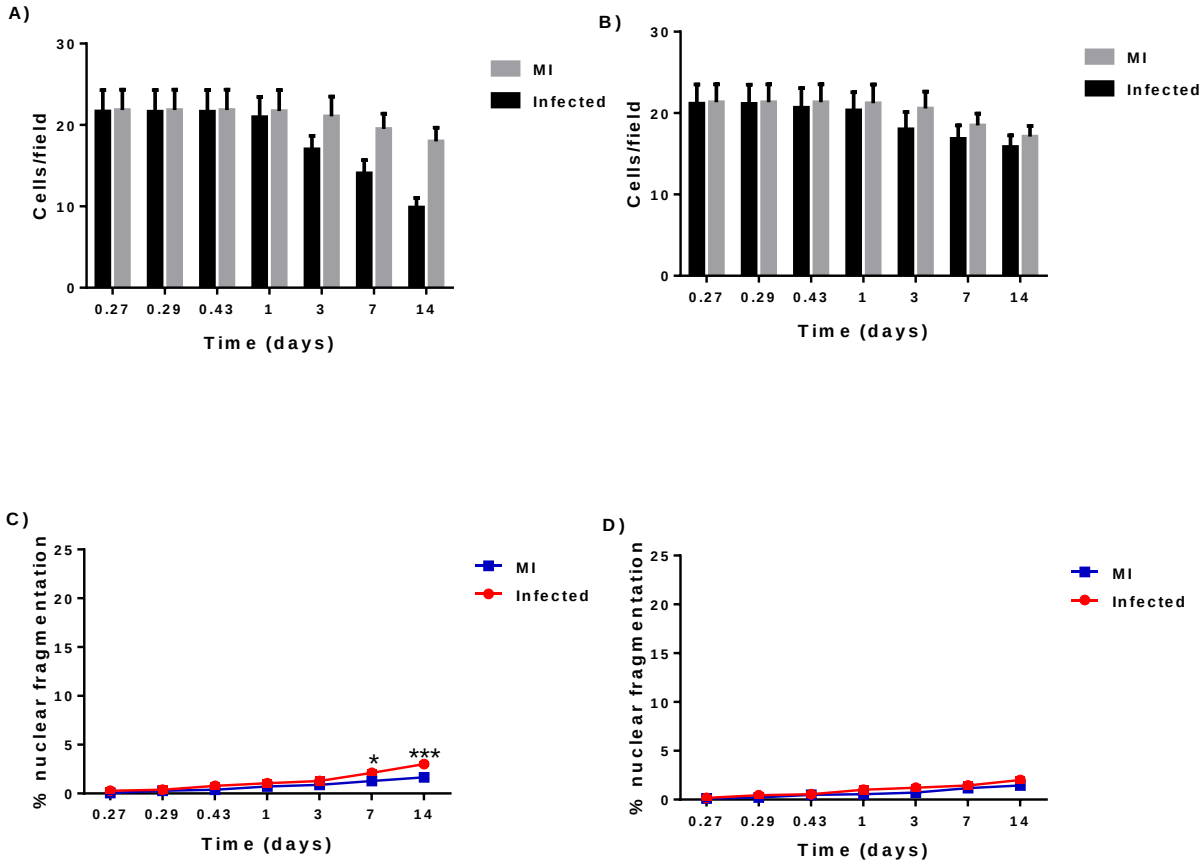

Figure 5.13: <u>Macrophage viability gradually declines over time in the presence of *S. aureus*.</u>

THP-1 differentiated macrophages were mock infected or challenged with *S. aureus* at an MOI of 0.05 for 6 hours and extracellular bacteria were killed with lysostaphin and macrophages were fixed and stained for apoptosis, defined by nuclear morphological features of apoptosis on microscopy, or maintained in lysostaphin for up to 48 hours post infection. Some cultures were then maintained without lysostaphin for up to 14 days post infection. **A)** Macrophage viability without lysostaphin, **B)** Macrophage viability with lysostaphin. **C)** The percentage of macrophage apoptosis without lysostaphin or **D)** The percentage of macrophage apoptosis with lysostaphin. The data represents the mean with the standard error of the mean; n=3, *p<0.05, ****p<0.0001, Two Way Anova with Bonferonni Post Test comparing mock infected to infected.

cytolytic death. As there are no further intracellular bacteria accumulating in these macrophages, despite what was already present, there is very minimal macrophage lysis over the next 11 days and macrophage numbers remain fairly constant throughout the remainder of the experimental culture.

These results were also shown by video time lapse microscopy (Figure 5.14).

5.2.7. The increase in intracellular bacteria in macrophages over extended periods requires repeated waves of cell lysis and re-uptake of released bacteria

Having demonstrated that macrophage cultures maintained without lysostaphin exhibited increases in intracellular viable *S. aureus* from 3 days post infection, I next determined if the late increase in intracellular burden required sustained phagocytosis. I reasoned that if phagocytosis was inhibited and the intracellular burden increased that this would suggest that the increase in intracellular bacteria was the result of intracellular replication. I hypothesised that since intracellular bacterial killing seemed exhausted that if bacteria did not replicate over an extended period of time, blocking internalisation with the actin cytoskeleton inhibitor cytochalasin D would lead to a gradual decline in intracellular viable bacteria over 14 days, as cells were lysed and released their bacteria.

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 0.05 bacteria per cell or MI for 6 hours and extracellular bacteria were killed with lysostaphin. Cultures were then either maintained in low dose lysostaphin with or without cytochalasin D from 2 days post infection for up to 14 days post infection or maintained in low dose lysostaphin for up to 2 days post infection and then cultured in lysostaphin free media with or without cytochalasin D for the remaining time. At each time point, cultures were either lysed for an estimation of intracellular CFU (extracellular CFU were also quantified) or fixed and stained as outlined previously for viability, apoptosis and detection of LAMP-1 staining.

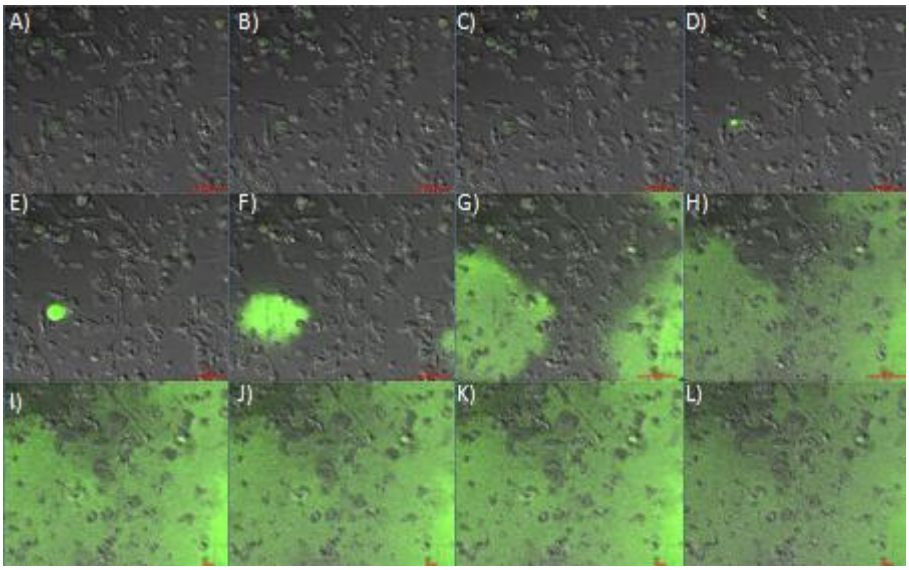

Figure 5.14: <u>Representative images of macrophage lysis by *S.aureus* from video time lapse microscopy.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* Newman~GFP at an MOI of 5 for 6 hours and extracellular bacteria were killed with lysostaphin. Cultures were either maintained without lysostaphin until 74 hours post infection. Imaging was started at 52 hours post infection. Images were taken using the x20 DIC and GFP magnification of the Nikon Ti inverted fluorescence microscope. **A)** 52 hours **B)** 54 hours **C)** 56 hours **D)** 58 hours **E)** 60 hours **F)** 62 hours **G)** 64 hours **H)** 66 hours **I)** 68 hours **J)** 70 hours **K)** 72 hours **L)** 74 hours post infection. Scale bar represents 100µm.

I found as outlined in chapter 3, that there was an initial decline in intracellular bacterial numbers over the first 30 minutes post internalisation and then a persistence of intracellular bacteria over the first 24 hours. Extracellular CFU were consistently undetectable over these time points. In cultures where lysostaphin was not maintained in the culture after 2 days I found that by 3 days post infection there was an increase in extracellular bacterial colony counts, irrespective of the presence of cytochalasin D, which increased further over the next 4 days and then plateaued between days 7 and 14 post infection.

For cultures maintained in lysostaphin, there were no detectable extracellular bacteria over the same time course. In accordance with these results for cultures maintained without lysostaphin or cytochalasin D there was an increase in intracellular viable bacteria from 3 days post infection (Figure 5.15A), whereas in cultures maintained without lysostaphin but with cytochalasin D the intracellular viable bacteria numbers gradually declined and became undetectable by 14 days post infection (Figure 5.15A). For cultures maintained with lysostaphin with or without cytochalasin D, there was also a gradual decline in intracellular viable bacteria over 14 days (Figure 5.15A). I verified these results by microscopy and showed for cultures maintained without lysostaphin or cytochalasin D there was an increase in the number of intracellular bacteria per cell from 3 days post infection (Figure 5.15B), not seen for cultures maintained with cytochalasin D (Figure 5.15B). Cultures maintained with lysostaphin with or without cytochalasin D showed a decrease in intracellular bacteria per cell becoming zero by day 14 (Figure 5.15B). This was matched by an increase in the number of macrophages with internalised bacteria from 3 days post infection for cultures without lysostaphin or cytochalasin D compared to a gradual decrease for cultures with lysostaphin with or without cytochalasin D and cultures without lysostaphin but with cytochalasin D (Figure 5.15C). Staining for LAMP-1 association revealed that in cultures maintained without lysostaphin the number of intracellular bacteria trafficking to LAMP-1 positive compartments increased from 3 days post infection (Figure 5.15D). In those cultures maintained in lysostaphin with or without cytochalasin D or without lysostaphin and with cytochalasin D the opposite occurred and the number of intracellular bacteria colocalising with LAMP-1 decreased and became zero by day 14 (Figure 5.15D). Despite this, the percentage of intracellular bacteria colocalising with LAMP-1 was fairly constant for cultures maintained without lysostaphin and decreased for cultures

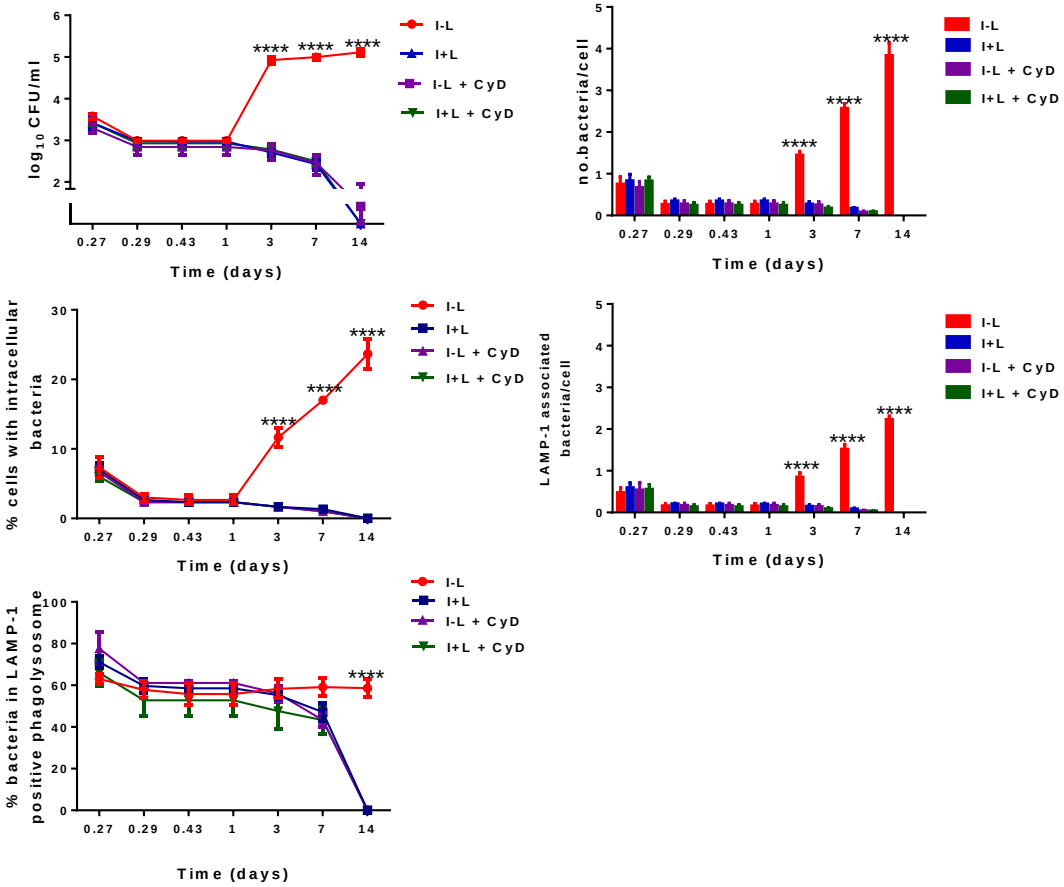

Figure 5.15: <u>The increase in intracellular viable bacteria is not the result of intracellular replication.</u>

THP-1 differentiated macrophages were challenged with *S. aureus* for 6 hours at an MOI of 0.05 and extracellular bacteria were killed with lysostaphin and macrophages were maintained in lysostaphin for up to 48 hours post infection. Some cultures were then maintained with (I+L) or without (I-L) lysostaphin for up to 14 days post infection either in the presence (with CyD) or absence of cytochalasin D. **A)** Intracellular viable bacteria depicted as logarithmic value of colony forming units, **B)** Number of intracellular bacteria per cell, **C)** The percentage of macrophages with intracellular bacteria. **D)** Number of intracellular bacteria in a LAMP-1 positive compartment. **E)** The percentage of intracellular bacteria in a LAMP-1 positive compartment. The data represents the mean with the standard error of the mean; n=3, ****p<0.0001, Two Way Anova with Dunnett's Post Test comparing with and without lysostaphin.

with lysostaphin with or without cytochalasin D and cultures maintained without lysostaphin and with cytochalasin D (Figure 5.15E).

When I looked at macrophage cell number, I found this to be fairly similar for infected *versus* MI cultures for all experimental setups for the first 2 days post infection but there was a non significant decline in all conditions involving *S. aureus* challenge by 3 days post infection (Figure 5.16A-B). However, the decrease in macrophage numbers became significant for cultures without lysostaphin whereas for cultures maintained in lysostaphin with or without cytochalasin D there was an initial 20% decrease by day 3 and then no further decrease in macrophage numbers, which was also similar for cultures without lysostaphin and with cytochalasin D (Figure 5.16A-B). Despite this, the percentage of apoptosis noted for all experimental conditions was markedly low (Figure 5.16C-D).

Taken together these results suggest that macrophages are lysed by intracellular bacteria and release them allowing them to then replicate extracellularly. There is then further internalisation by other macrophages allowing maintenance of a pool of intracellular bacteria. Treatment with cytochalasin D suggests the increase in intracellular viable numbers is not the result of intracellular replication of bacteria since it is dependent on ongoing phagocytosis. Also, the cell number results suggest cytochalasin D is not toxic to the macrophages over extended periods of culture and cell death is mainly occurring in the presence of ongoing ingestion of extracellular bacteria. Extracellular bacteria are by themselves not sufficient to induce cell loss since in the presence of cytochalasin D and extracellular bacteria there was only a non-significant decline in macrophage cell number.

5.2.8. The percentage of non-viable bacteria decreases with increasing dose

Having determined that intracellular *S. aureus* persisted within macrophages and were not in a compartment of low pH, I next investigated whether intracellular bacteria were dead. To do this I used the fluorescent dye DRAQ7, which enters permeable cells and binds to the DNA as a marker of death. Prior to carrying out this experiment, I used my data to hypothesise the percentage of non viable bacteria that should be present for each MOI (Table 5.1).

THP-1 differentiated macrophages were challenged with live or heat killed *S. aureus* at an MOI of 0.05 or 5 bacteria per cell 5 hours and extracellular bacteria were killed with

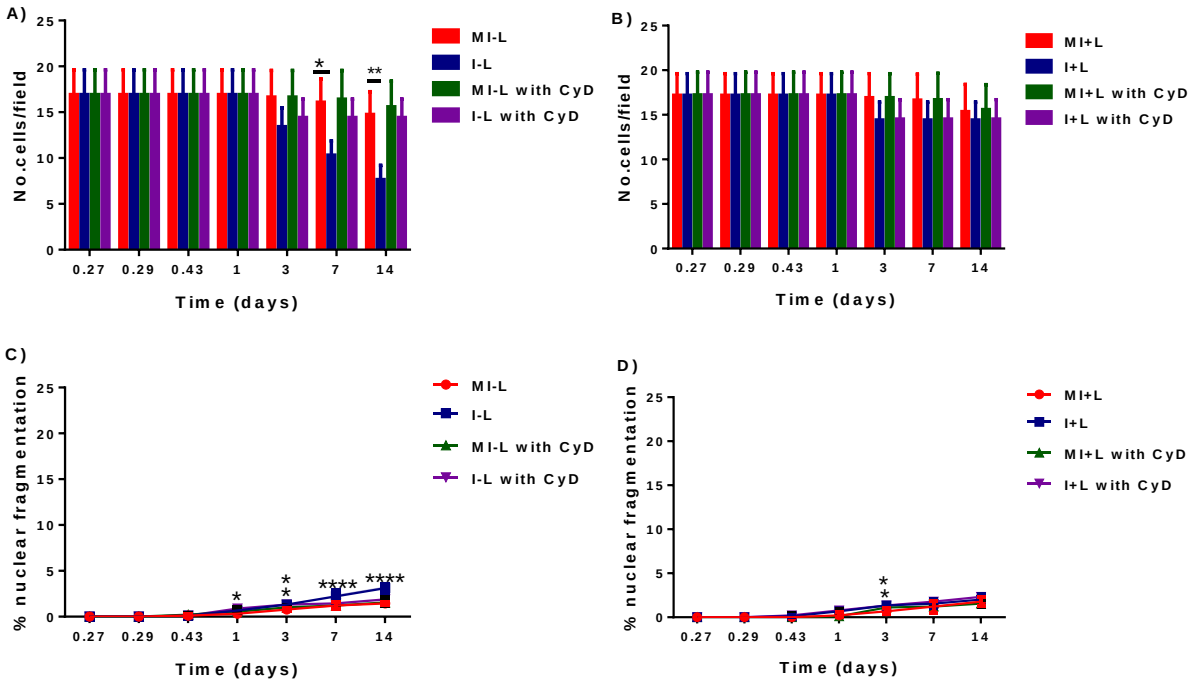

Figure 5.16: <u>Cytochalasin D does not affect macrophage viability.</u>

Macrophages were mock infected (MI) or challenged with *S. aureus* at an MOI of 0.05 (I) for 6 hours and extracellular bacteria were killed with lysostaphin and macrophages were fixed and stained for apoptosis or maintained in lysostaphin for up to 48 hours post infection. Some cultures were then maintained with (+L) or without (-L) lysostaphin for up to 14 days post infection either in the presence (with CyD) or absence of cytochalasin D. **A)** Macrophage viability without lysostaphin and with or without cytochalasin D, **B)** Macrophage viability with lysostaphin and with or without cytochalasin D. **C)** The percentage of macrophage apoptosis (depicted as % nuclear fragmentation) without lysostaphin and with or without cytochalasin D. **D)** The percentage of macrophage apoptosis with lysostaphin and with or without cytochalasin D. The data represents the mean with the standard error of the mean; n=3, *p<0.05, **p<0.01 ****p<0.0001, Two Way Anova with Bonferonni Post Test comparing MI+/-L to infected. In panels C) and D) the 2 x * above 3 days indicates significance in I-L and I –L with CyD *vs* MI-L and I +L and I +L with CyD *vs* MI+L.

MOI (5 hours)	Intracellular bacteria x macrophage numbers	Viable intracellular CFU (log)	Estimated non viable CFU (log)	Percentage killing (%)
0.05	200000	1200	199000	99.5
0.5	268000	2570	266000	99.3
1	380000	2700	377000	99.2
2	428000	3700	422000	99.1
5	516000	105000	410000	79.7

Table 5.1: Macrophages have a finite capacity for intracellular bacterial killing which is overwhelmed by increasing numbers of bacteria.

The number of intracellular bacteria per macrophage for each dose was multiplied by the total number of macrophages in culture to calculate the total intracellular burden, after 5 hours of challenge. The number of viable intracellular bacteria was subtracted from this to give an estimate of the non viable intracellular bacteria. This was then used to give an estimate of the percentage of intracellular bacteria that were killed.

lysostaphin. Cultures were then lysed in saponin and centrifuged using a combination of low and high spin speeds. The supernatants were then left unstained or stained with DRAQ7 and analysed by flow cytometry.

I found at a low MOI ~95% of intracellular bacteria were dead (Figure 5.17A, E) *versus* ~73% at a high MOI (Figure 5.17B, E). This suggested that macrophages were capable of killing nearly all intracellular *S. aureus* at the lower MOI but were increasingly accumulating viable intracellular bacteria, with a higher dose.

5.2.9. Intracellular bacterial accumulation over the initial period of bacterial challenge is not the result of intracellular replication

Having determined that macrophages accumulated intracellular bacteria over time, which persisted in an endosome which was not completely acidified, I next wanted to investigate whether this was the result of ongoing ingestion or of intracellular bacteria replicating.

THP-1 differentiated macrophages were challenged with *S. aureus* at an MOI of 5 bacteria per cell for 4 hours. At this point, they were either left untreated or incubated in either cytochalasin D to prevent ingestion, Trolox to prevent oxidative killing or both cytochalasin D and Trolox to inhibit both processes. Bacterial challenge was then continued for another 1-3 hours. At 5, 6 or 7 hours extracellular bacteria were killed with lysostaphin for 30 minutes and cultures lysed to estimate intracellular viable CFU. Results were confirmed using video time lapse microscopy, challenging THP-1 differentiated macrophages with *S. aureus* Newman~GFP for 1-6 hours and imaging over this time course.

I found compared to untreated controls, cytochalasin D treatment led to a decrease in intracellular CFU over time (Figure 5.18). In contrast both untreated and Trolox treated cultures showed a significantly higher increase in viable CFU over the same time course, as compared with cytochalasin D treated cultures, with the Trolox treated samples showing a significantly higher overall viable CFU *versus* untreated controls (Figure 5.18). Dual treatment with both compounds produced a straight line with no increase or decrease in intracellular CFU (Figure 5.18). These results suggested that macrophages were accumulating intracellular viable bacteria over these time points and this was not the result of intracellular replication. I confirmed this with video time lapse

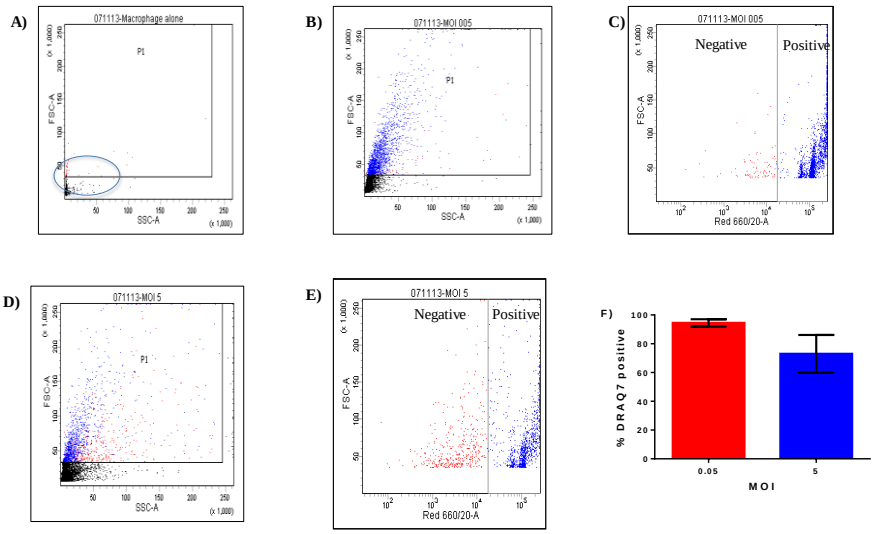

Figure 5.17: <u>Macrophages accumulate viable intracellular *S. aureus* with increasing dose.</u>

THP-1 differentiated macrophages were challenged *S. aureus* at an MOI of 0.05 or 5 for 5 hours and extracellular bacteria were killed with lysostaphin. Macrophages were lysed and stained with DRAQ7 and analysed by flow cytometry analysing 10,000 events per sample. **A)** Representative dot plot of lysed macrophages showing debris where gate is applied, **B)** Dot plot at an MOI of 0.05 before gating, **C)** Representative dot plot at an MOI of 0.05 after gating, **D)** Dot plot at an MOI of 5 before gating, **E)** Representative dot plot at an MOI of 5 after gating, **F)** The percentage of non viable bacteria, MOI of 0.05 *versus* an MOI of 5. The data represents the mean with the standard error of the mean; n=3.

microscopy which showed ingestion with no intracellular replication obvious over the first 6 hours of bacterial challenge (Figure 5.19).

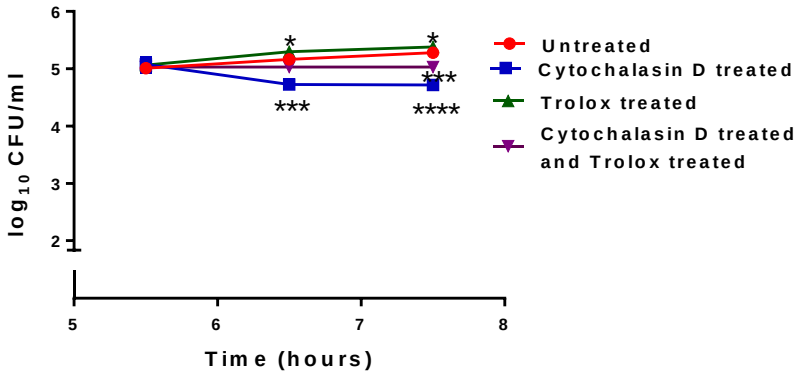

Figure 5.18: <u>Intracellular replication is not apparent with *S. aureus.*</u>

Macrophages were challenged with *S. aureus* at an MOI of 5 for 4 hours and then treated in various ways to inhibit either ingestion with cytochalasin D, intracellular killing with Trolox or both processes simultaneously for an additional 1-3 hours after which lysostaphin was added for 30 minutes to kill extracellular bacteria. Intracellular viable colony forming units were then followed and quantified between 5.5-7.5 hours post challenge. *p<0.05, ***p<0.001. Two Way Anova with Dunnett's Post Test *versus* untreated controls.

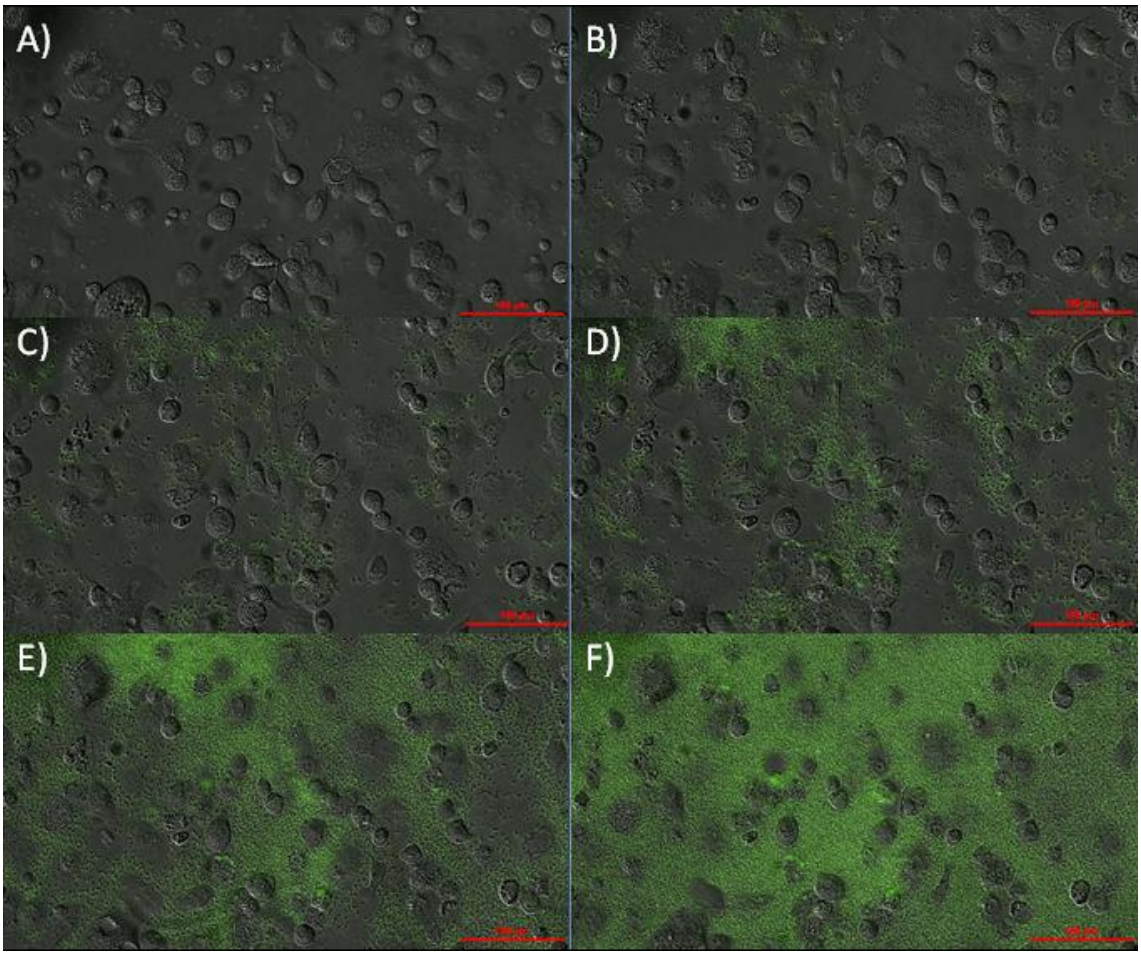

Figure 5.19: <u>Intracellular replication is not apparent with *S. aureus* as shown by video time lapse microscopy.</u>

Macrophages were challenged with *S. aureus* Newman~GFP at an MOI of 5 for 0-6 hours and imaged using the x30 GFP/DIC Nikon Ti inverted fluorescence microscope. **A)** T=1 hour, **B)** T=2 hours, **C)** T=3 hours, **D)** T=4 hours, **E)** T=5 hours, **F)** T=6 hours. Scale bar represents 100μm.

5.3. Discussion

The ability of *S. aureus* to escape host defence and survive intracellularly has been well documented (Gresham et al., 2000, Hess et al., 2003). The intracellular survival of *S. aureus* within macrophages has been a topic of interest over recent years (Kubica et al., 2008). The reason why *S. aureus* is able to survive in a rather hostile environment has not been fully explored. It is not known where intracellularly *S. aureus* resides and if there is a block in early processes during the formation of the phagolysosome. This prompted me to investigate the intracellular compartment *S. aureus* survived in and whether there may be a defect in phagolysosome formation. In this section I investigated the intracellular compartment in which *S. aureus* resided in more detail, whether macrophage associated apoptosis was engaged, whether intracellular bacteria replicated and what happened to bacteria and macrophages over extended periods of culture.

As LAMP proteins are required for optimal maturation of phagolysosomes (Huynh et al., 2007), I investigated if intracellular *S. aureus* resided in a LAMP positive compartment. I found that macrophages phagocytosed *S. aureus* into LAMP-1 positive phagolysosomes. LAMP-1 formed a tight protein ring around intracellular bacteria, suggesting that macrophages were not inhibited in their ability to fuse with early endosomes. Over 48 hours intracellular *S. aureus* persisted within LAMP-1 and LAMP-2 positive phagolysosomes. Interestingly the shapes of the graphs were different because the kinetics of LAMP-1 and 2 acquisition differ (Pitt et al., 1992, Desjardins et al., 1994). As both proteins were present over 48 hours the bacteria could be maintaining the proteins within the phagolysosomes or cause recycling of the protein (Rohrer et al., 1996) leading to its maintained expression. Alternatively, but less likely, there could have been movement of bacteria between macrophages and therefore constant fusion of phagosomes with lysosomes occurring and hence constant LAMP acquisition.

At both low and high bacterial MOI, macrophage viability was maintained with low levels of apoptosis. This was not strain dependent and *S. aureus* SH1000 did not cause a loss in macrophage cell number and macrophage apoptosis was not engaged. This suggested that *S. aureus* was either subverting or preventing macrophage apoptosis. To determine apoptosis I used DAPI staining, which although reasonable is not the most reliable technique because it determines live and dead nuclei. Other methods I could

have used to determine apoptosis include caspase 3 assay to determine caspase 3 activation which is a terminal phase in apoptosis activation; alternatively I could have done a Western blot to look for truncated apoptosis inducing factor (tAIF) which is also a terminal phase in apoptosis and when this protein is activated it moves to the nucleus and induces the nuclear changes of apoptosis. Other methods include flow cytometry to look for phosphatidylserine exposure on the surface of the macrophage using annexin V staining. Finally, in combination with the DAPI staining I could have performed TUNEL staining to fully prove the nuclei appearing apoptotic were apoptotic. These methods would have supplemented the DAPI staining results.

I found that for a low MOI, *S. aureus* was able to survive within macrophages for between 7-14 days post infection. Macrophage cell number was maintained and the levels of macrophage apoptosis were low. Culture without extracellular lysostaphin showed that from 3 days post infection there was extensive extracellular bacterial replication with a concomitant loss in macrophages compared to cultures with extracellular lysostaphin. This led to an increase in intracellular bacteria with increases in intracellular bacteria colocalising with LAMP-1, suggesting ongoing fusion was occurring. Cytochalasin D treatment did not prevent the loss in macrophages but did prevent any increase in intracellular bacterial numbers. Intracellular replication at these late time points could not be ruled out and was not proven directly. So it is possible at later time points there is a balance between macrophage internalisation and intracellular replication occurring. Similarly for a higher MOI intracellular replication was not occurring, at least at these early time points, as treatment with cytochalasin D led to a decrease in intracellular viable bacteria compared to untreated or Trolox treated cultures. Dual treatment also prevented any increase in intracellular viable bacteria further demonstrating that there was no intracellular replication. I verified this with video time lapse microscopy. Together these results suggest that the increase in viable intracellular bacteria over time was because the macrophages were internalising and accumulating bacteria. In these experiments I did not determine macrophage viability. One method I could have used to determine this is a dual stain for live and dead cells which is commercially available. This combines two different dyes that allow for the discrimination between live and dead cells. The theory is that permeabilised membranes permit entry of the non-viable stain and all cells permit entry of the viable stain, and therefore dead cells fluoresce one colour and live cells fluoresce another. Another method, is PI staining which would allow for the determination of compromised cellular

membranes. This could be combined with TUNEL staining to determine which non viable cells were apoptotic or necrotic.

The most likely explanation for the above result is that intracellular *S. aureus* are persisting in a 'silent' fashion. At some point, they are able to lyse the intracellular compartment where they reside and enter the cytoplasm and then go onto lyse the macrophage and replicate extracellularly, as shown for other cell types (Giese et al., 2009, Lam et al., 2010, Giese et al., 2011, Grosz et al., 2013). Extracellular bacteria are then ingested by other macrophages that may or may not have already ingested bacteria and the process repeats. In the presence of lysostaphin this stage of extracellular replication and the pool of extracellular bacteria required for re-uptake is removed and the cycle broken. The macrophages become exhausted from intracellular killing giving the bacteria an advantage, and they ultimately kill the exhausted macrophage and get released into the extracellular fluid. It is also possible that it is a subset of macrophages that lyse and release intracellular bacteria as I was able to demonstrate via video time lapse imaging that only some macrophages in the population lyse but my analysis was unable to determine whether this represented a majority of those that ingest bacteria or just a sub-population.

As I was interested in determining why intracellular *S. aureus* was able to survive within macrophages, I decided to examine whether intracellular *S. aureus* were in a compartment of low pH. As I was interested in determining whether or not intracellular *S. aureus* were in a compartment of low pH, I decided to use a stain that would label the bacteria and not the intracellular compartment where the bacteria resided. As this particular stain fluoresces maximally at a pH of 6 or below, it gave me an indication of whether intracellular bacteria were in a mature phagolysosome. If I wanted to determine the actual pH of the compartment where the bacteria resided I could have used a dye that directly determined the pH of the intracellular compartment such as Lysostracker. However, as the question I was interested in was whether or not *S. aureus* was in a compartment of low pH, a dye that labelled the bacteria and fluoresced at low pH but not neutral or high pH was deemed to be a suitable selection. I was able to show that only 30% of intracellular bacteria were in a compartment of low pH. This was not dose dependent with a similar result seen with low *versus* high MOI. I was also able to demonstrate that I had not missed the peak in acidification through my time course. This phenotype was *S. aureus* specific because both *E. coli* and *S. pneumoniae* demonstrated significantly higher levels of phagolysosomal acidification. These results suggested that

S. aureus was preventing phagolysosome acidification, in contrast to non-professional phagocytes (Giese et al., 2009, Lam et al., 2010), which could lead to intracellular persistence in a less hostile endosome. It is important to mention here why I used opsonised *S. pneumoniae* but non-opsonised *S. aureus*. Mainly, the opsonisation is important to allow internalisation because *S. pneumoniae* possesses a thick polysaccharide capsule around it. Although this is not a direct comparison it was chosen because the *S. pneumoniae* strain has been widely exploited by the research group and some of the results I was trying to verify had been done using this strain (D39). Therefore because it was known using this strain of *S. pneumoniae* that macrophage apoptosis occurred, I wanted to prove this was also linked into this strain residing in a mature phagolysosome. For a more direct comparison, it would be useful to repeat the experiments using a non-opsonised *S. pneumoniae* strain and this is definitely something for the future.

The reasons for the above result are varied. Firstly, it is possible that *S. aureus* could prevent the inclusion of the V-ATPase into the phagolysosome leading to a failure to acidify and activate proteases. Secondly it is possible that the V-ATPase is present but not functioning. Thirdly it is possible that the fusion and acidification has occurred and that the macrophages are over-producing ROS to kill intracellular bacteria causing the decrease in phagolysosomal pH. It is possible that there is a failure to fuse with late endosomes bearing markers like Rab7 or lysosome integral membrane protein 2 (LIMP-II) and hence downstream processes such as V-ATPase acquisition are blocked. Finally, since some of the acidification process results from fusion with acid carrying vesicles derived from early endosomes this fusion event may be inhibited (Hackam et al., 1997).

I next determined the percentage of viable and non viable bacteria within macrophages. I found that at low MOI there was ~95% of intracellular bacteria that were non viable, which decreased to ~73% for MOI of 5. These results suggested that over time macrophages accumulated viable bacteria and that as MOI increased the capacity for sustained intracellular killing started to become overwhelmed.

The findings presented in this section of my thesis demonstrate that macrophages efficiently phagocytose *S. aureus* into LAMP-1 positive phagolysosomes and intracellular bacteria are able to persist in a LAMP-1 and 2 positive phagolysosomes, but the majority of bacteria are not in a phagolysosome of low pH. Macrophages do not engage apoptosis as a response to *S. aureus*. Over time, intracellular bacteria escaped

from the macrophages and replicated extracellularly. There were then further increases in intracellular bacterial numbers and further decreases in macrophage cell numbers. Intracellular replication was not observed at least at early time points. I demonstrated that as dose increased, macrophage killing was becoming exhausted leading to a persistence of viable intracellular bacteria. The reasons for the failure of *S. aureus* to reside in a compartment of low pH and how the bacteria were escaping the macrophages were beyond the scope of this section, but the host and microbial factors responsible will pose interesting topics for future research.

Chapter 6 Discussion

6.1. Major findings

In this thesis I report that macrophages are able to control the growth of extracellular bacteria but demonstrate a limited ability to kill intracellular *S. aureus* leading to intracellular persistence, despite sustained phagocytic capacity. The bulk of intracellular killing occurs immediately after phagocytosis and after this there is very little further killing. The absolute level of killing reaches a maximal level and then remains fairly fixed till the late time points after which the level drops off. My mathematical model suggested these patterns were consistent with a model where macrophages ingest bacteria at a rate proportional to the bacterial population but are limited in their killing capacity, and therefore over time macrophages become unable to kill all the bacteria they have phagocytosed. I found that only a minority of phagocytosed bacteria entered an appropriately acidified phagolysosome, unlike other extracellular bacteria tested, despite the fact that the phagosome acquired late endosomal markers such as LAMP-1 or 2. There was loss in the number of macrophages by day 3 of culture suggesting macrophages were being killed. As apoptosis was low and cells died the death process appeared to be necrosis. This meant that intracellular persisted for up to 3 days post infection within a macrophage and were then released extracellularly as macrophages were lysed. These bacteria were then taken up by other macrophages and therefore a cycle of release and re-uptake was required to maintain the intracellular pool of bacteria.

6.2. *S. aureus* as an intracellular pathogen

It is well established that *S. aureus* is easily phagocytosed (Jonsson et al., 1985). The pathogen does not possess major virulence factors blocking phagocytosis. Therefore phagocytosis of *S. aureus* is not observed to be the rate limiting step in the macrophage's control of the bacterium. In contrast the pathogen possesses multiple attributes that resist intracellular killing by macrophages and this is thought to be the rate limiting step in bacterial clearance. It has been suggested that in AMs, macrophage killing of *S. aureus* is slower compared to other pathogens (Jonsson et al., 1985). Limited intracellular killing by macrophages results in *S. aureus* intracellular persistence. The reasons why *S. aureus* are able to subvert intracellular killing are varied. Firstly, *S. aureus* produces virulence factors that inhibit intracellular killing. Catalase production detoxifies hydrogen peroxide (Mandell, 1975, Das and Bishayi, 2009). Staphylokinase has the ability to neutralise ROS and allow *S. aureus* to survive

oxidative stress (Clauditz et al., 2006). It also produces lactate dehydrogenase to survive under nitrosative stress as well as a haem metalloprotease (Richardson et al., 2006, Richardson et al., 2008). *S. aureus* is inherently resistant to a range of antimicrobial proteins such as lysozyme (Bera et al., 2005) and defensins (Jin et al., 2004). This combination of virulence factors likely contributes to *S. aureus* success as an intracellular pathogen. It has been demonstrated that macrophages produce less ROS than neutrophils (Devalon et al., 1987). However, ROS production by macrophages requires optimal stimulation of macrophages with factors such as IFNγ and by phagocytosis (DeLeo et al., 1999), suggesting that without ongoing activation, macrophage killing of intracellular of *S. aureus* is limited. Ongoing killing of *S. aureus* by macrophages has been demonstrated to require dual signalling through TLRs at the cell surface and intracellularly (Wolf et al., 2011). There was an initial signal when TLR2 on the macrophage surface was engaged by *S. aureus* PAMPs and then a second intracellular signal provided amplification dependent on the timing of intracellular degradation of bacteria, which released microbial factors required to engage the pattern recognition receptor (Wolf et al., 2011). Resistance to intracellular degradation resulted in a failure to fully activate this amplification loop and *S. aureus* then persisted intracellularly until a time when it was ready to escape from lysed cells.

S. aureus can also alter its genetic and proteomic profile allowing it to persist intracellularly without activating the macrophage antibacterial responses in the form of small colony variants (Tuchscherr et al., 2011). Small colony variants are well adapted to survive within the harsh intracellular environment of the macrophage (Tuchscherr et al., 2010). They alter their genetic profile appropriately to survive intracellularly and maintain integrity of the host cell (Kriegeskorte et al., 2011). This affords *S. aureus* with the ability to lower its virulence and persist intracellularly. The Agr system becomes downregulated in small colony variants preventing host cell killing (Tuchscherr et al., 2011). By manipulating its genetic phenotype, the bacterium has the ability to either attack the host immune system or persist (Tuchscherr et al., 2011). Small colony variant formation allows intracellular *S. aureus* to both escape intracellular killing and to be shielded within a protective niche (von Eiff et al., 1997).

Once phagocytosed, the majority of intracellular *S. aureus* fail to traffic to a compartment of low pH. This could be an inherent defect in endosomal trafficking, preventing the mature phagolysosome forming. The majority of intracellular *S. aureus*

were in a LAMP positive compartment, suggesting they had fused with the early endosome because LAMP acquisition is downstream of early endosomal markers (Huynh et al., 2007). The failure to acidify, might be related to a failure of the phagosome to fuse with late endosomes containing markers like LIMP-II and Rab7 (Huynh et al., 2007). For certain microorganisms it has been demonstrated the phagosome can be remodelled and bacteria persist in an organelle with early and late lysosomal markers but exclude the V-ATPase which acidifies the phagolysosome (Asare and Abu Kwaik, 2007). These pathogens persist quietly, can also possibly replicate and at later stages of infection escape into the cytosol and ultimately are released from the host cell (Asare and Abu Kwaik, 2007). In the case of *S. aureus*, a subset of intracellular bacteria might be manipulating the endocytic pathway and some might end up in a mature phagolysosome. The macrophage would be able to detect and kill the bacteria in the mature phagolysosome but those in a phagosome or other compartment that had not fully matured would serve as a source for bacteria that persist and at later stages macrophage lysis and release from the host cell.

There could be exclusion of the V-ATPase leading to intracellular persistence as seen with *Mycobacterium tuberculosis*. Research demonstrates that the majority of *Mycobacterium tuberculosis* do not end up in an acidic compartment but there is still partial acidification taking place (Hackam et al., 1997). This is mediated by a sodium/hydrogen exchange (NHE) protein that is poised in the phagosome membrane to exchange phagosomal sodium ions for cytoplasmic protons leading to partial acidification (Hackam et al., 1997). If a similar mechanism holds true for *S. aureus* it could explain why some bacteria do end up in an acidic compartment but the majority do not.

In the case of *Francisella tularensis*, the phagosome becomes acidic prior to lysosomal fusion (Santic et al., 2008). The bacterial phagosome acquires the V-ATPase and there is an acidification of the phagosome prior to it fusing with the lysosome (Santic et al., 2008). This could lead to lysosomal membrane permeabilisation (LMP) and the release of bacteria into the cytosol to replicate (Santic et al., 2008). Research from the Dockrell group has shown that during *S. aureus* infection, there isn't LMP and activation of the lysosomal protease cathepsin D (Bewley et al., 2011). LMP and cathepsin D activation have been demonstrated to sensitise the macrophage for apoptosis (Bewley et al., 2011). It can be argued that the failure to detect LMP and cathepsin D activation during *S.*

aureus infection of macrophages may be due to a failure to acidify the phagolysosome or for it to mature appropriately, causing a reduction in the early phase macrophage killing response and ultimately reduced apoptosis. Failure to engage apoptosis would prevent apoptosis-associated killing, which is critical for the removal of persistent intracellular bacteria.

S. aureus again has the ability to manipulate macrophage apoptosis which has been shown to involve the anti apoptotic protein Mcl-1 (Koziel et al., 2009, Koziel et al., 2013). For certain pathogens, there is a decrease in Mcl-1 levels over time which correlates with induction of macrophage apoptosis (Marriott et al., 2005). In the case of *S. aureus* this does not occur. It has been found that Mcl-1 levels are maintained at a high level (Koziel et al., 2009). The ability of *S. aureus* to manipulate this pathway in macrophages and prevent apoptosis-associated killing would lead to increased persistence of bacteria which eventually escape the macrophage at a later period of time. My data suggests that failure to downregulate Mcl-1 may be linked to failure to appropriately mature the phagolysosome.

Finally, *S. aureus* has been shown to be able to escape intracellular confinement, which appears to be a final stage in its complex interaction with the macrophage, although these results require further characterisation (Kubica et al., 2008, Grosz et al., 2013). This ability is important during *S. aureus* infection whereby it spreads to distant tissues and re-establishes infection. The factors responsible for this phase of infection are only just beginning to be elucidated and include virulence factors such as phenol soluble modulins (Grosz et al., 2013). By turning on expression of these toxins at a crucial point in time, this allows *S. aureus* to escape the phagosome, enter the cytoplasm and escape the macrophage.

In the experiments presented in my thesis, I was able to record macrophages phagocytosing bacteria at different levels. This is not necessarily reflective of a difference in the capacity of macrophages to phagocytose and the actual reason behind the behaviour is unknown. One speculative reason is the activation status of different macrophages in culture at the start of the experiment, especially as we are dealing with a heterogeneous population, or alternatively the ability of the macrophages to respond to activation stimuli. There could be a concentration gradient occurring, e.g. of bacterial peptides meaning some macrophages can respond to the bacteria earlier and start phagocytosing and so appear to have more intracellular bacteria within them than those

that might respond later. Another possibility is that there is a defect in intracellular killing as time progresses. It can be suggested that within a macrophage there could be two compartments that I have termed 'fast killing' and 'slow killing.' When macrophages first phagocytose bacteria it is possible that some bacteria reside in a 'fast killing' compartment and some in a 'slow killing' compartment. Over time, the slow killing compartment cells begin to dominate when there are no bacteria in the fast killing compartment leading to increases in intracellular viable bacteria. Those macrophages that start phagocytosing later appear to have fewer bacteria if they still have the capacity to kill fast and slow. Ultimately those macrophages exhibiting very high numbers of intracellular bacteria could represent those that cannot kill bacteria. Therefore, this could represent why in a heterogeneous population there are macrophages with varying levels of intracellular bacteria. A final possibility is there is differing levels of recruitment of macrophages meaning there is always a pool of macrophages that do not respond until much later. This could account for differing levels of intracellular bacteria, especially if for example, some had been phagocytosing for 5 hours and some for 1 hour, if they were recruited later as the bacterial density increased. This would need to be tested experimentally and I have not explored this in my thesis.

6.3. Mathematical models of host: pathogen interactions

I used my biological data to develop a novel mathematical model to describe the interaction between macrophages and *S. aureus*. Mathematical modelling is a powerful tool that can both inform our understanding of biological systems and also help make predictions about unknown events (Callard and Yates, 2005, Kirschner and Marino, 2005). Having performed an in depth characterisation of the kinetics of macrophage phagocytosis and intracellular killing of *S. aureus* I used my experimental results to motivate and parameterise a mathematical model that would accurately portray the biological results and provide insight into the underlying biological process.

Consistent with what I observed *in vitro* I was able to demonstrate that as bacterial dose increased there was a progression towards macrophages not being able to successfully control extracellular replication over the observed time period. My results from the parameterised model show that macrophages are able to limit extracellular growth for at least 6 hours at low bacterial doses. *In vivo* this delay to exponential growth of bacteria may be an important factor as it may buy enough time for other cells to be recruited to

the site of infection. On their own however, the model shows that the macrophages are ultimately fighting a losing battle since bacterial growth will eventually outstrip phagocytosis.

The experimental work in my thesis has highlighted that there is a limit to intracellular killing of *S. aureus* by macrophages. In my model I therefore assumed that while macrophages phagocytose at a rate proportional to the extracellular bacteria numbers, the killing ability of each activated macrophage is fixed. In other words, macrophages will ingest more and more bacteria as the extracellular concentration increases, but will still kill internalised bacteria at a constant rate. Therefore while intracellular bacteria numbers may be kept low while the extracellular population is controlled, as the extracellular bacteria enter their exponential growth phase the intracellular numbers will ultimately increase.

In the absence of extracellular bacteria there is a gradual decline in intracellular bacteria, but even at later time points there was still some intracellular persistence, even at low bacterial doses. Therefore, the model highlighted that although the macrophages were able to kill intracellular bacteria they were not able to clear all of the intracellular load which allowed some bacteria to persist. However, the administration of an antimicrobial can help the macrophage clear intracellular bacteria, and over time, in the presence of extracellular antimicrobials intracellular bacterial numbers become zero.

I made a number of key assumptions when developing the model that should be considered in further modelling work. First of all the mathematical model assumes a homogeneous population while my experimental data suggests a subset of cells ingest and kill and a significant population remain naive although they can potentially become macrophages that phagocytose at later time points. Secondly, it is possible the macrophage needs additional help from other cells to achieve optimal control of infection, as evidenced through TB models (Warrender et al., 2006). Thus the investigation of macrophages in isolation of other cells may not fully represent their behaviour *in vivo*. Furthermore although my data showed there were two phases of killing, this initial model assumed there was a single constant rate of intracellular killing for mathematical tractability. The first phase is more dramatic and occurs promptly after phagocytosis, while the second is much more gradual. This model should now be manipulated, to include the two phases of killing, and, having done the necessary experiments, to begin to unravel whether the intracellular persistence is because there is

a depletion of an essential element of the intracellular killing machinery, or because the bacteria are subverting the macrophage response.

I feel it is important to briefly comment on how mathematical modelling can be used to predict experimental outcome as this is a key objective of any mathematical model. When a basic mathematical model is established it is important to determine if it holds up experimentally. This is usually done by selecting a parameter within the model to test e.g. macrophage intracellular killing and manipulating it experimentally. This then produces a result which can be used to calculate an alternative rate for the parameter under investigation. This new rate can be added into the model to see if it produces reasonable dynamics to what is observed experimentally. Alternatively, prior to carrying out the experiment, the model can be tested by running plots at for example a lower dose and determining the behaviour. This can then be compared to an experiment to see if the model is good at making predictions which can be verified. This ability to manipulate models is important clinically. In trials where certain drugs are known to target specific processes, these altered rates can be incorporated into the model to determine how useful the drug will be, e.g. will it provide long term health benefits. This has proven popular and has been done with HIV research where it was shown that treatment only benefits over a small window.

In my thesis, this manipulation was not carried out. The main reason was although I had produced a mathematical model that worked it did not fully take into account the heterogeneous nature of the population and assumed homogeneity. This is something that needs to be added in before manipulating the model to account for the differing behaviour of the population. Also, the main manipulation I wanted to perform was to look at intracellular killing. As the model is presented I was not able to estimate two different rates of intracellular killing meaning my model although fitting the data, it did not account for the slow and fast phases independently. Extensions to the model would be to look at the effect of macrophage recruitment on phagocytosis and add this into the model to account for the heterogeneous behaviour. However, what I have presented, I used my Se replication rate and varied it to show the responses on the model and also ran the model at a higher dose than I normally use to show how Se and Si bacteria and Ma macrophages behave and this shows that my initial model holds up, but requires some minor improvements and statistical analysis to further improve it. In summary, although I did not get round to manipulating my mode owing to time, I was able to briefly show, at least by altering the model, that it could be used to make predictions.

6.4. Future directions

6.4.1. Characterisation of the compartment of intracellular persistence

I have shown that intracellular *S. aureus* persist in a compartment that is LAMP positive and not of a low pH. This leaves an open ended question as to what the nature of this intracellular compartment. One possible mechanism that requires further characterisation is whether or not there is a defect in lysosomal/endosomal fusion post LAMP acquisition. It would be interesting to see whether the bacteria are manipulating the endocytic pathway and therefore persisting in a phagosome like compartment. Also it would be interesting to see if there is a failure to recruit the V-ATPase, if the V-ATPase is functioning or if a subset of bacteria acquire an alternative acidifying machinery leading to a subset being in a 'mature phagolysosome' but the majority not.

6.4.2. The mechanism of macrophage death by intracellular *S. aureus*

I also demonstrated that over extended periods of culture, intracellular bacteria escaped from the macrophage, replicated extracellularly and were reingested by other macrophages. Given this interesting observation, it would be interesting to unravel how intracellular bacteria are able to escape from the macrophage. It would be interesting to determine the mode of cell death occurring. I have defined it as cytolytic but does not involve apoptosis. It is likely to involve necrosis or necroptosis but it would be interesting to determine to what extent it is programmed by the innate response or whether it is activated by a bacterial leukocidin or other toxin. It would also be interesting to phenotype the bacteria at the start of infection, during persistence and after macrophage lysis to determine if there are any changes to them when persisting and whether they express particular virulence factors to lyse the macrophage.

6.5. Conclusion

In summary, the data presented in this thesis demonstrate that the rate limiting step in the macrophage response to *S. aureus* is at the level of intracellular killing. Macrophage killing of bacteria becomes exhausted before phagocytosis. Intracellular persistence eventually led to the expulsion of intracellular bacteria from the macrophage and concomitant cell death. These extracellular bacteria were then re-phagocytosed. I have demonstrated that intracellular bacteria do not reside in a compartment of low pH and macrophage apoptosis is not engaged during *S. aureus* infection. I have translated my data into a mathematical model to describe the extracellular and intracellular behaviour

of macrophages towards *S. aureus*. The mechanisms surrounding some of this data require further characterisation to fully understand the biological reasons behind them.

Chapter 7 References

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Appendix 1 Script for extracellular mathematical model

```
rm(list=ls())

staph.dynamics<-function(t,var,par)
{
Mf<-var[1]
Ma<-var[2]
Sf<-var[3]
Si<-var[4]
gamma<-par[1]
r<-par[2]
k<-par[3]
beta<-par[4]
mu<-par[5]
K<-par[6]
dMf=(-gamma*Mf*Sf)+(mu*Ma)*(Si/Ma<K)
dMa=(gamma*Mf*Sf)-(-mu*Ma)*(Si/Ma<K)
dSf=(r*Sf*(1-(Sf/k)))-(gamma*Mf*Sf)-(beta*Ma*Sf)
dSi=(gamma*Mf*Sf)+(beta*Ma*Sf)-(-mu*Ma)
list(c(dMf, dMa, dSf, dSi ))
}

staph.par<-c(0.00000011, 0.7, 100000000, 0.00000011, 0.16,1)
staph.init<-c(80000, 1, 10000, 1)
staph.t<-seq(0,6,by=0.2)
library(odesolve)
staph.sol<-lsoda(staph.init, staph.t, staph.dynamics, staph.par)
fix(staph.sol)

plot(staph.sol[,1],staph.sol[,2],type='l', main=' MOI 5 ', xlab='Time(h)', ylab='Macrophage count', ylim=c(0,80000), xlim=c(6,16), col='blue')
points(staph.sol[,1],staph.sol[,3],type='l', col='black')
plot(staph.sol[,1],log10(staph.sol[,4]),type='l', main=' MOI 5 ', xlab='Time(h)', ylab='log10 Extracellular bacterial CFU', ylim=c(2,9), xlim=c(0,16), log="y",
points(staph.sol[,1],staph.sol[,],type='l', col='purple')
```

Appendix 2 Script for intracellular mathematical model

```
rm(list=ls())

staph.dynamics<-function(t,var,par)
{
Mf<-var[1]
Ma<-var[2]
Si<-var[3]
mu<-par[1]
K<-par[2]
dMf=(mu*Ma)*(Si/Ma<K)
dMa=(-mu*Ma)*(Si/Ma<K)
dSi=(-mu*Ma)
list(c(dMf, dMa,  dSi ))
}

staph.par<-c(0.16,1)
staph.init<-c(35.87, 79964.130, 660180.800)
staph.t<-seq(6,16,by=0.2)
library(odesolve)
staph.sol<-lsoda(staph.init, staph.t, staph.dynamics, staph.par)
fix(staph.sol)

plot(staph.sol[,1],staph.sol[,2],type='l', main=' MOI 5 ', xlab='Time(h)', ylab='Macrophage count', ylim=c(0,80000), xlim=c(6,16), col='blue')
points(staph.sol[,1],staph.sol[,3],type='l', col='black')
plot(staph.sol[,1],(staph.sol[,4]),type='l', main=' MOI 5 ', xlab='Time(h)', ylab='Intracellular bacterial CFU', ylim=c(0,270000), xlim=c(6,16),   col='red')
```

Conference abstracts presented

(1) The role of the alveolar macrophage in *Staphylococcus aureus* pneumonia, Jubrail J, Marriott H, Boots M and Dockrell D (Staph GBI 2011)

(2) The interaction of *Staphylococcus aureus* with macrophages, Jubrail J, Marriott H and Dockrell D (EMBO Conference 'The subversion of host defences by pathogens 2012).